

Patterns of Evolutionary Rate Variation Among Genes of the Anthocyanin Biosynthetic Pathway

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The anthocyanin biosynthetic pathway is responsible for the production of anthocyanin pigments in plant tissues and shares a number of enzymes with other biochemical pathways. The six core structural genes of this pathway have been cloned and characterized in two taxonomically diverse plant species (maize and snapdragon). We have recently cloned these genes for a third species, the common morning glory, *Ipomoea purpurea*. This additional information provides an opportunity to examine patterns of evolution among genes within a single biochemical pathway. We report here that upstream genes in the anthocyanin pathway have evolved substantially more slowly than downstream genes and suggest that this difference in evolutionary rates may be explained by upstream genes being more constrained because they participate in several different biochemical pathways. In addition, regulatory genes associated with the anthocyanin pathway tend to evolve more rapidly than the structural genes they regulate, suggesting that adaptive evolution of flower color may be mediated more by regulatory than by structural genes. Finally, for individual anthocyanin genes, we found an absence of rate heterogeneity among three major angiosperm lineages. This rate constancy contrasts with an accelerated rate of evolution of three CHS-like genes in the *Ipomoea* lineage, indicating that these three genes have diverged without coordinated adjustment by other pathway genes.

Introduction

A general pattern that has emerged from analysis of molecular evolution is that proteins differ, often markedly, in their rates of evolution (Nei 1987, chapters 4 and 5; Li 1997, chapter 7). This variation has generally been ascribed to differences in selective constraint among proteins (Kimura 1977; Gillespie 1991, pp. 40–42; Li 1997, chapter 7), although support for this explanation is based largely on anecdotal inferences regarding the degree to which different proteins are thought to be constrained (Gillespie 1991, p. 41). Moreover, in most cases, it is virtually impossible to predict a priori whether any particular protein will exhibit a fast or slow evolutionary rate. One possible reason for this uncertainty is that investigation of evolutionary rates has focused almost exclusively on individual unrelated genes.

Most proteins, however, do not operate in isolation, but as components of complex metabolic networks that operate as functional units. There is thus the potential for evolutionary rates of proteins within a network to vary in predictable ways as a function of network properties. In this report, we examine how some of these network properties may produce patterns of evolutionary rate variation that are not discernible when proteins are examined in isolation. Specifically, we consider three potential patterns of rate variation that might be expected among enzymes in a biochemical pathway. Our hope is that identifying such patterns will provide insight into the causes of variation in selective constraint.

The first pattern is systematic variation in evolutionary rate with enzyme position in a pathway. In particular, we ask whether upstream enzymes evolve more

slowly than downstream enzymes in a linear pathway with upstream branch points. Two properties of metabolic networks suggest that this pattern may be expected: (a) the impact of an individual enzyme of a network on metabolic flux and end product concentration (and hence on the phenotype) depends not only on its own kinetic properties, but also on those of other proteins in the network (Cornish-Bowden 1990; Savageau 1990); and (b) some enzymes (those above pathway branch points) potentially influence a greater number of end products than others (those below branch points). The importance of property a is suggested by classical biochemical theories of metabolic regulation, which assert that enzymes at upstream branch points of metabolic networks control flux largely through particular pathways (Atkinson 1977, chapter 6; Crabtree and Newsholme 1987). In such circumstances, structural variation in these rate-controlling enzymes is likely to have a greater impact on fitness, and hence be subject to greater selective constraint, than variation in the remaining “equilibrium” enzymes, for which variation in kinetic parameters affects flux minimally. This reasoning suggests that upstream, rate-controlling enzymes in a pathway are likely to evolve more slowly than downstream equilibrium enzymes. Property b is expected to produce a similar pattern, since mutations in upstream enzymes, which are more likely to be above branch points, are expected to have more extensive pleiotropic effects and are thus less likely to be effectively neutral than mutations in downstream enzymes. A primary objective of this study was to determine whether this expected pattern is exhibited by the anthocyanin pathway.

A second pattern of evolutionary rate variation that appears to be emerging from recent studies is that regulatory genes tend to evolve more rapidly than structural genes (Tucker and Lundrigan 1993; Whitfield, Lovell-Badge, and Goodfellow 1993; Purugganan and Wessler 1994). However, evolutionary rates of regulatory genes have generally not been directly compared with those of the structural genes they control. Apparently high rates

Key words: anthocyanin, metabolic pathways, evolutionary rates, flavonoids.

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Mol. Biol. Evol. 16(2):266–274. 1999

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of regulatory gene evolution may thus simply reflect rapid evolutionary change in many genes, both regulatory and structural, of pathways associated with strongly selected traits. A more instructive comparison is therefore between the evolutionary rates of regulatory and structural genes from the same pathway. A second objective of this investigation was to conduct such a comparison for regulatory and structural genes of the anthocyanin pathway.

