Control of expression patterns of anthocyanin structural genes by two loci in the common morning glory

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Two loci producing white flowers have been identified in the common morning glory, *Ipomoea purpurea*. At the W locus, ww individuals produce flowers with white corollas and pigmented rays, while at the A locus, aa individuals produce flowers with white corollas, and a^*a^* individuals produce variegated corollas. To determine whether these two loci correspond to regulatory or structural genes, six structural genes required for anthocyanin synthesis were cloned and their expression pattern was examined in genotypes with white and pigmented flowers. In ww flower buds, expression of all six structural genes was greatly reduced or eliminated, indicating that the W locus encodes a regulatory gene. By contrast, genotype at the A locus did not affect expression of any of the structural genes, suggesting that the A locus may encode one of those structural genes. An evolutionary comparison of structural gene regulation in maize, snapdragons, petunias, and morning glories suggests that regulatory control of the anthocyanin pathway is evolutionarily labile.

INTRODUCTION

The flavonoid pathway, and in particular its anthocyanin branch, has served as a paradigm for understanding gene regulation in plants (Dooner et al., 1991; van der Meer et al., 1993; Holton and Cornish, 1995). In addition, recent evolutionary studies suggest that this pathway may also serve as a model for elucidating the evolution of plant metabolic pathways at the molecular level (Koes et al., 1994; Shirley, 1996). In both of these areas, however, investigation has concentrated primarily on three species: maize (*Zea mays*), petunia (*Petunia hybrida*), and snapdragon (*Antirrhinum majus*), primarily because until recently it was only in these three species that most or all of the six known structural genes required for anthocyanin production have been cloned and characterized. While investigations of these three species have already led to some important generalizations, such as the conservation of some regulatory genes across a wide range of taxa (Goodrich et al., 1992; Quattrocchio et al., 1993), further generalizations regarding both regulation and evolution will be more robust if these processes can be examined in additional species. For this reason, we have undertaken an attempt to characterize the anthocyanin pathway structural genes in a species of morning glory, *Ipomoea purpurea* (Convolvulaceae).

Like its close relative, the Japanese morning glory *I. nil*, *I. purpurea* exhibits a variety of genetic variants that differ in flower color (Ennos and Clegg, 1983; Epperson and

Clegg, 1987, 1988, 1992). In particular, mutations at two loci produce white flowers rather than the usual blue or pink flowers. At the W ("white") locus, individuals that are homozygous WW produce normally pigmented flowers, while ww individuals produce flowers with white corollas and pigmented rays and Ww individuals produce lightly pigmented flowers. There are no apparent pleiotropic effects of the w allele on pigmentation in other tissues (e.g., no effects on stem or leaf pigmentation). At the A ("albino") locus, the flowers of AA individuals are normally pigmented, while those of aa plants have a completely white corolla without pigmented rays. Heterozygous Aa individuals produce lightly pigmented flowers. By contrast with the W locus, homozygous aa individuals exhibit pleiotropic effects on stem pigmentation: AA individuals have red to brown stems, whereas aa individuals have green stems. In addition, a third, unstable allele, a^*, has been characterized at the A locus. Individuals that are a^*a^* exhibit variegation of flowers and stems. The rates of mutation to and from a^* suggest that the unstable nature of the pigment pattern associated with the a^* allele results from the presence of a transposable element (Epperson and Clegg, 1992).

The lack of pleiotropic effects of the W locus, along with the production of pigmentation in rays of ww individuals, suggests that this locus may regulate the expression of one or more of the structural genes needed for pigment production. In these properties, this locus is similar to the *Delila* locus of *Antirrhinum* (Goodrich et al., 1992) and the An2 and An4 loci of *Petunia* (Quattrocchio et al., 1993), regula-

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tory loci apparently homologous to the *R* and *C1* family of transcriptional activators of maize. By contrast, the complete elimination of pigmentation from both flowers and some non-floral tissue suggests that the *A* locus may correspond to a structural gene that is expressed in several tissues. A similar phenotypic pattern is seen in *I. nil* due to inactivation of the *DFR* gene by a transposable element (Inagaki et al., 1994,1996).

