Altered *trans*-Regulatory Control of Gene Expression in Multiple Anthocyanin Genes Contributes to Adaptive Flower Color Evolution in *Mimulus aurantiacus*

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A fundamental goal in evolutionary biology is to identify the molecular changes responsible for adaptive evolution. In this study, we describe a genetic analysis to determine whether the molecular changes contributing to adaptive flower color divergence in *Mimulus aurantiacus* affect gene expression or enzymatic activity. High performance liquid chromatography analysis confirms that flower color differences are caused by the presence versus absence of anthocyanin pigments. Cosegregation analysis and in vitro enzymatic assays rule out mutations that affect enzymatic function in the anthocyanin pathway genes. By contrast, cosegregation of gene expression with flower color suggests that tissue-specific differences in pigment production are caused by the coordinated regulatory control of three anthocyanin pathway genes. We provide evidence indicating that these expression differences are caused by a locus that acts in *trans*- and explains 45% of the phenotypic variance in flower color. A second locus with sequence similarity to the R2R3 MYB family of transcription factors explains 9% of the variation but does so in a complex fashion. These results demonstrate one of only two examples where we have clear evidence of both the adaptive nature of a flower color transition and evidence for its genetic basis. In both cases, mutations appear to affect expression of the anthocyanin structural genes. Future studies will allow us to determine whether these differences represent a real bias in favor of mutations that affect gene expression.

Introduction

A central goal in evolutionary biology is to identify, at the molecular level, the genetic changes that are associated with adaptive evolution. Such characterization is important because it should allow us to explore several long-standing questions about the genetics of adaptation, such as how many genes underlie an adaptive trait? What are their average effect sizes? What types of mutations are most frequently involved in adaptation? Have the genetic changes been fixed due to natural selection? And have they arisen from novel mutations or from standing genetic variation? Despite much recent effort to address these questions in several systems (Orr 2005; Hoekstra and Coyne 2007; Barrett and Schluter 2008; Stern and Orgogozo 2008; Stinchcombe and Hoekstra 2008), we still have much to learn about the nature of the genes contributing to adaptive evolution. Therefore, additional examples that dissect the genetics of adaptive traits are needed before we will be able to provide definitive answers to these types of questions.

Examples exist from a variety of organisms in which the genetic bases of character differences have been identified, but in a majority of these, either adaptation has been a response to human intervention or manipulation (e.g., Doebley et al. 1997; Newcomb et al. 1997) or there is little evidence indicating that the character differences are actually adaptive in their natural environments (Sucena and Stern 2000; Sucena et al. 2003; Witkopp et al. 2003; Gompel et al. 2005; Prud'homme et al. 2006). One trait that is often thought to be adaptive due to its role in the attraction of different animal pollinators is floral pigmentation (Grant 1993). Flower coloration is a main component of a species' pollination syndrome (Faegri and van der Pijl 1971), which is a suite of floral traits that is believed to have evolved in a correlated fashion to increase specialization by particular classes of pollinator (Fenster et al. 2004). Despite a common belief in the adaptive nature of flower color evolution, a surprising lack of direct evidence exists in most species, and in many cases, nonadaptive hypotheses of variation in floral color cannot be ruled out (Armbruster 2002; Strauss and Whittall 2006; Rausher 2008).

By contrast, for *Mimulus aurantiacus* (Phrymaceae), there is strong evidence that flower color variation between two parapatrically distributed races is adaptive. In San Diego County, CA, western populations contain exclusively red-flowered plants, and eastern populations consist of only yellow-flowered individuals. Along a narrow transition zone, both pure and intermediate forms are found. Previous work has demonstrated that flower color has a genetic component and that geographic differentiation is being maintained because of strong selection despite gene flow at neutral loci (Streisfeld and Kohn 2005). In addition, hummingbird and hawkmoth pollinators show strong preference for these different floral types in each habitat, suggesting that pollinators contributed to the differentiation of floral traits in these races (Grant 1993; Streisfeld and Kohn 2007). Although these previous results implicate a direct role for natural selection maintaining this geographic variation in flower color, little is known about the underlying genes that contribute to this variation. Our goal in this report is to present an analysis of the genetic basis of adaptive divergence in flower color in *M. aurantiacus*.

Among angiosperms, anthocyanins are the predominant floral pigments (Winkel-Shirley 2001), and the flavonoid biosynthetic pathway that is responsible for anthocyanin production is highly conserved (Holton and Cornish 1995). As a consequence of the extensive investigation into the function and regulation of the genes comprising this pathway (Grotewold 2006), we have a reasonable expectation of the effects of particular types of mutations on organismal fitness. These expectations derive from our knowledge of the potential effects of these mutations on deleterious pleiotropy, thus allowing us to make predictions about the likely genetic targets responsible for changes in flower color.

For example, the six core anthocyanin structural genes (those coding for anthocyanin pathway enzymes; fig. 1) are

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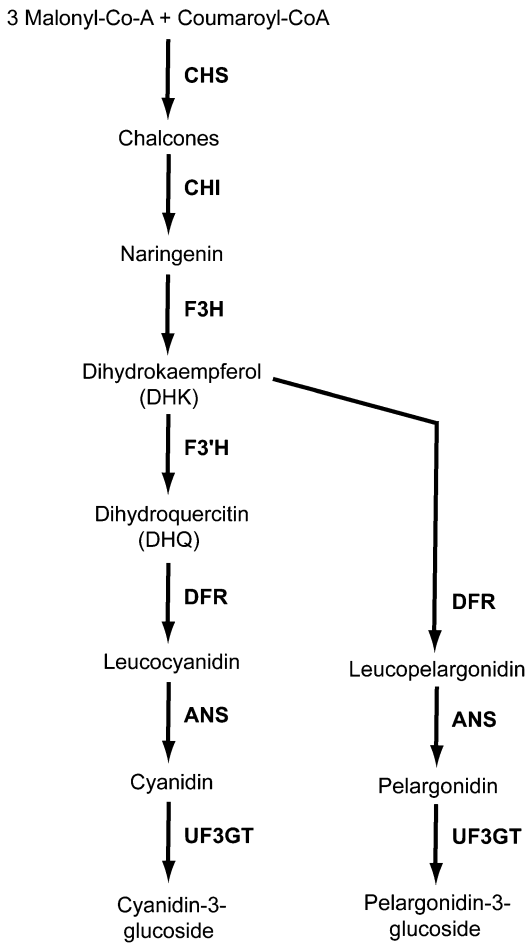

FIG. 1.—A schematic diagram of the anthocyanin pathway, with the core structural enzymes listed alongside the arrows. The substrate for each enzymatic reaction is the product of the previous reaction and is listed below the arrows.

required not only for the production of anthocyanin pigments, but many also are required for the production of non-anthocyanin flavonoids, which perform many vital physiological and ecological functions (Koes et al. 1994; Shirley 1996; Winkel-Shirley 2002). In some plant species, many of these structural genes exist as single copies. Consequently, eliminating or altering the function of these genes is likely to have adverse pleiotropic effects because flavonoid production will be reduced throughout the plant. This argument may not pertain to structural genes that exist as small gene families if one copy is expressed in flowers and other copies in vegetative tissue. However, in several species that have been examined (e.g., chalcones synthase in Petunia, *Ipomoea*), one copy is widely expressed in most tissues, including flowers, whereas other copies have more narrow expression domains (Koes et al. 1989; Inagaki et al. 1999; Durbin et al. 2003). Although this pattern is not necessarily universal, it suggests that even when there are multiple copies of a structural gene, there is commonly a potential for knockouts of the floral copy to exhibit deleterious pleiotropy (e.g., Coberly and Rausher 2008).

