

The Genetic Basis of a Flower Color Polymorphism in the Common Morning Glory (*Ipomoea purpurea*)

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Abstract

The common morning glory (*Ipomoea purpurea*) is highly polymorphic for flower color. Part of this phenotypic variation is due to allelic variation at the *P* locus. This locus determines whether flowers will be purple or pink, where purple is dominant to pink. We have determined that the anthocyanin biosynthetic gene *flavonoid 3'-hydroxylase* (*f3'h*) corresponds to the *P* locus. In the pink allele of *f3'h* there is a large insertion in the third exon, which results in the production of a truncated transcript. This shortened transcript produces a nonfunctional F3'H enzyme, resulting in the production of pink flowers rather than purple. In addition, we describe a polymerase chain reaction (PCR)-based assay that can be used to determine the genotype of a plant at this locus.

Natural populations of the common morning glory (*Ipomoea purpurea*) in southeastern North America are highly polymorphic for flower color, with approximately 20 hue-intensity combinations having been identified (Ennos and Clegg 1983; Epperson and Clegg 1988). All of this variation is accounted for by allelic variation at four Mendelian loci (Epperson and Clegg 1988). Two of these loci, *A* and *W*, control whether pigments are expressed in floral tissue. At each of these loci, pigment and nonpigment alleles are codominant, therefore heterozygotes are easily recognizable phenotypically, which has facilitated experimental analysis of the evolutionary forces responsible for maintenance of polymorphisms at these loci (e.g., Fry and Rausher 1997; Rausher and Fry 1993).

In contrast, at the other two loci influencing flower color, *P* and *I*, one allele is completely dominant (Ennos and Clegg 1983). This dominance impedes examination of the evolutionary processes affecting these loci because it makes it difficult, without performing extensive crosses, to distinguish between heterozygote and dominant homozygote individuals when examining pollinator interactions and estimating reproductive success in natural populations (e.g., Brown and Clegg 1984). In addition, performing multi-generation crosses to generate seeds of known genotype, as has been done extensively in the experimental analysis of the *A* and *W* loci (Coberly and Rausher 2003; Fry and Rausher 1997; Rausher and Fry 1993; Subramaniam and Rausher

2000), is also difficult because of the additional crosses required to distinguish heterozygote and dominant homozygote parental plants. In an effort to alleviate these problems, we have identified the gene corresponding to the *P* locus and have characterized at the molecular level the allelic variants at this locus. In doing so we have developed a simple polymerase chain reaction (PCR)-based assay that can be used to determine the genotype of any individual at this locus. Moreover, identification of the gene corresponding to the *P* locus will permit the employment of standard population genetic analyses of patterns of sequence variation in this gene to learn about the historical forces that have acted on the polymorphism at this locus (e.g., MacDonald and Kreitman 1991; Tajima 1989).

The genotype at the *P* locus determines whether flower color is purple or red-pink. Individuals that are either *PP* or *Pp* have purple flowers, whereas individuals that are *pp* are pink or red, depending on the genotype at the *I* locus. This difference in pigment color is due to a difference in degree of hydroxylation of the anthocyanidin core of the pigment: in purple-flowered genotypes, all anthocyanins are derivatives of cyanidin, which is hydroxylated at the 3' position, whereas in the pink-flowered genotype, all anthocyanins are derivatives of pelargonidin, which is unhydroxylated at the 3' position (Saito et al. 1995, 1996; Schoen et al. 1984).

The anthocyanin biosynthetic pathway in plants is well characterized and serves as a model system for examining