A final pattern that might be anticipated is that a set of genes from a particular metabolic pathway may experience correlated acceleration of evolutionary rates within particular lineages. Such correlated evolution may be expected for several reasons. On the one hand, particular taxa may experience repeated changes in an external environmental factor, such as temperature (Hochachka and Somero 1984, chapter 11; Pierce and Crawford 1997), that could independently select for modification of the kinetic properties of pathway enzymes. A correlated response of this type is possible if several enzymes in a pathway modulate flux or end product concentration, as suggested by metabolic control theory (Kacser and Burns 1973; Savageau 1990; Clark 1991). On the other hand, correlated evolutionary divergence may be promoted by interactions among enzymes in a pathway. For example, where different pathway enzymes associate as multienzyme complexes (Keleti 1990), beneficial evolutionary modifications of the kinetic properties of one enzyme might also produce accompanying detrimental effects on associations with other enzymes, which may select for compensatory modification of those enzymes. The final objective of this investigation was to determine whether correlated evolution could be detected among anthocyanin pathway genes.

The anthocyanin pathway is responsible for the production of floral pigments in many plants, and it also includes enzymes involved in the synthesis of a diverse array of other flavonoids that serve several ecological functions, including protection of plants against UV light, pathogens, and insects, facilitation of pollen germination and interactions with symbionts, and mediation of hormone transport (Koes, Quattrocchio, and Mol 1994; Shirley 1996). The six core structural genes believed to be common to this pathway in all angiosperms (fig. 1) can be roughly divided into two groups. Upstream genes (e.g., chalcone synthase [CHS], chalcone-flavanone isomerase [CHI], and flavanone 3-hydroxylase [F3H]) code for enzymes that tend to produce precursors for one or more important non-anthocyanin flavonoid pathways, while downstream genes (e.g., anthocyanidin synthase [ANS] and UDPglucose flavonoid 3-oxy-glucosyltransferase [UF3GT]) code for enzymes that are specific to the anthocyanin pathway. All six of these genes have previously been cloned and characterized for *Zea* (maize) and *Antirrhinum* (snapdragon). Recently, we cloned representatives of all six genes from a third species, the common morning glory, *Ipomoea purpurea* (L.) Roth (Tiffin, Miller, and Rausher 1998). In addition, homologous representatives of two classes of transcriptional activators have been reported

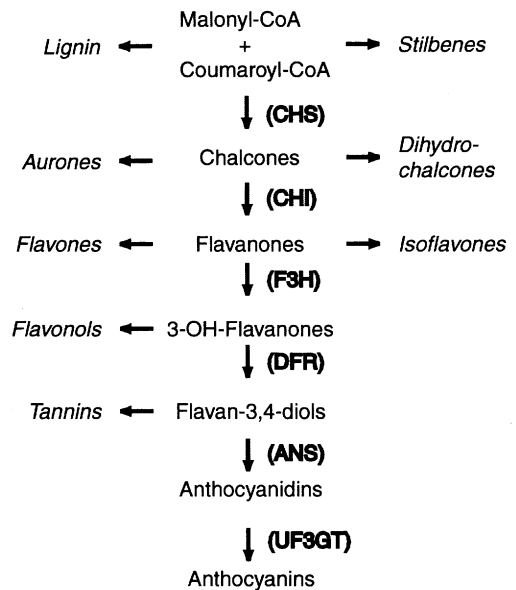


FIG. 1.—A generalized depiction of the anthocyanin biosynthetic pathway in angiosperms. Pathway enzymes are indicated in parentheses in boldface: CHS, chalcone synthase; CHI, chalcone-flavanone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; UF3GT, UDP glucose flavonoid 3-oxy-glucosyltransferase. Other products produced by branches of the pathway are indicated in italics. Not all of these alternative products are necessarily produced by any particular plant species.

from *Zea* and *Antirrhinum* (Dooner, Robbins, and Jorgensen 1991; van der Meer, Stuitje, and Mol 1993; Holton and Cornish 1995). One class, the R family, consists of genes that encode *myc*-like proteins containing a basic helix-loop-helix (bHLH) domain characteristic of other eukaryotic transcriptional activators such as Max and MyoD1 (Purugganan and Wessler 1994). The second class, the C family, consists of genes that encode proteins containing an *myb*-like binding domain similar to that of other eukaryotic regulatory genes, including the *c-myb* proto-oncogene of vertebrates and the glabrous gene that controls trichome formation in *Arabidopsis* (Paz-Ares et al. 1987).

Using reported sequences of these anthocyanin genes, we address the three objectives described above. First, we demonstrate that over long evolutionary timescales representative of the divergence between monocots and dicots, genes that code for upstream enzymes have evolved more slowly than genes that code for downstream enzymes. A similar, although not as pronounced, trend is exhibited by the two dicot species that have diverged for only half as long a period. Second, we demonstrate that over similar timescales, anthocyanin regulatory genes have evolved more rapidly than the structural genes they regulate. Finally, we examine whether correlated evolution among anthocyanin structural genes has occurred. Specifically, Durbin et al. (1995) recently reported that three members of the CHS gene family in the genus *Ipomoea* have diverged substantially from CHS in other plant taxa. This apparently accelerated rate of evolution affords an opportunity to ask whether other genes in the anthocyanin pathway exhibit a correlated acceleration of evolutionary rates in

the *Ipomoea* lineage, and we report that this has not occurred.