By contrast, transcription factors that activate anthocyanin structural genes tend to be highly specific, with activities often localized to individual tissues (Ludwig and Wessler 1990; Cone et al. 1993; Koes et al. 2005). Therefore, alterations of function or expression of these transcription factors can potentially affect floral-anthocyanin production without altering anthocyanin or flavonoid production in other tissues (Quattrocchio et al. 2006). Similarly, because *cis*-regulatory regions are commonly modular in nature (Wray et al. 2003), mutations in these regions of anthocyanin structural genes may often be capable of altering anthocyanin production in flowers without causing similar alteration in vegetative tissues (e.g., van Tunen et al. 1989, 1991). However, major alteration of promoter regions (e.g., by transposon insertion) may affect flavonoid production in vegetative tissues as well as flowers (Galego and Almeida 2007). Because of this localized action, mutations that alter either *cis*-regulatory regions or transcription factor function are often expected to have fewer deleterious pleiotropic effects, and thus a greater net selective advantage, than phenotypically similar flower color mutations in structural genes. In turn, because the probability of fixation is proportional to the net selective advantage, mutations in anthocyanin regulatory sequences are expected to be disproportionately selected.

As a consequence of this understanding of the function and regulation of the anthocyanin pathway, several investigators have suggested previously that evolutionary transitions in flower color will frequently involve mutations that directly affect the expression of one or several anthocyanin structural genes (Quattrocchio et al. 1999; Durbin et al. 2003; Rausher 2006; Schwinn et al. 2006; Whittall et al. 2006; Coberly and Rausher 2008). These mutations could be located in the *cis*-regulatory regions of the structural genes or in the coding sequences or *cis*-regulatory regions of the genes that encode anthocyanin-regulating transcription factors.

Although there are several examples consistent with this prediction, in many of these cases, the researchers have not ruled out the possibility that coding sequence mutations in the structural genes were also involved (Durbin et al. 2003; Zufall and Rausher 2004; Whittall et al. 2006; Hoballah et al. 2007). Therefore, it is not clear which of the genetic changes were causally related to the evolutionary transition in flower color and which were subsequent, redundant changes. By contrast, two definitive studies have documented mutations in transcription factors as being primarily responsible for changes in flower color between species and thus support this expectation (Quattrocchio et al. 1999; Schwinn et al. 2006). In addition, some evidence supports the assumption that alteration of an anthocyanin structural gene incurs greater deleterious pleiotropy than a mutation in the coding sequence of a transcription factor that produces a similar novel flower color (Coberly and Rausher 2008). In this study, we use our understanding of the ecology of *M. aurantiacus*, as well as our functional knowledge of the anthocyanin pathway, to assist us in determining whether the genetic changes contributing to variation in flower color in this system affect gene expression or enzymatic activity of the structural anthocyanin genes.

Materials and Methods

Anthocyanin Pathway and Genes Examined

At least six different enzymatic reactions must occur to produce pigments, and the genes coding for these structural enzymes have been characterized in several species (fig. 1; Holton and Cornish 1995). These enzymes also serve as branching points for the nonpigmented flavonoids, including lignins, flavonols, and other phenolic compounds such as tannins (Shirley 1996). Although no known regulatory function has been ascribed to the products of these genes, mutations in their coding regions can completely eliminate the accumulation of pigmented and nonpigmented flavonoids in all tissues that express them, leading to potentially severe ecological consequences (Koes et al. 2005; Rausher 2006). Many spontaneous flower color mutations among horticultural accessions involve these genes (Holton and Cornish 1995; Rausher 2006).

In addition to the structural backbone of the pathway, much is known about the regulation of the genes coding for these enzymes. In all species that have been examined, gene regulation occurs at the level of transcription via the interaction of *cis*-regulatory elements in the structural anthocyanin genes and functional transcription factor complexes. These complexes comprise members of the R2R3 MYB, basic helix–loop–helix (bHLH), and WD40 repeat families. These three classes of transcription factors are represented by large gene families, with the identification of 125 putative R2R3 MYBs, 133 bHLH, and more than 200 presumed WD40 proteins in the Arabidopsis genome (Stracke et al. 2001; Heim et al. 2003; van Nocker and Ludwig 2003). For proper transcriptional activation, the MYB protein is dependent on physical interactions with bHLH proteins, whereas the WD40 proteins are believed to serve as an anchor to support the complex (Broun 2005; Ramsay and Glover 2005).

In this study, we focused on the genes for the six anthocyanin pathway core enzymes: chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid-3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-flavonoid-3-glucosyl-transferase (UF3GT). In addition, we identified three transcription factors: an R2R3 MYB protein, a bHLH protein, and a WD40 protein. One enzyme, F3′H (coding for flavonoid-3′-hydroxylase) serves as a branching point between the cyanidin and pelargonidin anthocyanins (fig. 1). Because F3′H directs flux down the cyanidin branch and because both red and yellow floral races produce cyanidin in their stems (see below), we did not investigate the role of F3′H in causing flower color differences.

Genetic Crosses

Previous genetic crosses have indicated that flower color is a quantitative character that appears to be controlled by a minimum of two genetic loci (Streisfeld 2005). In this study, we grew open-pollinated *M. aurantiacus* seed collected from a red-flowered population (ELT) and a yellow-flowered population (INJ; Streisfeld and Kohn 2005) in the Duke University greenhouses in standard potting soil under natural day lengths. A single individual from each population was crossed to produce an F_1 that was subsequently self-fertilized to produce 378 F_2 offspring that were grown until flowering.

Phenotyping

Flower color of first-day flowers was measured spectrophotometrically as the absorbance of anthocyanin pigment in petal extracts of 1% acidic methanol. Details of the methods have been described elsewhere (Streisfeld and Kohn 2005).