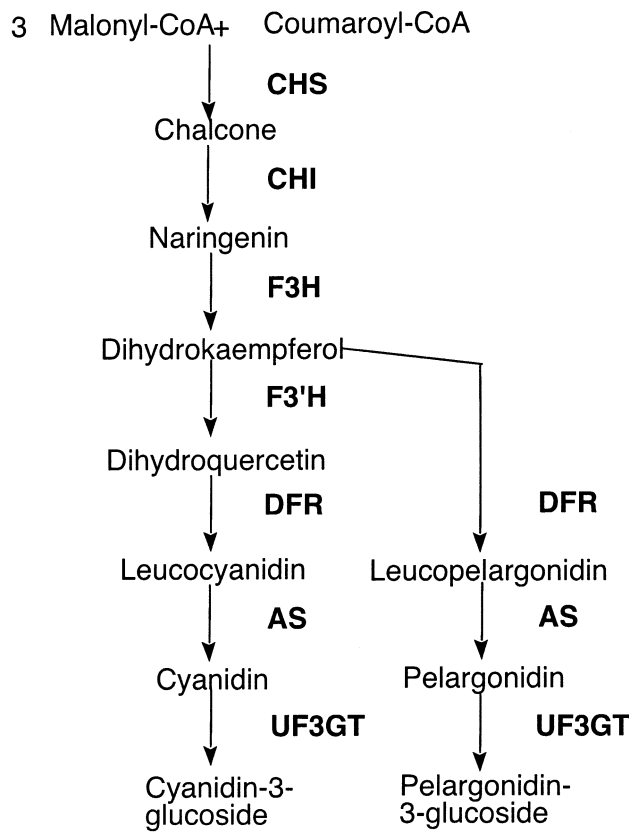


Figure 1. Anthocyanin biosynthetic pathway. Enzyme abbreviations: AS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavonone 3-hydroxylase; F3'H, flavanoid 3'-hydroxylase; UF3GT, UDP glucose flavonoid 3-glucosyltransferase

transcriptional regulation of plant genes (Dooner et al. 1991; Harbourn 1967; Holton and Cornish 1995; van der Meer et al. 1992). Molecular and genetic analyses of the pathway in the taxonomically disparate model organisms maize (*Zea mays*), snapdragon (*Antirrhinum*), *Petunia*, and *Arabidopsis* indicate that the pathway consists of the same set of seven core enzymes in most, if not all, angiosperms (Dooner et al. 1991; Holton and Cornish 1995; Mol et al. 1998). Examination of the topology of the pathway (Figure 1) suggests two ways by which a mutation could result in the production of pelargonidin rather than cyanidin. The most obvious way is by inactivation of the gene coding for the enzyme F3'H. This gene was first characterized from *Petunia* by Brugliera et al. (1999). The gene produced by this enzyme is a member of the cytochrome P-450 oxidase multigene family and is directly responsible for hydroxylation of anthocyanidin precursors at the 3' position. Mutational inactivation of this enzyme would thus prevent hydroxylation, causing the production of pelargonidin rather than cyanidin.

Alternatively, production of pelargonidin rather than cyanidin could be achieved by a change in specificity of

either of the downstream enzymes dihydroflavonol reductase (DFR) or anthocyanidin synthase (AS). In many plant species, these enzymes utilize both hydroxylated and nonhydroxylated precursors to produce anthocyanidins, and are thus substrate generalists (Holton and Cornish 1995). In contrast, in other species these enzymes are substrate specialists. For example, *Arabidopsis thaliana* produces only cyanidin-based anthocyanins because its DFR enzyme is unable to use the unhydroxylated dihydrokaempferol (DHK) as a substrate (Dong et al. 2001). Similarly petunia cannot produce pelargonidin-based anthocyanins because the petunia DFR does not use DHK as a substrate (Forkmann and Ruhnau 1987). Moreover, in petunia a single amino acid change in DFR alters substrate specificity dramatically (Johnson et al. 2001). In the purple (*P*) genotypes of *I. purpurea*, DFR and ANS are both able to metabolize hydroxylated cyanidin precursors. Because at least one of these enzymes is unaffected by the mutation at the *P* locus, this enzyme must be a generalist, since all *P* locus genotypes produce anthocyanins. A mutation, corresponding to the *p* allele, in the other enzyme that converts it to a specialist on DHK would then result in the production of pelargonidin and pink flowers rather than cyanidin and purple flowers.

Morita et al. (1999a,b) report, without documentation, that a similar pink phenotype in *I. purpurea* is due to a mutation in the *f3'h* gene. In this article we demonstrate that the *P* locus in *I. purpurea* corresponds to the gene encoding the enzyme F3'H, and that production of pink flowers by *pp* individuals results from inactivation of this gene rather than alteration of the substrate specificity of AS or DFR. Inactivation of F3'H results from a 400-bp insertion in the 3' end of the gene. Because of this insertion, PCR products of the third (last) exon are recognizably different in size on agarose gels, which provides a simple assay for determining an individual's genotype.