In this report, we attempt to determine whether the *W* and *A* loci correspond to regulatory or structural loci. To do so, we describe the cloning and characterization of the genes chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanin synthase (*ANS*), and UDP glucose: flavonoid 3-O-glucosyltransferase (*UF3GT*) from a cDNA library of *I. purpurea* floral-bud tissue. We also describe the expression patterns of these genes, which indicates that the *W* locus regulates the expression of all six structural genes in flower buds, whereas the *A* locus does not affect the expression pattern of these genes. Finally, we discuss the implications of these results for the evolution of regulation of the anthocyanin pathway in angiosperms.

MATERIALS AND METHODS

Isolation of structural genes. Structural genes were isolated from a cDNA library constructed from the distal half of flower buds of *I. purpurea* using the ZAP cDNA synthesis kit (Stratagene). Our general strategy for isolation was to use PCR to obtain a small fragment of a gene, then use this fragment as a probe of the library to isolate the entire cDNA for the gene. Degenerate PCR primers were designed based on conserved sequences in the appropriate genes, as determined by comparing sequences reported in GenBank, and are listed in Table 1. PCR reactions were performed under one of two conditions, depending on the particular gene (Table 1). Fragments obtained were sequenced to verify homology to the gene sought and hence appropriateness for use as a probe. For each gene, approximately 300,000 plaques were probed and one to ten highly hybridizing clones were isolated and sequenced. For isolating the *UF3GT* gene, we probed the cDNA library with a fragment of the homologous snapdragon gene. This fragment was produced using the PCR primers indicated in Table 1 with the entire snapdragon gene (supplied by Cathie Martin) as a template.

Analysis of expression patterns. We examined the expression patterns of the six structural genes in the distal portion of *I. purpurea* flower buds using RNA (Northern) blotting. Total RNA was extracted from the distal half of flower buds using a Promega isolation kit. For each gene, a 500-1000 bp fragment was used as a probe for its transcript. To make the blots, approximately 15 μg of total RNA was subjected to denaturing electrophoresis (Sambrook et al., 1989), then transferred to a nylon membrane (Hybond N$^+$, Amersham). Probing was conducted at 65°C, with washing at 60°C, and probed membranes were read using a phosphorimager. We also probed blots with a fragment of the *I. purpurea* 18S ribosomal RNA gene as a control for RNA loading.

Strains examined were from our collection of inbred lines and included: line J (pigmented); line N (*aa*, white flowers); line W (*a*a**, with mostly white flowers and minimal flecking); and line P (*ww*). For the *ww* genotype, bud tips were dissected and the pigmented ray tissue removed before RNA extraction. As a control, pigmented (*WW*) buds were also dissected and extracted in a similar way. For the remaining genotypes, including *WW*, RNA was extracted from undissected bud tips.

Table 1. Accession numbers and PCR primers and conditions used to obtain gene fragments used as probes

Gene	Accession No.	PCR Primers	PCR Conditions[a]
CHS	U74082	ATGATGTA[TC]CA[AG]CA[AG]GGNTG GCNGG[AG]TGNGC[ACG]ATCCA[AG]AA	1
CHI	AF028238	TT[AC]CTNG[GC]NGGNGCNGGN ATNGANGCNCCNGGNGG[AG]AA	2
F3H	U74081	GTNTCNAG[TC]CA[TC]CTNCA[AG]GG TT[TC]TG[AG]TCCAT[AG]TCNAC[AG]CA	2
DFR	AF028601	GA[AG]AA[TC]GA[AG]GTNAT[ACT]AA[AG]CC GG[ATG]AT[ATG]AT[AG]CT[ATG]AT[AG]AA[AG]TC	1
ANS	AF028602	GA[AG]TGGGA[AG]GA[TC]TA[TC]TT[TC]TT TCNGT[AG]TGNGC[TC]TCNACNCC	2
UF3GT	AF028237	AAAGCCCTACCACAAGGATT ATCTCTAACCCTAACCCCAA	1

[a] Condition 1: 35 cycles of 94°C for 1 min., 37°C for 1 min., 72°C for 30 sec.
Condition 2: 15 cycles of 94°C for 1 min., 37°C for 1 min., 72°C for 30 sec., followed by 25 cycles of 94°C for 1 min., 50°C for 1 min., 72°C for 30 sec.