Identification of Anthocyanidins in Flowers and Stems

We extracted anthocyanidins, direct precursors of anthocyanin pigments, according to the methods in Harborne (1984) using approximately 1 g of fresh corolla tissue and 5 g of stem tissue from several red- or yellow-flowered plants. Extracts were resuspended in 1% acidic methanol, and 30 μl was injected on a Shimadzu LC-10AT liquid chromatograph with a 4.6 $\times$ 150 mm Alltech Prevail reverse phase C18 column (Alltech Associates, Deerfield, IL) at a flow rate of 1 ml min^{-1}. The polar solvent was 0.1% trifluoroacetic acid (TFA) in high performance liquid chromatography (HPLC)-grade water, whereas the organic solvent was 1-propanol/0.1% TFA. Anthocyanidins were eluted at 30 °C beginning at 4 min based on two linear increases from 15% to 20% (propanol/TFA:TFA) over 6 min and 20–22.5% over 2 min. A constant flow at 20% for 4 min separated these gradients. Upon reaching 22.5% propanol/TFA:TFA, there was an instantaneous increase to 27.5% for 2 min. The propanol/TFA then decreased to 15% and remained there for the final 3 min. Spectrophotometric detection was carried out at 520 nm. A mixture of pure standards of six anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin; Polyphenols Laboratories, Sandnes, Norway) was run as a control, and we compared the retention times of the peaks from the stem and flower extracts against these standards, which were run separately for each sample.

Cloning of Anthocyanin Pathway Genes

Partial coding sequences from the six core structural anthocyanin genes plus three anthocyanin-regulating transcription factors were cloned using degenerate primers and reverse-transcribed polymerase chain reaction (RT-PCR). Total RNA was extracted from floral buds using the RNeasy kit (Qiagen, Valencia, CA). First strand cDNA synthesis was performed using M-MLV reverse transcriptase according to the manufacturer's specifications (Invitrogen, Carlsbad, CA). Degenerate PCR primers were designed based on conserved regions in a multiple species alignment consisting of plant species from the orders Lamiales and Solanales (supplementary table S1, Supplementary Material online) or were taken from Whittall et al. (2006). PCR products were gel extracted (Qiagen gel extraction kit), cloned into the pCR 2.1 TOPO vector (Invitrogen), and multiple clones were sequenced for each gene (using primers M13F and M13R). In all cases, Blast searches

against the National Center for Biotechnology Information protein database yielded the highest similarity with sequences of previously characterized anthocyanin structural genes and their known transcriptional regulators from model species. Moreover, in order to evaluate whether the R2R3 MYB gene that we cloned clustered with previously identified floral-anthocyanin regulators from several model species, we constructed a gene tree that included a subset of known copies of R2R3 MYB genes from various species (supplementary fig. S1, Supplementary Material online).

Qualitative Gene Expression Assays

From these cloned sequences, *M. aurantiacus*-specific primers were designed for each of the structural genes for expression analysis (supplementary table S1, Supplementary Material online). To test for the presence versus absence of expression of these genes, RT-PCR was performed on samples collected from four natural red-flowered populations and four natural yellow-flowered populations from across their range in San Diego County, CA. Floral bud and stem tissue were collected separately from the same developmental stages, and RNA was extracted using the Spectrum Total RNA extraction kit (Sigma, St Louis, MO), which included an on-column DNase digestion to remove traces of genomic DNA. Total RNA (500 ng) was used to make cDNA, followed by 30 cycles of PCR. PCR products were visualized on 1% agarose gels stained with ethidium bromide. As a positive control for the presence of cDNA in the PCR, the constitutively expressed gene *Ef1α*, which encodes the translation elongation factor, was included. In order to ensure that we amplified the same gene copy in both tissues, PCR products from genomic DNA and cDNA synthesized from floral and stem RNA from the same individuals were sequenced.

Quantitative RT-PCR

Total RNA was extracted from floral buds collected one day before anthesis from 45 F_2 plants with varying flower colors. Specific PCR primers were designed to amplify fragments of no more than 250 bp at *Chs*, *Chi*, *F3h*, *Dfr*, *Ans*, *Myb*, and *Ef1α* (supplementary table S1, Supplementary Material online). Specificity of the primers was determined by visualizing single bands on 2% agarose gels stained with ethidium bromide. Total RNA (250 ng) was used to synthesize 20 μl of cDNA using M-MLV reverse transcriptase (Invitrogen). cDNA (1 μl) was used in 20 μl quantitative RT-PCR (qPCR) reactions containing 10 μl of Dynamo SYBR green qPCR mix (Finnzymes, Espoo, Finland), 0.2 μM of each primer, and 1× ROX passive reference dye. qPCR reactions were run on an ABI Prism 7000 sequence detection system using the following conditions: 94 for 10 min, followed by 40 cycles of 94 for 20 s, 55 for 30 s, and 72 for 45 s. A single red-flowered F_2 plant was included in every run to control for interassay variation, and each sample was run in triplicate to control for intra-assay variation. Threshold values (Ct) were first corrected for differences in PCR efficiency among genes, according to Peirson et al. (2003). Relative expression was calculated for each target gene as the logarithm of the ratio of differences between experimental and control Ct for the target gene to differences between experimental and control Ct values for the constitutively expressed *Ef1α* gene. A two-parameter exponential regression of relative expression levels of *F3h*, *Dfr*, and *Ans* was fitted against flower color measurements using Sigma Plot v. 9.01.

Cosegregation Analysis

Total genomic DNA was isolated from parental, F_1, and F_2 plants according to the methods in Streisfeld and Kohn (2005). Single-nucleotide polymorphism (SNP) markers in either exonic or intronic regions that differentiated the parents were generated by direct sequencing of PCR products from each of nine candidate anthocyanin genes (six structural genes + three transcription factors; see above). For each marker, the 30 F_2 individuals with the most extreme phenotypes were genotyped by sequencing, and we determined whether any of the markers were statistically associated with red or yellow flower colors using Fisher's Exact test. For the *Dfr* and *Myb* markers, all 378 F_2s were genotyped using a PCR-RFLP approach called Derived Cleaved Amplified Polymorphic Sequence (Neff et al. 2002), and alleles were separated with 3% superfine resolution agarose gels (Bio-Rad, Hercules, CA) stained with ethidium bromide. Assessment of the phenotypic effect of allelic variation of these loci was determined with PROC GLM (SAS v. 9.1; SAS Institute, Cary, NC).

Cloning of Full-Length Coding Sequences

Full-length coding regions from *Dfr* and *Myb* were identified using 5′ and 3′ rapid amplification of cDNA ends (RACE) procedures (Invitrogen). The entire coding region from red- and yellow-flowered parents was PCR amplified from floral cDNA and cloned into the pCR 2.1 TOPO vector (Invitrogen). The *Myb* coding region was directly sequenced from PCR products of floral cDNA from 12 individuals from red- and yellow-flowered natural populations.