Materials and Methods

Study System

Ipomoea purpurea Roth (Convolvulaceae) is a weedy annual vine found in disturbed habitats throughout the southeastern United States and Mexico. Purple and pink individuals used in this study were obtained from collections made by one of us (MDR) from a population near Durham, North Carolina. Inbred lines propagated by single-seed descent were created from the original collections, and plants used in our analyses represent the 11th generation of inbreeding.

Pigment Identification

Total flavonoids were extracted from flowers by soaking corolla tissue in 2 N HCl for 1 h to overnight, then boiling for 1 h. Anthocyanidins were extracted from the HCl with

a few drops of isoamyl alcohol. This extract was dried to a powder in a speed-vac and resuspended in acidic methanol. Anthocyanidins were separated using thin-layer chromatography on cellulose-coated glass plates in either forestal (acetic acid:HCl:water, 30:3:10) or "isopropanol" (water:isopropanol:HCl, 5:6:1) (Harbourne 1967). Identities of anthocyanidins were determined by comparison with cyanidin and pelargonidin standards run at the same time.

Cloning of *f3'h* from the Pink-Flowered Genotype

The gene *f3'h* was previously sequenced from *I. purpurea* by Morita et al. (1999a,b), who provided us with the cDNA sequence. The genomic sequence of this gene and the corresponding sequence in pink-flowered plants was obtained by PCR of overlapping fragments using the primer pairs listed in Table 1 (genomic DNA was obtained using the DNEasy kit, Qiagen). Conditions for PCR were 35 cycles of 95°C for 30 s, 50°C for 1 min 30 s, and 70°C for 1 min, followed by a 7 min extension at 72°C. A 15 µl reaction contained 8.94 µl water, 1.5 µl 10× buffer (containing 15 mM MgCl₂), 0.6 µl of each 10 µM primer, 0.3 µl 10 mM dNTPs, 3 µl DNA, and 0.06 µl *Taq* (AmpliTaq DNA polymerase, Applied Biosystems). PCR fragments were cloned (TA-cloning kit, Invitrogen) and sequencing reactions were performed using BigDye terminators (Perkin-Elmer). Sequences were read using an ABI 3700 automated sequencer.

To obtain full-length clones of the purple and pink alleles of *f3'h* for complementation analysis, RNA was extracted (Promega Total RNA kit) from floral buds collected 1 day prior to opening and stored at -80°C. Total RNA was reverse transcribed with M-MLV reverse transcriptase (Invitrogen) and the resulting single-stranded cDNA was used in PCR reactions to obtain full-length clones. Primers for this reaction, which were designed from the sequences of the DNA fragments containing the 5' and 3' ends of the gene, are listed in Table 1. Conditions for PCR were the same as above, with the addition of 1 M GC-Melt (Advantage-GC PCR kit, Clontech). The PCR products were cloned and sequenced as above.

Cosegregation Analysis

Cosegregation analyses were performed by crossing *PP* plants with *pp* plants and allowing the F₁ progeny to self-pollinate. A total of 40 F₂ progeny were scored for flower color and for genotype at the *f3'h* locus. To score genotypes, genomic DNA was extracted from leaves and used in PCR reactions with primers N3 and N4. This primer pair amplifies a 509 bp fragment from the *P* allele of *f3'h* and a 910 bp fragment from the *p* allele. Fragments were scored on 1% agarose gels. A subset of the PCR products were cloned and sequenced to verify that they corresponded to *f3'h*. In addition, RNA was extracted from a subset of the scored F₂ individuals, converted to cDNA, and used in PCR reactions with primers N1 and N4 to clone and subsequently sequence the resulting fragments.

Table 1. PCR primers used in cloning *f3'h*

| Primer name | Primer sequence (5' → 3') |
|-------------|----------------------------------|
| P15 | AAGACCAAGTATGGCTACC |
| P30 | ATCTCCTCCACCATTGACTT |
| P23 | CACCTCTGTGCATCTCTTCTC |
| N2 | CTCATCCCAGAGCATATTCTACGCC |
| N3 | CTCAGGGTTGCGAGATCAATGGC |
| N4 | GGTCGAAAGCTAGACCTCTGTTTAAAGTG |
| N1 | CGTTGTCCAAAGATGTGGACTTCTTGAGCACC |

Overlapping regions from genomic DNA were amplified with the following pairs: P15-P30, P23-N2, and N3-N4.