RESULTS

Isolation of structural genes. For the genes *CHI, F3H, DFR*, and *ANS* we were able to obtain complete coding sequences, as indicated by the presence of the expected start codon and a poly-A tail. We obtained a truncated *CHS* gene, the sequence of which was identical to that of the *CHS-E* gene subsequently reported by Fukada-Tanaka et al. (1997), except that it lacked 249 bp at the 3' end of the sequence, though it included a poly-A tail. To date we have obtained only a partial sequence for *UF3GT*. This sequence is 1512 bp long and contains a poly-A tail, but lacks approximately 115 bp from the 5' end, compared to the snapdragon *UF3GT* sequence. Sequences of these genes have been deposited in GenBank and accession numbers are listed in Table 1.

Generally, the nucleotide and inferred amino acid sequences of the genes we have isolated exhibit high similarity to the sequences of the corresponding petunia genes, and very high similarity (greater than 95%) to genes isolated from the closely related *I. nil* (Table 2). Moreover, this high similarity exists along the entire length of each gene. In the case of *CHI*, where the percent identity with *Petunia* is lower than 70%, comparisons of the sequences among other species (e.g., petunia *vs.* snapdragon, proportion identity of amino acid sequences = 0.627, *vs.* 0.653 for *I. purpurea* and snapdragon) also exhibit similarly reduced

homology, indicating more rapid divergence of this gene. As expected, the proportion identity is lower, but still appreciable, when *I. purpurea* is compared with the more distantly related snapdragon and maize.

For *UF3GT*, no comparison is possible with either *I. nil* or petunia, since cloning of this gene has not been reported for these species. Homology of our clone is lower with snapdragon and maize *UF3GT* than in similar comparisons with the other genes; but the sequence identity between snapdragon and maize *UF3GT* (0.434 for nucleotide sequence, 0.305 for amino acid sequence) is similar to that seen between *I. purpurea* and maize, as would be expected because the snapdragon and *I. purpurea* lineages have been separated the same amount of time from the maize lineage. As in the case of *CHI*, these results suggest that *UF3GT* has an elevated rate of evolutionary change, compared to the other anthocyanin structural genes.

Table 2. Degree of homology between isolated *Ipomoea purpurea* sequence and homologous sequence from other species

Gene	Ipomoea nil	Petunia	Antirrhinum	Zea
CHS	0.959	0.856	0.845	0.801
	0.955	0.766	0.717	0.713
CHI	NA	0.661	0.613	0.569
		0.688	0.653	0.618
F3H	0.978	0.814	0.675	0.735
	0.979	0.757	0.656	0.700
DFR	0.953	0.717	0.629	0.567
	0.964	0.735	0.663	0.590
ANS	NA	0.770	0.740	0.508
		0.725	0.702	0.572
UF3GT	NA	NA	0.423	0.270
			0.528	0.451