DFR Enzyme Assay

In order to determine whether the DFR enzyme was functional in yellow flowers, we performed in vitro enzymatic assays using recombinant, overexpressed DFR protein. *Dfr* in pCR 2.1 from red and yellow floral cDNA was subcloned into the pENTR-SD/D vector and recombined into the Gateway pDEST-14 overexpression vector, which placed *Dfr* under the control of the T7 promoter. This construct was then transformed into BL21 Star DE3 *Escherichia coli*, and expression was induced at 25 °C by the addition of 0.5 μM IPTG. Crude protein extracts were assayed in vitro for their ability to reduce the dihydroquercitin (DHQ) substrate (fig. 1), via the conversion of the products into the pink-colored cyanidin (Stafford and Lester 1982; Des Marais and Rausher 2008). The results from three independent clones from red- and yellow-flowered plants were compared

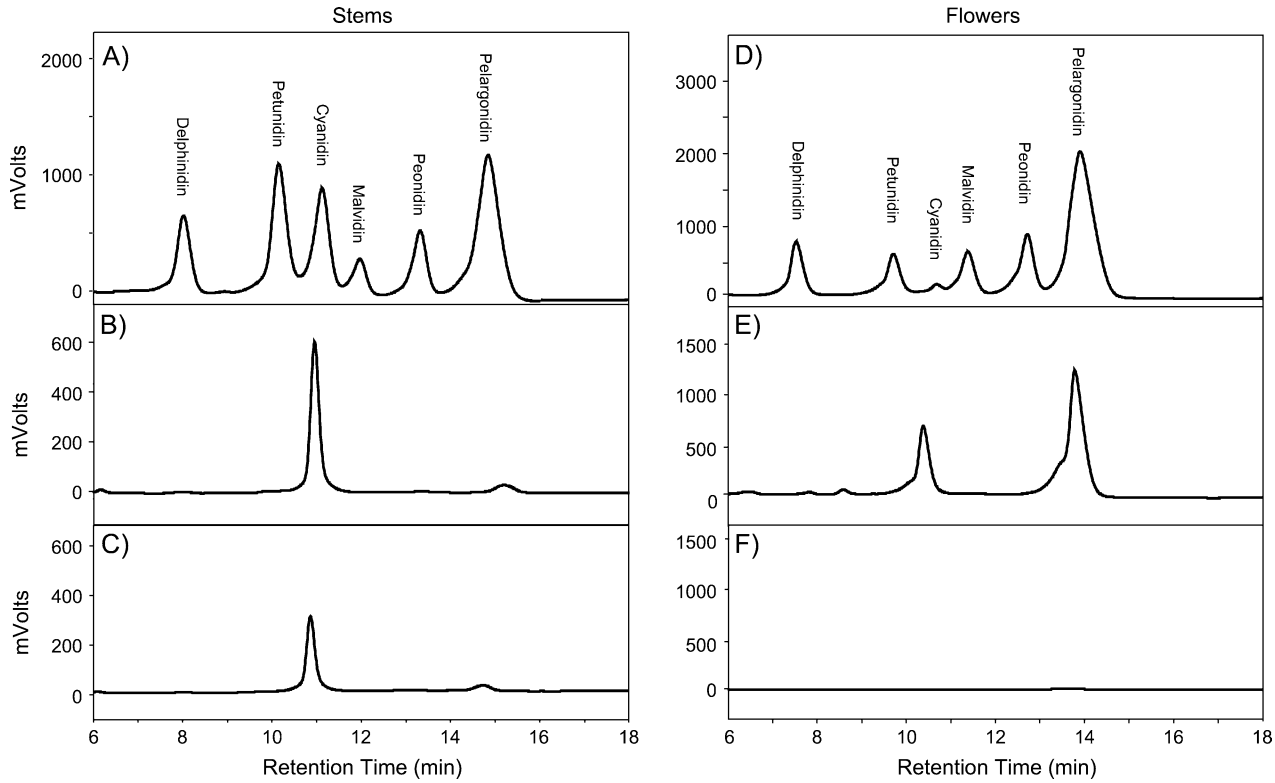

FIG. 2.—HPLC of anthocyanidins from stem (*A–C*) and flower petal (*D–F*) extracts from red- and yellow-flowered *Mimulus aurantiacus*. Panels *A* and *D* represent a mixture containing pure standards of six anthocyanidins and was run separately for stem and floral samples to determine the position of each peak. Panels *B* and *C* show the results from stem extracts of red- and yellow-flowered plants, respectively. (*E*,*F*) Results from red and yellow flower extracts, respectively.

spectrophotometrically, and differences in enzymatic activity were analyzed using a *t*-test.

Allelic Expression of *Dfr*

To distinguish between *cis*- and *trans*-acting regulation of *Dfr* expression, we used pyrosequencing to examine relative expression levels of the red- and yellow-flowered parental *Dfr* alleles when they were placed in a common *trans*-genetic background. We PCR amplified a 92-bp fragment surrounding an A/T heterozygous polymorphism in the fourth exon of *Dfr* from floral cDNA and genomic DNA from two F_1 individuals. Both F_1 plants were full-sibs generated from the original cross between the red-flowered ELT and yellow-flowered INJ populations. This included the same individual that was selfed to create the F_2 mapping population used in this study. For each individual, we ran two pyrosequencing reactions, starting with independent PCRs with four cDNA replicates and two genomic DNA replicates each. No-template controls and no-sequencing-primer controls were included in each run. Expression of each allele has been shown to be directly proportional to the peak height obtained during a pyrosequencing reaction (Witkopp et al. 2004). Genomic DNA samples were used to check for variation in the PCR efficiency of each allele by first testing for significant differences in the peak heights of the red ("R") and yellow ("Y") alleles. We then tested the null hypothesis that similar levels of expression of each allele is indicative of a *trans*-regulatory effect by using PROC GLM (SAS v 9.1), with the fixed effects of allele (R or Y), reaction replicate, and F_1 individual. Similar analyses could not be performed on the *Myb* locus because no coding-region substitutions were detected between the red- and yellow-flowered alleles (see below).

Results

Pigment and Expression Differences between Races

Pigment Chemistry

To characterize the pigment differences between the races, we used HPLC to assay for the presence and identity of the anthocyanidin precursors to the anthocyanin pigments in flowers and stems of both races. Extracts from stems of both races exhibited peaks corresponding to cyanidin and traces of pelargonidin (fig. 2). Extracts from red flowers produce cyanidin and pelargonidin, but yellow flowers do not produce any detectable anthocyanidin. This confirms previous light microscope observations that although both races produce the yellow, carotenoid pigments, color differences between the races are due to the presence of anthocyanins in red but not yellow flowers (Streisfeld and Kohn 2005). The additional observation that anthocyanidins are produced in the stems of yellow-flowered plants but not in their flowers suggests, but does not prove, that changes in gene expression, rather than in enzymatic function of the structural genes, are