Full-length cDNA clones were obtained using P15 and N4.

Complementation Analysis

Full-length clones of *f3'h* from both purple- and pink-flowered plants were subcloned into the vector pBI 1.4t containing the constitutive CaMV 35S promoter. These vectors were then transformed into the *Agrobacterium tumefaciens* strain GV3101 using a freeze-thaw protocol (An et al. 1988). Transformation of *A. thaliana* *tt7* mutants, which are homozygous for a loss-of-function mutation in *f3'h* (Schoenbohm et al. 2000), was achieved using the standard dip protocol (Clough and Bent 1998). Transformants were selected by growing on MS medium containing 50 mg/L kanamycin. Anthocyanin phenotypes of T₂ plants were scored by growing on sand under high light and extracting anthocyanins. To confirm the success of the transformations, T₂ plants were transplanted to soil and allowed to mature. DNA was extracted from these plants and scored for the presence of the transgene by PCR and sequencing. In addition, RNA was extracted from seedlings grown in sand and high light to confirm expression of the transgene.

Results

Chromatographic Analysis

Thin-layer chromatographic analysis of floral pigments indicates that pink flowers produce anthocyanins derived from pelargonidin, whereas purple flowers produce anthocyanins derived from cyanidin. This analysis confirms the previous results of Schoen et al. (1984) and indicates that the pink-purple variation we examined is likely caused by allelic variation in the same gene they examined.

Sequences of *f3'h* Alleles

The DNA sequence of the intact purple allele of *f3'h*, corresponding to *P* (henceforth designated *F3'h*), is 3801 bp long from start to stop codon and contains three exons and two introns (Figure 2). In contrast, in the pink allele, corresponding to *p* (*f3'h-GG*), there is an approximately 400 bp insertion in the third intron that begins 480 bp 3' of the boundary between the second intron and third exon. Within this insertion is an approximately 25 bp stretch of repeated adenine nucleotides which proved impossible to sequence