Top number indicates fraction identity in amino-acid composition, while bottom number indicates fraction identity in nucleotide composition. Comparisons are with *CHS-E* and *DFR-B* genes from *Ipomoea nil, CHS-A* gene from *Petunia*, and *CHS C2* gene from *Zea*. NA: sequence not available for comparison with *I. purpurea* sequence. Accession numbers for compared sequences: (1) *Ipomoea nil CHS*–AB001819, *F3H*–D83041, *DFR*–AB006792; (2) Petunia *CHS*–X14591, *CHI*–Y00852, *F3H*–X60512, *DFR*–X79723; (3) *Antirrhinum CHS*–X03710, *CHI*–M68326, *DFR*–X15536; (4) *Zea CHS*–X60205, *CHI*–Z22760, *F3H*–U04434, *ANS*–X55314, *UF3GT*–X13502. *Antirrhinum* sequences for *F3H, ANS*, and *UF3GT* were provided by Cathie Martin. *Petunia ANS* and *Zea DFR* sequences were obtained from the literature.
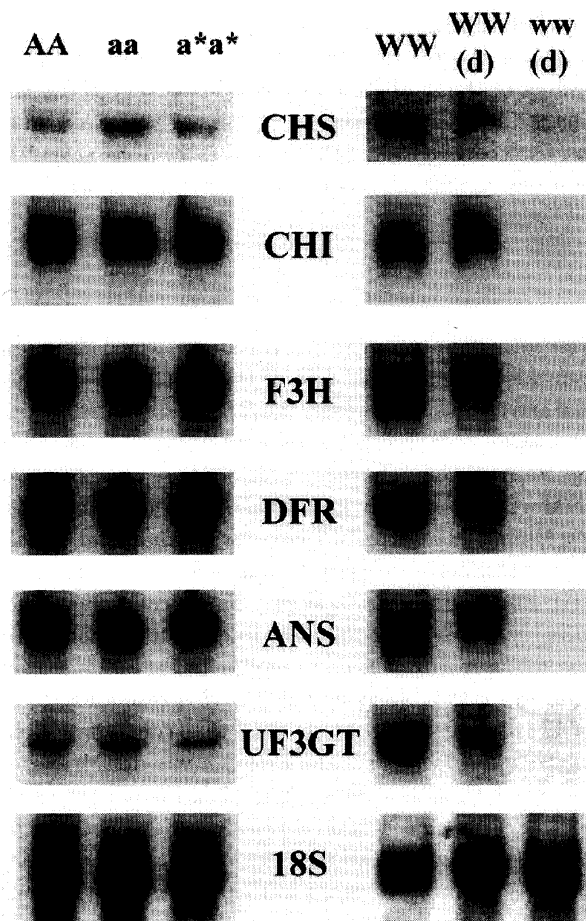

Fig. 1. RNA gel blot analysis of anthocyanin structural genes of *Ipomoea purpurea*. Total RNA (15 μg) from the distal half of flower buds were hybridized with the indicated cDNA probe as described by Sambrook *et al.* 1989. Genotypes corresponding to individual lanes are indicated at top. WW(d) and ww(d) lanes correspond to RNA extracted from bud tips with the pigmented ray tissue dissected out, while all other lanes represent RNA from entire bud tips. 18S RNA fragment is control for RNA loading.

Analysis of expression patterns. Expression of each of the six structural genes was comparable for the *AA*, *aa*, and *a*a** genotypes (Fig. 1). By contrast, all six genes had greatly reduced expression in the *ww* genotype. *CHS* and *CHI* exhibited faint expression, while for the other genes no transcript was detected. The general intensity of *CHS* expression in all genotypes appears lower than that of the other genes. We suspect that the reason for this reduced intensity is that the primary *CHS* transcript expressed in corolla tissue (corresponding to the distal portion of the bud we have examined) is *CHS-D*, while the probe we used is *CHS-E*. While both genes are expressed in corolla tissue, *CHS-D* predominates, constituting approximately 85% of the *CHS* transcripts (Fukada-Tanaka et al., 1997). Moreover, the nucleotide sequence identity between *CHS-D* and *CHS-E* is only approximately 80%, which could reduce the specificity of the *CHS-E* probe to the predominant *CHS-D* transcript, thus weakening the signal. Fukada-Tanaka et al. (1997) report such signal weakening in their probing of their cDNA library. What is not clear is whether the faint expression of *CHS* detected in *ww* tissue reflects lack of an effect of the *w* allele on expression of one of the *CHS* genes, or simply represents a baseline "leakiness" in the expression of *CHS*. In either case, however, it is clear that expression of at least one of the *CHS* genes is affected by the *W* locus.

DISCUSSION

The current model of anthocyanin structural gene regulation acknowledges the role of two classes of transcriptional activators. One class, the *R* family, consists of genes that encode *myc*-like proteins containing a basic Helix-Loop-Helix domain characteristic of other transcriptional activators such as *Max* and *MyoD1* (van der Meer et al., 1993; Holton and Cornish, 1995; Purugganan and Wessler, 1994). This class includes the maize *R* and *B*, snapdragon *Delila*, and petunia *An4* genes. The second class, the *C* family, consists of genes encoding proteins containing an *myb*-like binding domain similar to that of eukaryotic regulatory genes such as the *c-myb* proto-oncogene of vertebrates and the *glabrous* gene that controls trichome formation in *Arabidopsis* and *Petunia* (van der Meer et al., 1993; Paz-Ares et al., 1987). This class includes the maize *C1* and *Pl*, the snapdragon *myb305* and the petunia *An2* genes. In maize, these two classes of genes appear to regulate all structural genes in the anthocyanin pathway, while in petunia and snapdragon they regulate only the three or four most downstream genes (e.g., *F3H*, *DFR*, *ANS*, and *UF3GT*; Holton and Cornish, 1995). This difference in regulation has led Koes et al. (1994) to outline a simple model of the evolution of the anthocyanin pathway in angiosperms involving at least one episode of evolutionary loss of regulatory control over *CHS* and *CHI*, in addition to loss of regulatory control of *F3H* in the petunia lineage.