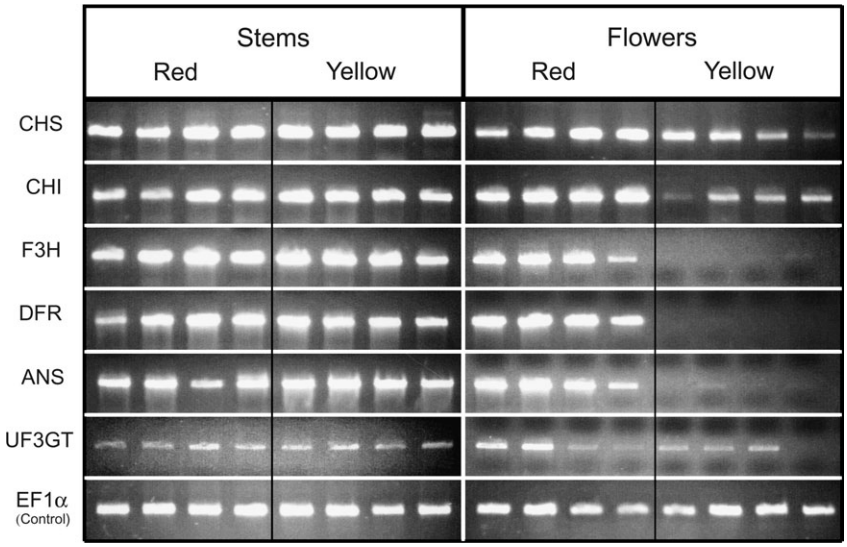

FIG. 3.—Qualitative expression levels of six core anthocyanin structural genes from stem and floral RNA from four natural red- and yellow-flowered populations. The genes are presented in the order they occur in the pathway. A constitutively expressed gene (*Ef1α*, coding for the translation elongation factor) was included in all experiments as a positive control.

likely responsible for the floral color difference between the races (see below).

Gene Expression

The absence of anthocyanidins from yellow flowers indicates that the anthocyanin pathway is blocked in these flowers. Blockage can occur either through a nonfunctional mutation in the coding region of a structural gene or through downregulation of one or more structural genes. In an attempt to determine whether changes in gene expression might be involved, we cloned partial coding regions from the six core structural genes in the anthocyanin pathway from stem and flower tissue and examined expression levels of these genes in different tissues. In all cases, identical sequences were obtained from the different tissue samples and from genomic DNA from the same individual, suggesting that we compared single copies of each of the structural genes in our assays.

Qualitative assays using RT-PCR document the production of abundant transcript from all six structural genes in the stems of both races as well as from flowers of the red floral race (fig. 3). Likewise, the two most upstream genes (*Chs* and *Chi*) and the most downstream gene (*Uf3gt*) in the pathway appear to be expressed in yellow flowers. By contrast, the middle three genes (*F3h*, *Dfr*, and *Ans*) show dramatically reduced expression in yellow flower tissue, even though the ubiquitously expressed control gene (*Ef1α*) produces bands of comparable intensity for all floral and stem samples. These results suggest that the differences in flower color between races may be due largely to mutations that affect gene expression, but they do not rule out the possibility that mutations in the coding sequence of the structural genes may also be involved. Because total expression varies so dramatically between the races at these three genes, it is clear that the developmental stage at which we collected flower buds is appropriate to capture the full extent of differences in expression.

Cosegregation Analysis

In order to determine more definitively the nature of the type of mutations that are involved, we performed a cosegregation analysis on an F_2 hybrid population segregating for flower color. In particular, absence of segregation of the structural genes with flower color would allow us to rule out their involvement in causing flower color differences. On the other hand, cosegregation of expression levels with flower color would point to regulatory change.

We used single-nucleotide differences between the two races in the six core structural genes and three anthocyanin transcription factors to deduce the nature of the genetic changes causing differences in expression and flower color between the red and yellow races. We found that allelic variation in both *Dfr* and a *Myb* transcription factor cosegregated with flower color (fig. 4). In a sample of 378 F_2 plants, *Dfr* genotype accounted for 44.8% of the phenotypic variation in floral-anthocyanin content, whereas *Myb* genotype explained an additional 9.0%. *Dfr* and *Myb* segregated independently of each other, thus suggesting that these loci are unlinked. The remaining unexplained phenotypic variation in this quantitative character can be attributed either to environmental variation or to additional loci that modify floral pigmentation. Of the remaining seven candidate genes, none cosegregated with flower color (supplementary table S2, Supplementary Material online), and none exhibited linkage with any of the other genes.

In addition, we found a highly significant interaction between alleles at the *Dfr* and *Myb* loci ($F = 8.0$; df = 4; $P < 0.0001$). Comparison of the means of each genotype demonstrated that when the allele derived from the yellow-flowered parent (*Y*) was in the homozygous state at either locus, its effect on flower color masked the effect of the other allele (fig. 4). Moreover, flower color did not vary significantly among homozygous "*YY*" genotypic classes at either locus (fig. 4), and there was no significant difference in flower color between these genotypes and the

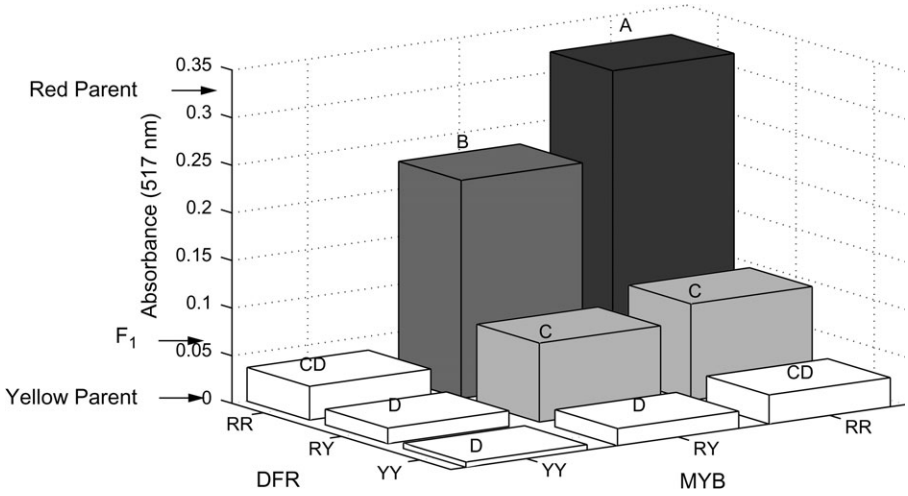

FIG. 4.—Effect of genotype at *Dfr* and *Myb* loci on flower color (anthocyanin content) in 378 F_2 individuals. For both loci, R and Y denote the alleles in the F_2 plants derived from the red- and yellow-flowered grandparents, respectively. Genotypic classes with different letters indicate significant differences in mean flower color, as determined by a Tukey posterior test. Arrows show mean phenotypes of the red- and yellow-flowered parents and the F_1 hybrid.

yellow-flowered parent (*t*-test; $P > 0.05$). In addition, there was no significant difference in mean flower color between F_2 plants that were doubly homozygous for the red allele (*RR*/*RR*) and parental red flowers (*t*-test; $P > 0.05$). These results indicate that these two loci play a large part in determining flower color in this system and that a functional copy of each is needed for anthocyanin production.