Materials and Methods

Anthocyanin Genes Used for Analysis

We obtained sequences of anthocyanin pathway genes of *Zea* and *Antirrhinum* from sequence databases. For *Ipomoea*, we used sequences of genes that we and others have recently isolated (Durbin et al. 1995; Fukuda-Tanaka et al. 1997; Tiffin, Miller, and Rausher 1998). Because several anthocyanin genes often occur in multigene families (i.e., CHS, CHI, and DFR), we attempted to ensure that only orthologous sequences were compared. Of the several members of the CHS multigene family reported for *Zea* and *Ipomoea*, we used *c2* from *Zea*, which is the sole gene involved in anthocyanin biosynthesis in seeds (Dooner, Robbins, and Jorgensen 1991), and CHS-D from *Ipomoea*, which is the predominant CHS transcript in flower petals. The gene tree obtained for 90 CHS and related stilbene synthase sequences from a large number of plant families (Fukuda-Tanaka et al. 1997) indicates that these copies, along with the single CHS from *Antirrhinum*, are orthologous. In addition, we examined evolutionary rates at the CHS-A, CHS-B, and CHS-C genes from *Ipomoea*. These genes form a tightly linked cluster that has diverged in sequence from most other CHS genes (Durbin et al. 1995). Despite this divergence, the Fukuda-Tanaka gene tree shows that these three CHS genes cluster with CHS-D, indicating that they arose by duplication of CHS-D (or vice versa) after the split of the *Antirrhinum* and *Ipomoea* lineages.

While CHI occurs as a small gene family in some plants (van Tunen et al. 1988, 1989), it is known only as a single copy from *Zea*, *Antirrhinum*, and *Ipomoea* (Martin et al. 1991; Grotewald and Peterson 1994; Tiffin, Miller, and Rausher 1998). Consequently, these copies are presumably orthologous. Finally, DFR is known only as a single copy in *Zea* (Schwarz-Sommer et al. 1987) and *Antirrhinum* (Martin et al. 1985; Coen, Carpenter, and Martin 1986). In *Ipomoea nil* (Inagaki et al. 1994; Hoshino, Inagaki, and Iida 1995; Inagaki, Hisatomo, and Iida 1996) and *I. purpurea* (unpublished data), it occurs, as a result of recent duplication, as a cluster of three tightly linked genes. Of these, one, *DFR-B*, is responsible for pigmentation in the flowers, leaves, and stems (Hisatomi et al. 1997), and we used this gene in our analysis. The other two have not been detected as transcripts and may be pseudogenes. The three DFR genes used in this analysis are therefore most likely orthologous.

For comparison of substitution rates of anthocyanin structural genes with regulatory genes, we used reported sequences for regulatory genes from *Zea* and *Antirrhinum*: R-family genes (*Lc* from *Zea* and *Delila* from *Antirrhinum*) (Holton and Cornish 1995) and C-family genes (*C1* from *Zea* and *Rosea* from *Antirrhinum*) (Holton and Cornish 1995; C. Martin, personal communication). Relevant properties of all genes analyzed are presented in table 1.

Table 1
Properties of Genes Analyzed

| Gene | Taxon | Accession Number | Coding Length (bp) | Length of Sequence Compared (bp) |
|-------|--|------------------|--------------------|----------------------------------|
| CHS | <i>Ipomoea purpurea</i> (A) ^a | U15946 | 1,191 | 1,161 |
| | <i>I. purpurea</i> (B) | U15947 | 1,191 | 1,161 |
| | <i>I. purpurea</i> (C) | U15949 | 1,055 | 1,055 |
| | <i>I. purpurea</i> (D) | AB001826 | 1,167 | 1,161 |
| | <i>Antirrhinum majus</i> | X03710 | 1,173 | 1,161 |
| | <i>Zea mays</i> | X60205 | 1,203 | 1,161 |
| CHI | <i>I. purpurea</i> | AF028238 | 724 | 537 |
| | <i>A. majus</i> | M68326 | 621 | 537 |
| | <i>Z. mays</i> | Z22760 | 693 | 537 |
| F3H | <i>I. purpurea</i> | U74081 | 1,104 | 945 |
| | <i>A. majus</i> ^b | — | 1,197 | 945 |
| | <i>Z. mays</i> | U04434 | 1,119 | 945 |
| DFR | <i>I. purpurea</i> | AF028601 | 1,161 | 969 |
| | <i>A. majus</i> | X15536 | 1,341 | 969 |
| | <i>Z. mays</i> | X05068 | 1,073 | 969 |
| ANS | <i>I. purpurea</i> | AF028602 | 1,116 | 1,065 |
| | <i>A. majus</i> ^b | — | 1,119 | 1,065 |
| | <i>Z. mays</i> | X55314 | 1,188 | 1,065 |
| UF3GT | <i>I. purpurea</i> | AF028237 | 1,263 ^c | 1,149 |
| | <i>A. majus</i> ^b | — | 1,347 | 1,149 |
| | <i>Z. mays</i> | X13502 | 1,416 | 1,149 |
| R | <i>A. majus</i> | M84913 | 1,830 | 1,830 |
| | <i>Z. mays</i> | M26227 | 1,935 | 1,830 |
| C | <i>A. majus</i> ^b | — | 666 | 312 ^d |
| | <i>Z. mays</i> | X06333 | 837 | 312 ^d |

^a Letters in parentheses indicate members of the CHS gene family in *I. purpurea*.