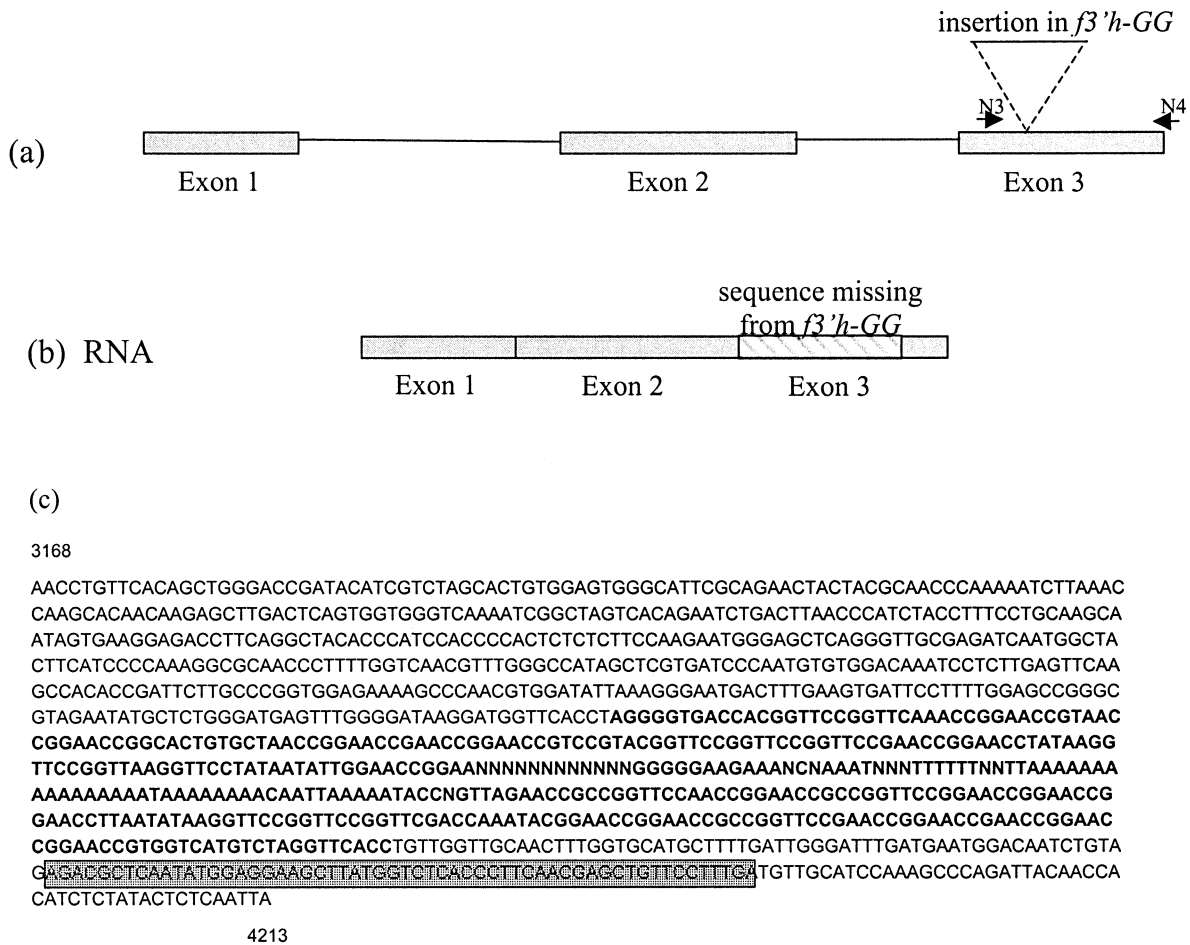


Figure 2. Structure of the $\beta 3' b$ gene in *I. purpurea*. **(a)** The DNA sequence of the $\beta 3' b-GG$ shows a 400 bp insertion relative to the $F 3' b$. The primers used for the genotype assay are shown labeled N3 and N4. **(b)** This insertion results in an mRNA molecule lacking the first 537 bp of exon 3, represented by the striped box. **(c)** The DNA sequence from $\beta 3' b-GG$ corresponding to exon 3 of $F 3' b$. The region of inserted DNA is shown in bold and the shaded box indicates the sequence corresponding to the translated region of exon 3 in $\beta 3' b-GG$.

accurately. This repetitive sequence suggests that this insertion may be a remnant of a retrotransposon insertion, though a BLAST search of GenBank using the insertion sequence revealed no similarity to any documented transposon sequences. It is likely that this is the same element found by Morita et al. (1999a). The insertion in $\beta 3' b-GG$ appears to result in missplicing of mRNA and a resultant frameshift, producing a transcript that lacks the first 538 bp of the third exon and encounters an early stop codon (Figure 2). Although the molecular mechanism that results in this missplicing is unclear, it may be due to a change in RNA secondary structure caused by the large insert. These sequences have been deposited in GenBank (accession nos. $F 3' b$, AY333419; $\beta 3' b-GG$, AY333420).

Cosegregation Analysis

Analysis of F_2 progeny indicates that $F 3' b$ and $\beta 3' b-GG$ cosegregate with flower color. PCR fragments of the 3' end

of $\beta 3' b$ using genomic DNA as template yielded PCR products of two lengths. The longer fragment (910 bp) was obtained from all pink-flowered individuals examined, whereas the shorter fragment (509 bp) was obtained from all purple-flowered homozygous individuals. Both fragments were obtained from purple-flowered heterozygotes. Cloning and sequencing a subsample of these fragments confirmed that they corresponded to the expected portion of the $\beta 3' b$ gene.

Analogous PCR fragments obtained from floral cDNA again yielded PCR products of two lengths. The shorter fragment (322 bp) was obtained from all pink-flowered individuals examined, while the longer fragment (860 bp) was obtained from all homozygous purple-flowered individuals. As with fragments from genomic DNA, cloning and sequencing a subsample of these fragments confirmed they corresponded to the expected 3' end of the $\beta 3' b$ gene.