With the exception of *Dfr*, the absence of linkage between all structural genes and flower color indicates that mutations affecting enzymatic activity of the structural genes do not contribute measurably to flower color divergence. As will be shown below, such mutations also can be ruled out for *Dfr*. By contrast, our cosegregation analyses provide strong indications that flower color variation is controlled largely by expression differences. Among a sample of 45 F_2 plants, quantitative variation in relative expression levels of *F3h*, *Dfr*, and *Ans* significantly predicted variation in flower color ($r > 0.53$; $P < 0.0001$; fig. 5), whereas quantitative expression levels of *Chs* and *Chi* from a subset of these plants showed no relationship with flower color (data not shown). Moreover, expression levels of *F3h*, *Dfr*, and *Ans* were highly correlated with each other but not with *Chs* and *Chi* (fig. 6), suggesting that *F3h*, *Dfr*, and *Ans* were coordinately regulated at the level of transcription. These results provide a direct link between changes in gene expression and flower color and demonstrate that mutations affecting gene expression of the structural genes contribute to flower color differences in this system.

An interesting feature of figure 5 is that for individuals with expression levels greater than −1.8-log units relative to a red flower, floral-anthocyanin production appears to occur in three discrete classes (see fig. 5). Individuals in class 1 effectively produce no anthocyanins, and all are homozygous for the yellow-race allele (*YY*) at the *Myb* locus. By contrast, *RY* and *RR* individuals at *Myb* are roughly

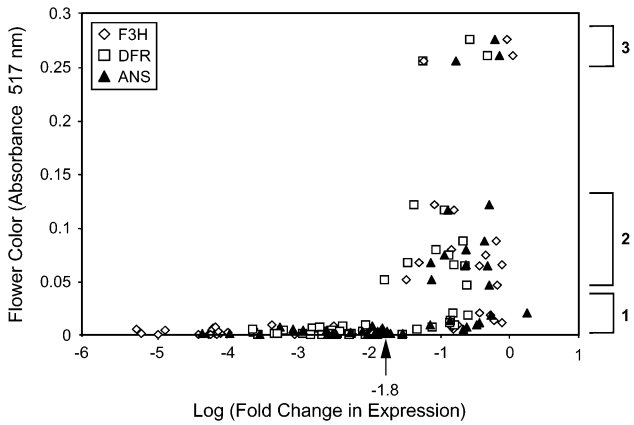

FIG. 5.—Association between quantitative expression levels of *F3h*, *Dfr*, and *Ans* and flower color (measured as the absorbance of anthocyanin pigment from petal extracts at 517 nm) from 45 F_2 flowers. Expression levels are presented as the logarithm of the fold change in expression for each gene relative to a red-flowered F_2 plant. The three discrete pigment classes for individuals with expression levels above −1.8-log units are indicated to the right of the figure.

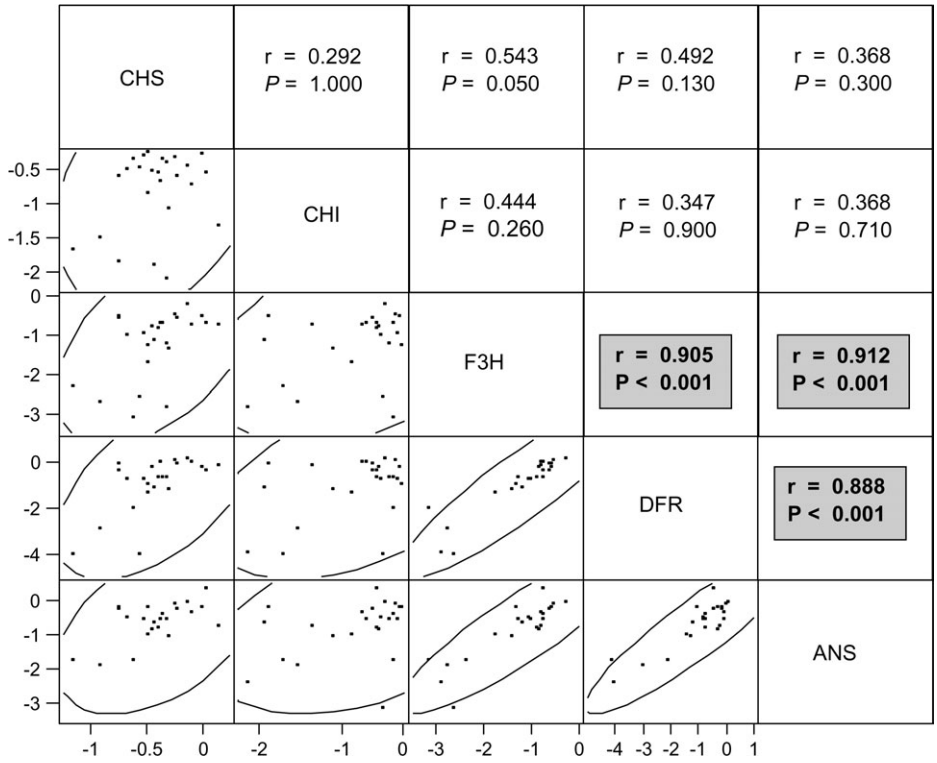

FIG. 6.—Pairwise correlations in relative expression levels between *Chs*, *Chi*, *F3h*, *Dfr*, and *Ans* from 25 F_2 flowers. Correlation coefficients and Bonferroni-corrected *P* values are listed above the diagonal and the raw data below the diagonal. Expression levels are presented as the logarithm of the fold change in expression for each gene relative to a red-flowered F_2 plant. The 99% bivariate normal density ellipse surrounds each set of points, with the width of each ellipse representing the relative strength of each correlation. Shaded cells indicate those correlations that are significant following Bonferroni correction.

equally represented in categories 2 and 3. These observations support our prior inference that at least one copy of the *Myb R* allele is necessary (but not sufficient) for anthocyanin production. In addition, the discrete nature of classes 2 and 3, along with the absence of differences in *Myb* genotype and structural-gene expression levels among these classes, suggests the involvement of a third locus that affects pigment intensity.

Analysis of *Dfr* Function and Expression

Because the cosegregation analysis revealed a link between flower color and a marker in the *Dfr* gene, we performed additional analyses to assess the nature of *Dfr*'s effect on color. The *Dfr* gene cloned from *M. aurantiacus* contains six exons and five introns, totaling 1,871 bp and encodes a predicted protein of 471 amino acids. Locations of the five introns are similar for other species from which *Dfr* has been characterized. There are three amino acid differences between red- and yellow-flowered *M. aurantiacus*. To determine whether these substitutions were functionally important for enzymatic activity, we performed in vitro enzymatic assays of DFR function. Overexpressed, recombinant DFR enzyme from both red- and yellow-flowered plants shows no difference in its ability to reduce DHQ and convert it into the colored cyanidin (mean [±2 standard errors (SE)] μmol cyanidin formed per 1 μmol DHQ substrate: red = 0.015 [0.003]; yellow = 0.012 [0.001]; *t*-test; $P > 0.05$). Although this result demonstrates that the DFR enzyme from yellow-flowered plants is functional, we note that another formal possibility requiring future investigation is that *Dfr* alleles may show epistatic effects with other loci that affect flower color. However, to the extent that these amino acid substitutions directly affect DFR enzyme activity, we have demonstrated that mutations in the coding regions of the structural anthocyanin genes do not contribute to the absence of anthocyanins in yellow flowers.