^b Sequence provided by Cathie Martin.

^c The cDNA clone for this gene is missing approximately 135 bp on the 5' end, based on comparisons with other UF3GT genes.

^d Only the DNA-binding domains were compared; see text.

Calculation of Genetic Distances

For estimation of evolutionary rates of anthocyanin genes, we calculated pairwise synonymous and nonsynonymous genetic distances for the coding regions of all six structural genes for two sets of species representing different divergence times: (1) long-term evolutionary divergence, represented by the divergence between monocots and dicots (for this category, we calculated genetic distances between *Zea* and *Antirrhinum* and between *Zea* and *Ipomoea*, as well as the average distance between *Zea* and the other two species), and (2) shorter-term evolutionary divergence, represented by the divergence between the two dicots *Antirrhinum* and *Ipomoea*. The monocot–dicot divergence is estimated to have taken place approximately 120 MYA in the early Cretaceous, while the oldest known fossils of the families represented by the two dicot species are approximately 40 and 50 Myr old, indicating a maximum divergence time for these two lineages of approximately 50 Myr (Lower Eocene) (Collinson, Boulter, and Holmes 1993; Crane, Friis, and Pedersen 1995). Distances for CHS were calculated separately for the four *Ipomoea* CHS genes. Because many of the genes analyzed exhibit highly divergent sequences at the 5' and 3' ends, yielding no detectable homology and precluding alignment, these regions were eliminated from our analysis (table

Table 2
Relative-Rate Test for Divergence of Anthocyanin Pathway Genes in *Ipomoea* vs. *Antirrhinum*

| Gene | Difference ^a | SE ^b |
|-------|-------------------------|-----------------|
| CHS-A | 0.178* | 0.023 |
| CHS-B | 0.179* | 0.023 |
| CHS-C | 0.186* | 0.025 |
| CHS-D | -0.001 | 0.012 |
| CHI | 0.018 | 0.026 |
| F3H | -0.049 | 0.017 |
| DFR | 0.010 | 0.018 |
| ANS | -0.015 | 0.022 |
| UF3GT | 0.028 | 0.057 |

^a Difference in numbers of nonsynonymous substitutions per nonsynonymous site, calculated using the method of Wu and Li (1985).

^b Standard error of the difference.

* $P < 0.0001$, using Bonferroni correction.

1). Genetic distances for synonymous and nonsynonymous substitutions, along with their variances, between pairs of taxa were calculated using the method of Li, Wu, and Luo (1985), except that for codons exhibiting more than a 1-bp substitution, we did not correct for differences in the probabilities of different substitution pathways. However, because such corrections usually have little effect on estimated substitution rates (Nei and Gojoberi 1986), our estimates are not likely to be biased. All calculations were performed using programs written by the senior author (M.D.R.).

Assessment of Rate Heterogeneity

To determine whether rates of substitution varied among lineages, we employed the relative-rate test as described by Wu and Li (1985). This test relies on knowledge of the phylogenetic relationship of the taxa being compared. For our analysis, we used established phylogenies (Chase et al. 1993), which group *Ipomoea* (Asteridae, Solanales) and *Antirrhinum* (Asteridae, Scrophulariales) as the most closely related taxa and *Zea* (Monocotyledoneae) as an outgroup. Using this phylogeny, we applied the relative-rate test to assess whether evolutionary rates of the anthocyanin genes differed in the *Ipomoea* and *Antirrhinum* lineages. The statistical significance of differences in relative rates is based on comparison of the rate difference with the estimated standard error of that difference using a standard z -test (Muse and Weir 1992). To correct for the effects of multiple comparisons, a Bonferroni correction was applied to each test to maintain an analysis-wide rejection probability of 0.01.

Results

Assessment of Rate Heterogeneity

The relative-rate test confirms Durbin et al.'s (1995) report of accelerated evolution in the CHS-A, CHS-B, and CHS-C genes of *Ipomoea*. Estimated excesses of nonsynonymous substitution rates in the *Ipomoea* lineage relative to the *Antirrhinum* lineage are highly significant for each of these genes, even after the Bonferroni correction is applied (table 2). In contrast, for CHS-D, there are no significant rate differences be-

Table 3
Estimated Nonsynonymous Replacement Rates Between Pairs of Species for Anthocyanin Pathway Genes

| COMPARISON | GENE | | | | | |
|------------|-------|-------|-------|-------|-------|-------|
| | CHS-D | CHI | F3H | DFR | ANS | UF3GT |
| I-Z | 0.135 | 0.299 | 0.147 | 0.325 | 0.423 | 0.739 |
| | 0.007 | 0.018 | 0.008 | 0.014 | 0.017 | 0.029 |
| A-Z | 0.136 | 0.282 | 0.195 | 0.315 | 0.439 | 0.714 |
| | 0.007 | 0.016 | 0.010 | 0.013 | 0.017 | 0.026 |
| (I+A)-Z | 0.136 | 0.290 | 0.171 | 0.320 | 0.431 | 0.732 |
| | 0.007 | 0.017 | 0.010 | 0.014 | 0.017 | 0.027 |
| I-A | 0.094 | 0.197 | 0.154 | 0.140 | 0.149 | 0.549 |
| | 0.006 | 0.013 | 0.009 | 0.008 | 0.008 | 0.022 |