In addition to individuals from populations in North Carolina, we amplified this same fragment of DNA from

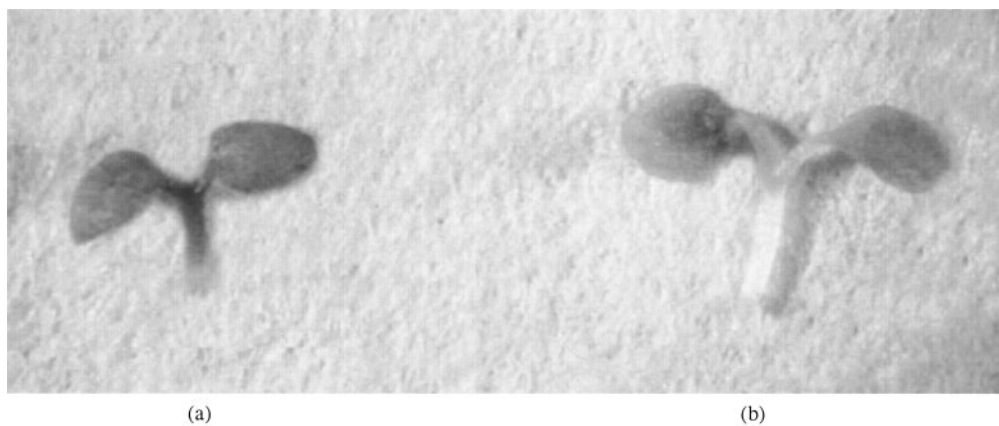


Figure 3. *Arabidopsis* seedlings growing in conditions that induce expression of anthocyanins. **(a)** T₂ transformants of #7 plants containing *F3'b*. **(b)** T₂ transformants of #7 plants containing *f3'b-GG*.

three independent pink-flowered inbred lines from a population in Wakinsville, GA (collected and generously supplied by S. Chang). A band corresponding to a 910 bp fragment was obtained from all individuals. This suggests that the same mutation is likely responsible for the pink phenotype in these populations and that this mutation is geographically widespread in *I. purpurea* and accounts for many, if not all, pink-flowered individuals in this species.

Complementation Analysis

When subjected to light and nutrient stress, *A. thaliana* seedlings containing a functional copy of *f3'b* produce anthocyanins in the seedlings, rendering them dark purple. In contrast, the *A. thaliana* mutant #7 lacks F3'H function due to a knockout mutation in the single copy of the gene coding for F3'H (Schoenbohm et al. 2000; Shirley et al. 1995). Homozygotes for this mutant fail to synthesize anthocyanins and thus produce green cotyledons under stress. This difference in phenotype, due to the presence or absence of a functional copy of *f3'b*, served as the basis of a complementation test for the functionality of *f3'b* from pink *I. purpurea* (Dong et al. 2001).

Transformation of #7 with *F3'b* from purple *I. purpurea* served as a control for the complementation assay. Numerous T₂ transformants were isolated and all exhibited purple cotyledons when grown under high light on sand lacking nutrients (Figure 3a). Cloning and sequencing of PCR fragments obtained from genomic DNA from several of these transformants confirmed the integration of *I. purpurea f3'b* into the *A. thaliana* genome.

In contrast, although two independent transformations of #7 with *f3'b-GG* from pink *I. purpurea* again produced numerous T₂ transformants, none produced purple cotyledons under stress (Figure 3b). We confirmed that several individuals from each transformation had integrated a copy of *I. purpurea F3'b* into their genome by cloning and sequencing PCR products obtained from genomic DNA. In

addition, we confirmed that the inserted gene is expressed by cloning and sequencing PCR products obtained from cDNA from seedlings. The failure of these transformants to produce anthocyanic pigmentation thus appears to be due to lack of functionality of *f3'b-GG*.

Discussion

Properties of Anthocyanin Pathway Genes

Our results demonstrate that a single mutation is responsible for the difference between the purple and pink flowers used in this study of *I. purpurea*. The mutation is a large insertion in the third exon of the gene that encodes F3'H. This insertion appears to be large enough to interfere with proper mRNA splicing and produces a transcript, and a presumptive enzyme, that is shortened by 538 bp and 196 amino acids, respectively. As would be expected, our complementation test indicates that this truncated enzyme is incapable of converting dihydrokaempferol to dihydroquercetin. The failure to produce dihydroquercetin in turn means that the downstream enzymes DFR, AS, and UF3GT do not have the substrates available to produce purple cyanidin-based anthocyanins. Instead, they produce the pink pelargonidin-based anthocyanins.