Two lines of evidence demonstrate that mutations in *cis*-regulatory elements cannot account for differences in *Dfr* expression between the red and yellow races. First, we distinguished between *cis*- and *trans*-acting regulation by comparing relative expression of the parental red- and yellow-flowered *Dfr* alleles when placed in the same genetic background (Witkopp et al. 2004). Both alleles are expressed at equal levels in the genomic DNA replicates ($F_{(1, 15)} = 1.05$; $P > 0.2$), suggesting that PCR efficiency does not differ between the alleles. Both alleles were also expressed at similar levels among the cDNA replicates (supplementary table S3, Supplementary Material online), providing no reason to reject the null hypothesis of *trans*-acting regulation at the *Dfr* locus.

Second, results from the cosegregation analysis provide direct evidence for the influence of a *trans*-acting factor on differences in *Dfr* expression. Levels of *Dfr* expression do not segregate independently of *F3h* and *Ans* expression levels, as would be expected if variation in *Dfr* expression was due to *cis*-regulatory control. Instead, expression levels of these three structural genes are highly

correlated (fig. 6), indicating coordinated control by a common factor. Moreover, allelic variation at the *Dfr* genetic marker predicts the expression levels of all three genes (table 1), which further suggests that this factor is linked to the *Dfr* locus.

Analysis of *Myb* Expression and Function

The *Myb* gene from *M. aurantiacus* exhibits structural features common among anthocyanin-regulating R2R3 MYB transcription factors. For example, phylogenetic analysis of several R2R3 MYB subfamilies confirms the placement of the *M. aurantiacus* sequence among members of subfamily six (Stracke et al. 2001), which contains previously characterized floral regulators of the anthocyanin structural genes (supplementary fig. S1, Supplementary Material online). In addition, residues previously identified as necessary for interaction with bHLH transcription factors in *Zea mays C1* (Grotewold et al. 2000) are present in the *M. aurantiacus* copy (supplementary fig. S1, Supplementary Material online), suggesting that this copy is functionally similar to previously identified anthocyanin regulators (Mol et al. 1998; Quattrocchio et al. 1999; Koes et al. 2005; Schwinn et al. 2006).

We sequenced all three exons of the *Myb* gene from floral cDNA in a sample of 12 red- and yellow-flowered individuals from natural populations and found no nonsynonymous substitutions or other obvious inactivating mutations (i.e., frame shifts or premature stop codons) among individuals of the different races (supplementary fig. S2, Supplementary Material online). We restricted this analysis to the coding region, and we did not examine whether sequence differences in the 5′ or 3′ UTRs varied according to floral race. This result suggests that there are no functional protein differences in MYB between red and yellow floral races.

We also analyzed expression differences of *Myb* in natural populations using qPCR. Relative to red flowers, *Myb* expression in yellow flowers was significantly reduced (log[SE] relative mean fold change in expression: red, 0.34[0.34]; yellow, −1.28[0.20], *t*-test; $P = 0.002$). To examine whether a *cis*-regulatory mutation contributed to this expression difference, we also assayed *Myb* expression in 25 F_2 plants of known genotype (plants were heterozygous at *Dfr*, but segregating for genotype at *Myb*). We found that genotype at *Myb* did not predict its own expression level (one-way ANOVA: $F = 0.32$; df = 2, 22; $P = 0.73$), implying that an additional regulatory factor was responsible for expression differences in natural populations. Moreover, *Myb* genotype did not predict expression levels of the structural genes (*Chs*, *Chi*, *F3h*, *Dfr*, and *Ans*) in these same F_2 plants (one-way ANOVA; $P > 0.23$ for all comparisons), even though expression levels of three of the structural genes were highly correlated with flower color.

Table 1
Mean (±1SE) of the Logarithm of Relative Expression Levels of Three Structural Anthocyanin Genes Compared for Different Genotypes at the *Dfr* Locus

	DFR Genotype		
Gene	RY	YY	*P*
F3h	−1.11 ± 0.246	−3.65 ± 0.265	<0.0001
Dfr	−1.26 ± 0.146	−2.26 ± 0.160	<0.0001
Ans	−0.85 ± 0.163	−2.30 ± 0.178	<0.0001

Discussion

A Major Gene Acting in *trans* Contributes to Flower Color Divergence in *M. aurantiacus*

A primary objective of this investigation was to determine whether the flower color divergence between two races of *M. aurantiacus* was due to mutations that affected gene expression or enzymatic function of the anthocyanin structural genes. We have identified a major locus that explains approximately half of the phenotypic variation in flower color among segregating F_2 individuals. This locus acts in *trans* by controlling the expression patterns of three anthocyanin pathway genes in flower tissue but does not appear to have a measurable effect on expression of any of the genes in vegetative tissues (fig. 3). This result adds support to the notion that *trans*-acting factors affecting the anthocyanin pathway often operate in a tissue-specific fashion.

We have arrived at this conclusion based on several experiments intended to test three alternative explanations for the observation that an SNP in the *Dfr* anthocyanin gene cosegregates with flower color. The first possibility is that the copy of the DFR enzyme from the yellow race is nonfunctional due to a mutation in the coding region of *Dfr*. We have ruled out this explanation by demonstrating that the DFR enzyme from the yellow race is fully functional in enzyme activity assays. The second possible explanation for the statistical association between *Dfr* genotype and flower color is that the copy of *Dfr* from the yellow race is downregulated due to a mutation in its promoter or other *cis*-regulatory elements. This possibility is ruled out by our demonstration that in heterozygotes, the yellow- and red-derived copies of *Dfr* are equally expressed; if a *cis*-regulatory mutation were responsible for differences in expression levels, the yellow copy would be expressed at lower levels in heterozygotes (Witkopp et al. 2004). The third alternative is that a gene linked to *Dfr* causes flower color differences. All of our results support this explanation: 1) expression levels of *F3h*, *Dfr*, and *Ans* cosegregate with flower color in F_2's (fig. 5); 2) expression levels of these three genes are highly correlated with each other (fig. 6); and 3) expression levels cosegregate with the genetic marker in *Dfr* (table 1). These results indicate that a locus linked to *Dfr* and acting in *trans* controls the expression of the three anthocyanin structural genes and explains almost half of the variation in flower color between the races. If these three genes were independently regulated, we would not expect their expression levels to be correlated. Moreover, because SNPs in *F3h* and *Ans* do not cosegregate with flower color, downregulation of these genes in the yellow race only can be explained by the effect of an unlinked *trans*-acting factor.