NOTE.—The top number of each pair is the estimated distance (number of substitutions per nonsynonymous site); the bottom number is the estimated standard error of the distance. Both quantities were calculated using a modification of the technique described by Li, Wu, and Luo (1985). Symbols for taxa: I, *Ipomoea*; A, *Antirrhinum*; Z, *Zea*. (I+A)-Z represents the average of the distances between I and Z and between A and Z.

tween the *Ipomoea* and *Antirrhinum* lineages. These results suggest that the acceleration of substitution rates in *Ipomoea* occurred after the divergence of *Ipomoea* and *Antirrhinum*. Additional analyses in fact indicate that this acceleration occurred after the more recent divergence of *Ipomoea* and *Petunia* (unpublished data).

For other genes in the anthocyanin pathway, including CHS-D from *Ipomoea*, there is little evidence of rate heterogeneity among the lineages (table 2). In all cases but one, the estimated difference in nonsynonymous substitution rates was considerably less than twice its standard error. The one exception involved a nominally significant difference in the rate of evolution of F3H in the *Ipomoea* and *Antirrhinum* lineages. However, we suspect that this result is a false positive, since after the application of a Bonferroni correction, this difference is not significant at the $P = 0.01$ level. Even if this indication of rate heterogeneity is real, however, it does not represent a case of correlated acceleration of evolutionary rates with CHS-A, CHS-B, and CHS-C, since the rate for F3H is lower in the *Ipomoea* lineage.

Comparison of Nonsynonymous Substitution Rates Among Structural Genes

The absence of rate heterogeneity among lineages for genes in the anthocyanin pathway (excluding CHS-A, CHS-B, and CHS-C) suggests that replacement substitutions in these genes behave as a molecular clock. It is thus reasonable to compare the evolutionary rates of different genes in the pathway. In table 3, we present estimated genetic distances for the different pairs of species. Because for each gene the estimated nonsynonymous substitution rates are similar for the *Zea*-*Antirrhinum* and *Zea*-*Ipomoea* comparisons, we also calculated the average distance for both comparisons as the best estimate of evolutionary rates for divergences between monocots and dicots.

Pairwise comparison of genes using these best estimates indicates that over the period during which monocots and dicots have diverged, the three downstream enzymes in the pathway (DFR, ANS, and UF3GT) have evolved faster than the three upstream

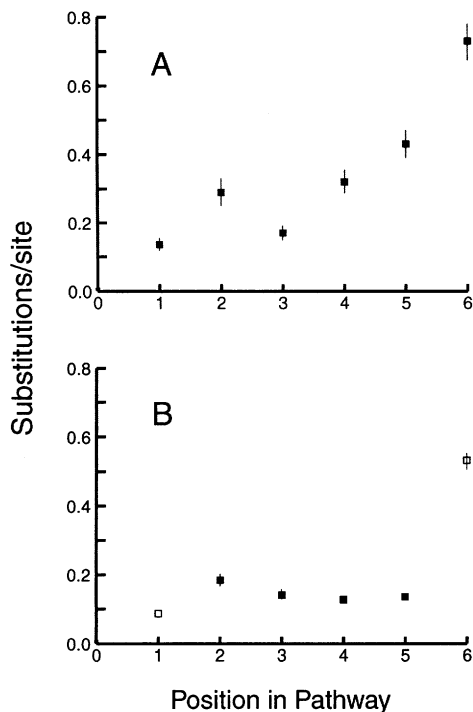


FIG. 2.—Correlation between evolutionary rates at nonsynonymous sites of anthocyanin pathway genes and positions in the pathway. Positions 1–6 correspond to CHS, CHI, F3H, DFR, ANS, and UF3GT, respectively (see fig. 1). Error bars represent 95% confidence intervals. *A*, Average of the number of substitutions per nonsynonymous site separating *Zea* and *Ipomoea* and the number separating *Zea* and *Antirrhinum*. *B*, Number of substitutions per nonsynonymous site separating *Ipomoea* and *Antirrhinum*. Open squares indicate values that are significantly different from values for all other genes.

enzymes (CHS, CHI, and F3H) (table 3). When compared pairwise statistically, all downstream genes exhibit a significantly greater divergence rate than all upstream genes at a probability level of $P < 0.0001$, except for the CHI–DFR comparison. Applying a conservative Bonferroni correction for the fact that nine comparisons are being made yields a probability level for each individual comparison of $P < 0.001$. Moreover, there is a significant positive rank correlation between position in the pathway and nonsynonymous substitution rate (fig. 2A; Kendall's $\tau = 0.87$, $P < 0.01$).

A similar, although weaker, correlation is exhibited when genetic distances between the two dicots *Antirrhinum* and *Ipomoea* are examined (fig. 2B). In this case, the most upstream enzyme, CHS, exhibits the slowest nonsynonymous substitution rate, which is significantly different from that of all other genes ($P < 0.01$ in all cases, with Bonferroni correction). In contrast, the most downstream enzyme, UF3GT, exhibits the fastest rate, which is significantly different from the rate of all other genes ($P < 0.01$ in all cases, with Bonferroni correction). The remaining enzymes in the pathway exhibit intermediate rates of nonsynonymous substitution that generally do not differ significantly from each other (only CHI and DFR differ significantly at the $P = 0.01$ level).