Our results also imply that in *I. purpurea* these downstream enzymes are capable of using both DHK and DHQ as substrates and are thus substrate generalists, since these enzymes metabolize pigment precursors in both purple- and pink-flowered genotypes. Despite this capability, purple-flowered genotypes carrying a functional *f3'b* gene produce no detectable pelargonidin-based anthocyanins. Thus, in these genotypes, the entire metabolic flux of the anthocyanin pathway is directed through the cyanidin branch of the pathway. Presumably this pattern of flux arises because the kinetic affinity of the functional F3'H enzyme for DHK is much greater than that of the competing enzyme, DFR.

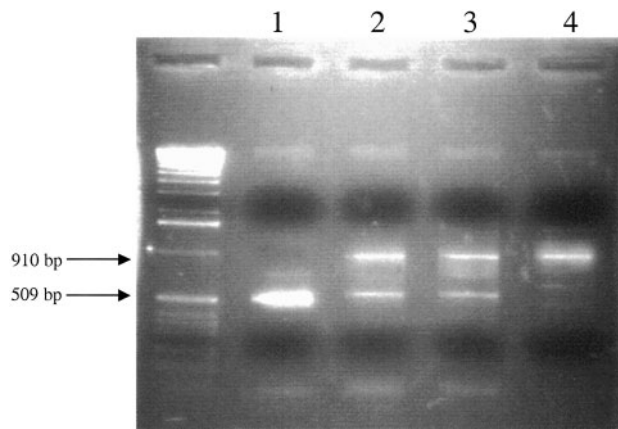


Figure 4. PCR-based genotype assay of $\beta^{\prime}b$. Lane 1 contains PCR product from a purple homozygote; lanes 2 and 3, purple heterozygotes; lane 4, a pink homozygote. The far left lane contains a 1 kb ladder

Transposon Activity

The insertion in $\beta^{\prime}b$ -GG has properties suggesting that it may be the remnant of retrotransposon activity. First, its size is within the range exhibited by known retrotransposons. Second, the poly-A repeat is characteristic of non-LTR retrotransposons (Feschotte et al. 2002). In addition, while there are no terminal repeats, there are four repeated sequences within the insertion, two direct and two inverted, each approximately 20 bp long. However, to our knowledge there have been no pink-flowered individuals described that would indicate active transposition at this locus, for example, purple sectoring of pink flowers resulting from somatic reversions.

The possibility that this mutation appears to be the result of transposon activity reinforces the pattern that the majority of flower color variants examined in *I. purpurea*, as well as in the closely related *I. nil*, appear to have been caused by transposition into genes of the anthocyanin pathway (Clegg and Durbin 2000; Durbin et al. 2001; Hoshino et al. 2001). However, the insertion in $\beta^{\prime}b$ -GG exhibits no sequence similarity to the transposons that have been detected in other anthocyanin genes in *Ipomoea*. This observation supports the notion that a variety of transposons are abundant and active in *Ipomoea* genomes. It is also interesting that all but one of the flower variants examined to date are due to mutations in structural, rather than regulatory, genes (Habu et al. 1998; Inagaki et al. 1994; Johzuka-Hisatomi et al. 1999), the exception being a mutation at the *W* locus in *I. purpurea* (Tiffin et al. 1998).

Genotype Assay

The marked difference in the lengths of genomic PCR fragments obtained from the 3' region of the $\beta^{\prime}b$ gene in *I. purpurea* provides the basis for a simple assay that can be used to discriminate between purple-flowered homozygotes and

heterozygotes. In particular, PCR of genomic DNA from heterozygotes using primers N3 and N4 consistently amplifies fragments of both sizes, whereas similar PCR analysis of homozygotes produces only the shorter fragments (Figure 4). Because this assay can be performed on genomic DNA collected from cotyledons or early leaves, it permits genotype determination of flower color weeks before flowering begins. Such early discrimination of heterozygosity should prove useful in selecting plants for specific crosses, as may be required for analyses of the effects of natural selection on variants at this locus.