The pattern of structural gene expression, as controlled by the *W* locus in *I. purpurea*, suggests that one or more aspects of this model are incomplete. In particular, the elimination of expression of all six structural genes in *ww* individuals indicates that the *W* locus is a major regulator of anthocyanin production in flowers of *I. purpurea*. However, the set of genes regulated differs from that controlled by known regulatory genes in petunia and snapdragon. Consequently, either the *W* locus represents a new class of anthocyanin regulatory genes or the control of structural gene expression by *myb*- and *myc*-like genes is more evolutionarily labile than previously envisioned.

Which of these possibilites is correct will only be settled definitively by cloning and characterizing the *W* locus. Nevertheless, several considerations suggest that the latter possibility is more likely. First, all anthocyanin regulatory genes that have been cloned were first characterized genetically as color mutants (van der Meer et al., 1993). It seems unlikely to us that if there are indeed additional classes of regulators, all known mutants should turn out to belong to only two classes of homologous regulators. Second, while the pattern of regulation exhibited by the *W* locus differs from that exhibited by regulatory genes in other dicots, it is similar to that seen in maize. Thus, no novel type of regulatory control, and hence no novel class of regulatory genes, need be invoked to explain the pattern exhibited by the *W* locus.

If, as we believe, this conclusion is correct, the pattern of gene regulation by the *W* locus indicates that the set of genes controlled by anthocyanin regulators is evolutionarily more labile than previously suspected. The model of Koes et al. (1994) suggests that coordinated regulation of the six major anthocyanin genes evolved early in the angiosperm lineage to ensure simultaneous expression of all genes needed for pigment production in the flower. It further envisions that in one or more dicot lineages, genes upstream in the biosynthetic pathway, particularly *CHS* and *CHI*, were removed from this common regulatory network. In particular, the minimally parsimonious tree under this model postulates a single loss of regulation of these two genes in the common lineage of petunias and snapdragons (Fig. 2c, ignoring the *Ipomoea* lineage). However, because this model was based on regulatory patterns observed in just three species (maize, petunia, and snapdragon), it can not be distinguished from an equally parsimonious alternative model in which absence of regulatory control of these two genes was the ancestral state (Fig. 2a, ignoring the *Ipomoea* lineage).