Table 4
Estimated Nonsynonymous Replacement Rates for Regions of R- and C-Family Regulatory Genes in Maize and Snapdragon

| REGION OF GENE | GENE | |
|-----------------------------|----------|----------|
| | R-family | C-family |
| Conserved domains | 0.288 | 0.326 |
| Variable domain | 0.015 | 0.025 |
| Entire gene | 0.041 | — |
| | 0.692 | — |
| | 0.019 | — |

NOTE.—The top number of each pair is the estimated distance (number of substitutions per nonsynonymous site); the bottom number is the estimated standard error of the distance. Both quantities were calculated using a modification of the technique described by Li, Wu, and Luo (1985). Distance values for the R-family genes are between *Lc* of *Zea* and *Delila* of *Antirrhinum*. Distance values for the C-family genes are between *Cl* of *Zea* and *Rosea* of *Antirrhinum*.

Comparison of Nonsynonymous Substitution Rates Among Regulatory Genes

Two classes of homologous transcription activators regulating structural genes in the anthocyanin pathway have been cloned for both *Zea* and *Antirrhinum*, allowing comparison of the rate of regulatory gene evolution with that of structural genes they regulate. The R-family, which consists of a small family of structurally related genes in *Zea* (van der Meer, Stuitje, and Mol 1993), including the *Lc* gene, is also represented in *Antirrhinum* by the *Delila* gene (Goodrich, Carpenter, and Coen 1992). These genes consist of two conserved domains and a more variable region. One conserved domain is an ~150-bp bHLH region that is presumed to be involved in DNA binding; the second is in an ~450-bp region of unknown function near the 5' origin of the coding region (Purugganan and Wessler 1994). For the entire gene, including the conserved regions, the estimated rate of nonsynonymous substitutions between *Lc* and *Delila* is comparable to that for UF3GT (table 4). However, as reported by Purugganan and Wessler (1994) in a general comparison of monocot and dicot copies of this regulatory gene, there is a substantial difference in nonsynonymous substitution rates between the conserved and variable regions (table 4). The substitution rate in the variable region is much higher than that in any of the anthocyanin structural genes, while the rate in the conserved regions falls in the middle of the range of rates exhibited by the structural genes.

A second set of anthocyanin regulatory genes identified in *Zea* and *Antirrhinum*, the C-family, belong to the myb class of transcription activators and include the *Cl* gene of *Zea* (van der Meer, Stuitje, and Mol 1993) and the *Rosea* gene of *Antirrhinum* (C. Martin, personal communication). In these genes there is also a conserved region, corresponding to a 312-bp DNA-binding domain, and a more variable region of approximately 340–520 bp (Jackson et al. 1991). Because there is no recognizable homology in the variable region, it is impossible to estimate rates of nonsynonymous substitution for either this region or the gene as a whole, indicating that these rates are much higher than those for any of the

Table 5
Estimated Synonymous Replacement Rates Between Pairs of Species for Anthocyanin Pathway Genes

| COM-PARI-SON ^a | GENE | | | | | |
|---------------------------|-------|-------|-------|-------|-------|-------|
| | CHS-D | CHI | F3H | DFR | ANS | UF3GT |
| I-Z | High | 1.273 | 1.465 | 2.727 | High | High |
| | | 0.230 | 0.221 | 1.362 | | |
| I-A | High | 1.451 | High | 1.488 | 1.893 | 1.827 |
| | | 0.319 | | 0.251 | 0.334 | 0.323 |

NOTE.—The top number of each pair is the estimated distance (number of substitutions per synonymous site); the bottom number is the estimated standard error of the distance. Both quantities were calculated using a modification of the technique described by Li, Wu, and Luo (1985). “High” indicates that substitutions were saturated and hence that replacement rates were not estimable but were higher than rates that are estimated.

^a Symbols for taxa are as in table 2.

structural genes. For the conserved region, however, the nonsynonymous substitution rate again lies within the range exhibited by the structural genes (table 4). The two regulatory genes thus exhibit similar patterns of rate variation.

Comparison of Synonymous Substitution Rates Among Structural Genes

Unfortunately, in many of the comparisons, synonymous substitutions reached saturation, precluding a valid estimate of synonymous substitution rates. It is thus not possible to compare the synonymous substitution rates for all six structural genes for any given pairwise comparison between taxa. However, sufficient information on synonymous rates is available to indicate that the pattern of differences between genes in synonymous substitution rates is not the same as the pattern of differences in non-synonymous rates (table 5). In particular, for comparisons both between *Ipomoea* and *Zea* and between *Ipomoea* and *Antirrhinum* (substitutions for all but one gene reached saturation in the *Antirrhinum*–*Zea* comparison), two patterns emerged. First, for genes for which the synonymous substitution rates could be estimated, there were no significant differences in synonymous substitution rates. For example, in the comparison between *Ipomoea* and *Zea*, CHI, F3H, and DFR do not differ when compared pairwise ($P > 0.30$ in all cases, without Bonferroni correction), even though two of the three pairs of genes exhibit significantly different nonsynonymous substitution rates. Similarly, in the comparison between *Ipomoea* and *Antirrhinum*, CHI, DFR, ANS, and UF3GT all exhibit similar synonymous rates that do not differ significantly ($P > 0.05$ in all cases, without Bonferroni correction), whereas the nonsynonymous substitution rate of UF3GT is substantially and significantly higher than those of the other genes (see above).