Coordinated expression of a block of anthocyanin structural genes appears to be a common feature of the transcriptional regulation of this pathway (Dooner 1983; Mol et al. 1998; Koes et al. 2005; Quattrocchio et al. 2006), although the set of loci coordinately controlled differs among

species (Martin et al. 1991). For example, in maize and *Ipomoea*, all the structural genes appear to be regulated by a single set of transcription factors (Mol et al. 1998; Tiffin et al. 1998; Morita et al. 2006). By contrast, in all other eudicots that have been examined, transcriptional control is partitioned among early and late pathway genes, but the breakpoint varies among species. The observed pattern in *M. aurantiacus* of combined downregulation of *F3h*, *Dfr*, and *Ans* suggests that regulation of these genes may be similar to the pattern in *Antirrhinum majus*, where control of *F3h* expression is shared with *Dfr*, *Ans*, and *Uf3gt* (Martin et al. 1991). A potential caveat to this point stems from our qualitative RT-PCR data, which show apparent expression of *Uf3gt* in yellow flower buds (fig. 3). This suggests that *Uf3gt* might be under separate regulatory control in *M. aurantiacus* flowers as compared with *A. majus* corolla tubes. However, we note that quantitative PCR was not performed on this gene, so we are unable to say with certainty that expression of *Uf3gt* is uncorrelated with the expression of *F3h*, *Dfr*, and *Ans* in *M. aurantiacus* flowers. It is also possible that we have failed to identify the proper copy of *Uf3gt*, that is, that the transcript we have identified as *Uf3gt* is not responsible for glycosylation of anthocyanidins in floral tissue, despite the fact that in a Blast search our sequence shows the highest similarities with confirmed *Uf3gt* sequences from *Perilla frutescens* (AB002818; percent amino acid identity in regions outside of in/dels = 67%). Even if this is the case, it does not affect our conclusion that *F3h*, *Dfr*, and *Ans* are coordinately regulated. Finally, expression levels of *Chs* and *Chi* are not correlated with each other, and their correlations with the other structural genes are greatly reduced in F_2 flower tissue (fig. 6), suggesting that these two genes are regulated separately from the rest of the pathway.

Unfortunately, we have not yet identified the gene that is involved in the coordinated downregulation of *F3h*, *Dfr*, and *Ans*. Therefore, we could not determine whether the genetic change in this gene between red and yellow races was due to a mutation in its coding or noncoding sequence. However, this does not invalidate our conclusion that a major gene controlling flower color differences between the races of *M. aurantiacus* affects gene expression of the structural genes rather than their enzymatic function. Although we suspect that this gene is a transcription factor, we cannot rule out the possibility that it encodes a micro RNA.

Despite the conclusion that flower color divergence in *M. aurantiacus* is caused largely by changes in this *trans*-factor, it appears that mutations in at least one, and perhaps a second, additional locus contributes to this divergence. Although the cosegregation of an SNP in the *Myb* locus with flower color suggests this may also represent a regulatory mutation, we cannot with current evidence rule out the possibility that the causal mutation may reside in another linked gene. Although lack of linkage between this genetic marker in *Myb* and any of the six core structural genes that we examined effectively eliminates them as causal contributors to flower color divergence in this system, there may be additional genes capable of influencing anthocyanin production. For example, it is common to find physical linkage among multiple copies of *Myb* genes (e.g., Schwinn et al. 2006), a possibility that requires future investigation. Alternatively, because several elements of the flavonoid network resulting in the production of aurones, flavonols, and proanthocyanidins branch off the anthocyanin pathway (Shirley 1996), it may be possible for coding-region mutations in the genes encoding enzymes at these subpathway branch points (e.g., flavonol synthase, auresidin synthase, and leucoanthocyanidin reductase) to shunt flux away from the anthocyanin branch, thus potentially affecting anthocyanin pigmentation.

Evolutionary Implications

The two most definitive prior investigations of the genetics of flower color evolution involve naturally occurring differences in the extent of floral-anthocyanin pigmentation between species (e.g., *Petunia integrifolia* and *Petunia axillaris*: Quattrocchio et al. 1999; Hoballah et al. 2007; *Antirrhinum* spp.: Schwinn et al. 2006; Whibley et al. 2006). In addition to demonstrating the direct involvement of putatively orthologous R2R3 MYB transcription factors, these studies effectively ruled out the possibility that redundant mutations in structural genes might also contribute to color differences. Moreover, they demonstrate how both coding and noncoding mutations in the same transcription factor can yield similar phenotypes among species. Our findings in this study add further support to the notion that changes affecting the expression of anthocyanin structural genes are typically involved in evolutionary transitions in flower color. However, until more data are collected from additional systems, we note that we cannot at this time determine whether particular types of mutations are overrepresented in nature. Several other investigations have pointed to the involvement of regulatory changes in floral color evolution by showing that variation among species in the type or quantity of anthocyanin pigments is correlated with expression differences of the anthocyanin structural genes (Durbin et al. 2003; Zufall and Rausher 2004; Whittall et al. 2006). However, unlike the studies in *Antirrhinum* and *Petunia*, as well as the current study in *Mimulus*, these latter studies have not ruled out the possibility that the mutations directly responsible for flower color evolution occurred in structural genes.

In assessing the types of genes that are used in adaptive phenotypic evolution, it is essential to demonstrate that the phenotypic change is in fact adaptive. Although it generally has been assumed that the tremendous diversity in flower color among angiosperms is largely a result of adaptive change associated with attracting animal pollinators (Grant 1981; Schemske and Bradshaw 1999; Fenster et al. 2004; Streisfeld and Kohn 2007), very few studies provide direct evidence that flower color divergence is adaptive, and in most cases, nonadaptive change cannot be ruled out (Rausher 2008). Of the two previous studies that have provided convincing evidence regarding the types of genetic changes involved in flower color evolution, only for *Antirrhinum* is information on adaptive divergence available (Schwinn et al. 2006; Whibley et al. 2006). A major contribution of our investigation is that we have previously documented that flower color divergence in *M. aurantiacus* is adaptive (Streisfeld and Kohn 2005, 2007), thereby

providing a second example in which flower color adaptation involves regulatory change.

Finally, the predominant role of changes that affect gene expression in *M. aurantiacus* supports theoretical predictions that deleterious pleiotropic effects associated with alternate products of the flavonoid pathway prevent the fixation of structural gene mutations. Flavonoid-based compounds can account for up to 30% of the dry weight of leaves in *M. aurantiacus*, are found in all of the races throughout the species complex, and are believed to be important in protecting plants from desiccation and herbivore damage (Lincoln 1980; Lincoln and Walla 1986; Hare 2002). The structural anthocyanin genes encode enzymes that control the production of both anthocyanins and nonpigmented flavonoids, suggesting that if a shift in flower color that attracted a different type of pollinator were favored by selection, a mutation in a region that only affected expression of flower pigments might have fewer deleterious pleiotropic effects than would inactivating enzymatic function throughout the plant. The action of two putative transcription factors in controlling these pigmentation differences suggests that they may be located in portions of the regulatory network that have few additional functions aside from regulating floral pigmentation.

Supplementary Material

Supplementary tables S1–S3 and supplementary figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/). Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession numbers EU305679–EU305689.

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