With the addition of *Ipomoea* to the phylogeny, it is still impossible to distinguish whether or not coordinate regulation of all six genes is ancestral in angiosperms. However, regardless of ancestral state, all minimally parsimonious phylogenetic trees require at least two evolutionary changes in the regulation of *CHS* and *CHI* (Fig. 2). Consequently, adding one additional species to the known pat-
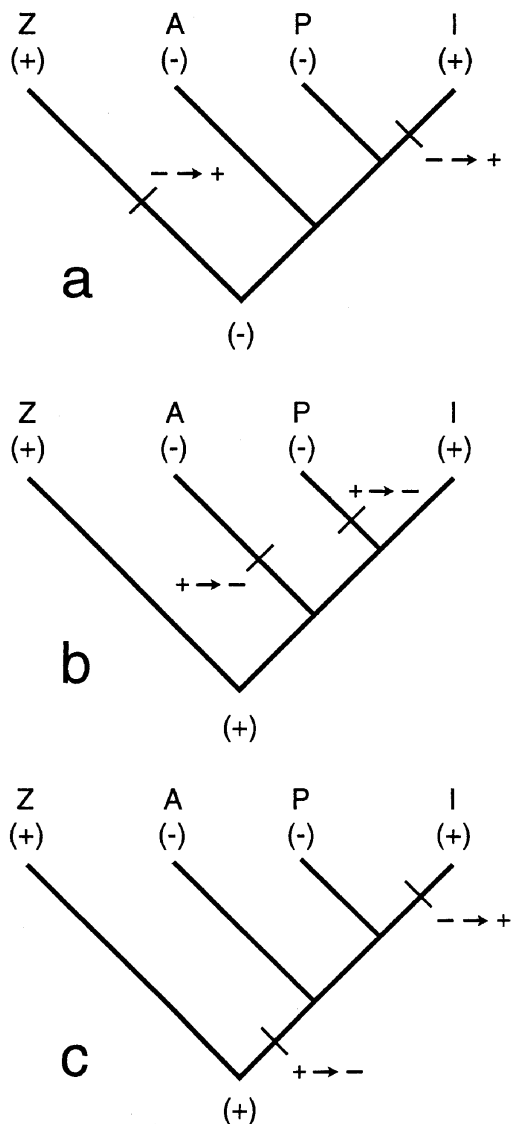
Fig. 2. Evolutionary transitions in regulatory control of *CHI* and *CHS* by known or suspected *myb*- and *myc*-like genes superimposed on known phylogeny (Chase et al., 1993) of the four species in which control has been examined: *Zea mays* (Z), *Antirrhinum majus* (A), *Petunia hybrida* (P), and *Ipomoea purpurea* (I). The three maximally parsimonious arrangements of transitions are shown. (+) and (−) refer to control and lack of control, respectively.

terns of regulation of *myb*- and *myc*-like genes indicates that alteration of regulatory control of these two genes could not have occurred just once in angiosperm evolution. Given a minimum of two evolutionary changes in regulation of these two genes when only four taxa are examined, we suggest that examination of the pattern of regulation in other taxa will reveal even greater evolutionary lability of regulation of these and other anthocyanin genes.

Unlike the *W* locus, the *A* locus appears to play no role in the regulation of the six structural genes examined, suggesting that *A* may encode a structural gene rather than a

regulatory gene. The phenotypes of *aa* and *a*a** individuals are very similar to those reported by Inagaki et al. (1994, 1996) for the Japanese morning glory, *I. nil*, having white (*aa*) or variegated (*a*a**) flowers and lacking pigmentation in the stem. In the closely related *I. nil*, these phenotypes result from the insertion of the transposable element *Tpn1* into an intron in the *DFR-B* gene. Thus, the structural gene *DFR* seems a likely candidate for the *A* locus in *I. purpurea*. Although absence of an apparent difference in *DFR* transcript length in the the *AA*, *aa*, and *a*a** genotypes may seem to mitigate against this inference, cases are known in which transposon splicing yields non-functional transcripts similar in length to functional transcripts produced by gene copies lacking transposon insertions (Wessler et al., 1987). Nevertheless, we can not rule out the possibility that *A* regulates either structural genes that produce precursors of the anthocyanin pathway, such as phenylalanine ammonia lyase (*PAL*) (van der Meer et al., 1993), or anthocyanin processing steps such as those involving vacuolar transport (Marrs et al., 1995).

A major unanswered issue in evolutionary biology is ascertaining the relative roles of structural and regulatory genes in causing phenotypic change. Recent comparative work in animals suggests that much evolutionary change in morphology is accomplished primarily by evolutionary change at regulatory loci (Akam et al., 1994; Gerhart and Kirschner, 1997). Whether this is also true of evolution in important plant characters, including flower color, is currently unknown. In this context, however, the results presented here demonstrate that naturally occurring mutations in regulatory genes have the potential to serve as the raw material for adaptive flower color change. The genus *Ipomoea* consists of more than 600 species (Austin and Huáman, 1996) of vines and shrubs that exhibit a tremendous diversity of flower colors, including many white-flowered species, and thus offers an opportunity to determine whether structural or regulatory genes play the primary role in evolutionary diversification of flower color. Our cloning of the six major structural genes of the anthocyanin pathway in this genus should facilitate determination of the relative importance of regulatory and structural genes in the evolutionary diversification of this important plant character.

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