The second pattern exhibited by both the *Ipomoea*–*Zea* and the *Ipomoea*–*Antirrhinum* comparisons was that synonymous substitutions were saturated for CHS but not for intermediate genes. This saturation implies that synonymous substitution rates were too high to be estimated, and in particular higher than those of the intermediate genes. This pattern is the opposite of that seen

for nonsynonymous substitutions, for which CHS had the lowest rate in both comparisons.

Discussion

Lack of Coordinated Evolution

The accelerated rate of evolution of the common ancestor of CHS-A, CHS-B, and CHS-C in *Ipomoea* contrasts markedly with the absence of rate heterogeneity among lineages for any of the other structural genes in the anthocyanin pathway, including CHS-D. There were thus no coordinated accelerations (or decelerations) in these genes that accompanied rapid divergence of CHS-A, CHS-B, and CHS-C. In particular, this divergence apparently did not alter kinetic properties of the anthocyanin pathway sufficiently to require any form of compensation or adjustment by other pathway enzymes. Moreover, the absence of rate heterogeneity indicates that divergence of CHS-A, CHS-B, and CHS-C is not due to a general acceleration of the rate of evolution in *Ipomoea*. Rather, these genes seem to have evolved on a trajectory independent of the remaining pathway genes. This pattern supports Durbin et al.’s (1995) suggestion that these genes have acquired, or are in the process of acquiring, a new function, analogous to the divergence of CHS duplications to produce stilbene synthases (Tropf et al. 1994) and other related enzymes (Helariutta et al. 1996). The recent discovery of additional CHS genes in *Ipomoea*, specifically CHS-D and CHS-E (Fukuda-Tanaka et al. 1997; Tiffin, Miller, and Rausher 1998), that show much greater sequence similarity to CHS genes from other plant taxa indicates that CHS-A, CHS-B, and CHS-C may be free to diverge in function while other CHS gene copies provide any needed chalcone synthase function. Confirmation of functional divergence of these genes, however, awaits analysis of their enzymatic properties.

More generally, the lack of rate heterogeneity among lineages for the other pathway genes is consistent with the view that evolutionary change occurs independently in the different anthocyanin genes. If either common responses of several pathway genes to environmental change or compensatory adjustment by some pathway genes to evolutionary change in other pathway genes were occurring over long timescales, we would expect to see coordinated accelerations or decelerations within some lineages, which would also produce rate heterogeneity among lineages. It is possible, of course, that coordinated rate changes occurred over much shorter timescales than are represented by the lineages analyzed here, but that these “averaged out” to produce apparent rate homogeneity over longer periods among the lineages analyzed. Assessment of this possibility will require analysis of rate heterogeneity on a finer scale, e.g., among species or genera rather than among exemplars of different plant families.

Rate Variation Among Anthocyanin Structural Genes

Variation among genes in substitution rates is ubiquitous, and much of this variation has been attributed to variation in selective constraint (see *Introduction*). Not

surprisingly, our results indicate that anthocyanin pathway genes also exhibit differences in nonsynonymous substitution rates. More interestingly, our analysis has revealed a striking pattern to this variation: downstream pathway genes have evolved more rapidly than upstream genes. This pattern suggests that upstream enzymes are more constrained than downstream enzymes, a difference which may arise because upstream enzymes are more extensively associated with pathway branches and thus may have a greater likelihood than the downstream enzymes of controlling pathway flux (Atkinson 1977, chapter 6; Crabtree and Newsholme 1987). The limited evidence available on rate limitation in the anthocyanin pathway is consistent with this interpretation. For example, several investigators have demonstrated a close correlation between CHS activity and flavonoid accumulation, but not between accumulation and activity of other pathway genes (Knogge, Schmelzer, and Weissenböck 1986; Knogge and Weissenböck 1986; Peter et al. 1991). In *Ipomoea purpurea*, heterozygotes carrying one functional copy of CHS-D and one transposon-inactivated copy produce less pigment than homozygotes carrying two functional copies (S. Iida, personal communication), suggesting that this enzyme is the major rate-limiting step in the pathway in this species. In addition, while CHS activity does not appear to limit anthocyanin production in *Antirrhinum*, another upstream enzyme, F3H, does (Martin 1993). Alternatively, greater constraint on upstream enzymes may arise because, unlike the downstream enzymes, they generally catalyze intermediate steps in the production of flavonoids as well as anthocyanins (fig. 1). Consequently, amino acid substitutions that have slightly detrimental effects on enzyme function may be expected to have larger deleterious fitness consequences in upstream enzymes, since they affect more end products. In either case, a smaller fraction of mutations slightly reducing enzyme function are expected to be effectively neutral in upstream enzymes, producing greater selective constraint. Because these types of differences in constraint are likely to arise in any complex metabolic web, we suggest that this pattern may be expected in many branched biosynthetic pathways: upstream enzymes above major branch points will tend to be more constrained than downstream enzymes below the major branch points.

An alternative explanation for the observed trend is that the observed differences in rates of enzyme evolution are due to differences in mutation rates at the corresponding genes. If this hypothesis were true, downstream genes would be expected to have higher synonymous substitution rates than upstream genes, for which we found no evidence. Moreover, this explanation seems to us unlikely a priori, because there is no reason to expect downstream genes to have higher mutation rates than upstream genes.

Relative Evolutionary Rates of Anthocyanin Structural and Regulatory Genes

An additional suggestive pattern revealed by our analysis is that, of the genes involved in the anthocyanin pathway, structural genes evolve more slowly than reg-

ulatory genes. Interpretation of this pattern is complicated by the observation that many regulatory genes, including those examined here, exhibit strong differentiation among their domains in evolutionary rates. Some regions are highly conserved, while others are very variable across taxa (Scott, Tankum, and Hartzell 1989; Tucker and Lundrigan 1993; Whitfield, Lovell-Badge, and Goodfellow 1993; Purugganan and Wessler 1994). In contrast, the structural genes of the anthocyanin pathway do not exhibit any obvious differentiation into highly conserved and highly variable domains.

Purugganan and Wessler (1994) found that rates of substitution in the variable region of R-family genes are similar for synonymous and nonsynonymous substitutions. Two interpretations of this pattern are possible. One is that the variable region is functionally relatively unconstrained and that most substitutions in this region are effectively neutral. This interpretation implies a simple explanation for the more rapid overall rate of amino acid substitutions in anthocyanin regulatory genes compared with the structural genes: nonfunctional domains constitute a greater proportion of the coding region for regulatory genes than for structural genes. Under this interpretation, our analysis indicates that the functional portions of regulatory genes and structural genes evolve at roughly similar rates.

The second interpretation is that similar rates of synonymous and nonsynonymous substitution in the variable regions reflect a history of positive selection for amino acid substitutions in this region. Under this interpretation, all regions of the regulatory genes are functional. The low substitution rate in the conserved domains then represents the effect of strong constraint in these regions, consistent with their function in DNA binding. In contrast, at least some portions of the variable regions of these regulatory genes presumably represent sites of interaction with other regulatory factors, and the high substitution rate in these regions then presumably reflects positive selection for altered regulatory activity. Under this interpretation, our analysis indicates that the functional regions of regulatory genes evolve more rapidly, on average, than do those of structural genes.

Although the available evidence precludes distinguishing definitively between these interpretations, we believe that the positive-selection interpretation is more likely for two reasons. First, given the set of functions R- and C-family genes must perform, it is difficult to imagine that a large fraction of the sequence of these genes is essentially nonfunctional. In addition to binding to specific DNA sequences, the products of these positive-regulatory genes must interact with the polymerase complex, as well as any other coacting transcription factors (Lewin 1994, chapter 30). While facilitating these interactions may be the major function of the N-terminal conserved domain in the R-family genes (Goff, Cone, and Chandler 1992), no similar non-DNA-binding conserved domain has been identified in plant C-family genes, suggesting that these functions are carried out by the variable region. The observation that in vertebrate *myb* genes, as well as maize *C1* alleles, sequences out-

side the binding domain function in transcriptional activation, hormone binding, and negative regulation (Bishop et al. 1991; Goff, Cone, and Fromm 1991; Takahashi et al. 1995) supports this suggestion. Second, evolutionary modification of anthocyanin distribution within plants, particularly within flowers, is frequent in many taxa (e.g., Austin 1997). Because such evolutionary lability in an ecologically crucial character is likely achieved to a great extent by evolutionary modification of anthocyanin gene regulation (e.g., Quattrocchio 1994, chapter 6), we would expect frequent selection for flower color modification to result in natural selection producing evolutionary change in anthocyanin regulatory genes. We thus believe that the overall greater rate of amino acid substitution in the regulatory genes observed here likely reflects a greater role of regulatory genes in the adaptive evolution of the anthocyanin pathway. Further examination of this suggestion may help to clarify the relative roles of structural versus regulatory genes in phenotypic change (King and Wilson 1975; Dickenson 1991; Doebley 1993; Gould 1997, chapter 11).

Conclusions

Although these suggestions regarding the causes of the patterns revealed in our study are admittedly speculative, it is clear that these patterns exist. Moreover, these patterns were only revealed by examining evolutionary trends not of individual genes, but of a set of genes that function together in a common biosynthetic pathway. Understanding the cause of such patterns may ultimately help to reveal rules that govern the evolution of complex metabolic webs (Clark 1991) and, in doing so, may bring us much closer to understanding the evolution of ecologically important traits (Doebley 1993).

Acknowledgments

We thank two anonymous reviewers for comments on the original version of the manuscript. Cathie Martin kindly provided several unpublished sequences for anthocyanin genes in *Antirrhinum majus*. This work was supported by National Science Foundation grant DEB 9318919 to M.D.R., by Duke University School of Arts and Sciences, which provided postdoctoral support for R.E.M., and by an NIH training grant awarded to the Duke University Genetics Program, which supported P.T.

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BARBARA A. SCHAAL, reviewing editor

Accepted November 3, 1998