Acknowledgments

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References

- An G, Ebert PR, Mitra A, and Ha SB, 1988. Binary vectors. In: Plant molecular biology manual (Gelvin SB and Schilperoort RA, eds). Dordrecht, The Netherlands: Kluwer Academic Press. A3:1–19.
- Brown BA and Clegg MT, 1984. Influence of flower color polymorphism on genetic transmission in a natural population of the common morning glory, *Ipomoea purpurea*. *Evolution* 38:796–803.
- Brugliera F, Barri-Rewell G, Holton TA, and Mason JG, 1999. Isolation and characterization of a flavonoid 3'-hydroxylase cDNA clone corresponding to the Ht1 locus of *Petunia hybrida*. *Plant J.* 19:441–451.
- Clegg MT and Durbin ML, 2000. Flower color variation: a model for the experimental study of evolution. *Proc Natl Acad Sci USA* 97:7016–7023.
- Clough SJ and Bent AF, 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743.
- Coberly LC and Rausher MD, 2003. Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. *Mol Ecol* 12:1113–1124.
- Dong X, Braun EL, and Grotewold E, 2001. Functional conservation of plant secondary metabolic enzymes revealed by complementation of *Arabidopsis* flavonoid mutants with maize genes. *Plant Phys* 127:46–57.
- Dooner HK, Robbins TP, and Jorgensen RA, 1991. Genetic and developmental control of anthocyanin biosynthesis. *Annu Rev Genet* 25:173–199.
- Durbin ML, Denton AL, and Clegg MT, 2001. Dynamics of mobile element activity in chalcone synthase loci in the common morning glory (*Ipomoea purpurea*). *Proc Natl Acad Sci USA* 98:5084–5089.
- Ennos RA and Clegg MT, 1983. Flower color variation in the morning glory, *Ipomoea purpurea*. *J Hered* 74:247–250.
- Epperson BK and Clegg MT, 1988. Genetics of flower color polymorphism in the common morning glory (*Ipomoea purpurea*). *J Hered* 79: 64–68.
- Feschotte C, Jiang N, and Wessler SR, 2002. Plant transposable elements: where genetics meets genomics. *Nat Rev Genet* 3:329–341.
- Forkmann G and Ruhnau B, 1987. Distinct substrate specificity of dihydroflavonol 4-reductase from flowers of *Petunia hybrida*. *Z Naturforsch* 42:1146–1148.

- Fry JD and Rausher MD, 1997. Selection on a floral color polymorphism in the tall morning glory (*Ipomoea purpurea* L.): transmission success of the alleles through pollen. *Evolution* 51:66–78.
- Habu Y, Hisatomi Y, and Iida S, 1998. Molecular characterization of the mutable *flaked* allele for flower color variegation in the common morning glory. *Plant J* 16:371–376.
- Harbourne JB, 1967. Comparative biochemistry of the flavonoids. London: Academic Press.
- Holton TA and Cornish EC, 1995. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7:1071–1083.
- Hoshino A, Johzuka-Hisatomi Y, and Iida S, 2001. Gene duplication and mobile elements in the morning glories. *Gene* 265:1–10.
- Inagaki Y, Hisatomi Y, Suzuki T, Kasahara K, and Iida S, 1994. Isolation of a suppressor-mutator enhancer-like transposable element, Tpn1, from Japanese morning glory bearing variegated flowers. *Plant Cell* 6: 375–383.
- Johnson ET, Ryu S, Yi H, Shin B, Cheong H, and Choi G, 2001. Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. *Plant J* 25:325–333.
- Johzuka-Hisatomi Y, Hoahino A, Mori T, Habu Y, and Iida S, 1999. Characterization of the chalcone synthase genes expressed in flowers of the common and Japanese morning glories. *Genes Genet Syst* 74: 141–147.
- McDonald JH and Kreitman M, 1991. Adaptive protein evolution at the ADH locus in *Drosophila*. *Nature* 351:652–654.
- Mol J, Grotewold E, and Koes R, 1998. How genes paint flowers and seeds. *Trends Plant Sci* 3:212–217.
- Morita Y, Hoshino A, Tanaka Y, and Iida S, 1999a. Mutations in the *flavonoid 3'-hydroxylase* genes of the Japanese and common morning glories [abstract]. *Genes Genet Syst* 74:333.
- Morita Y, Hoshino A, Tanaka Y, Kusumi T, Saito N, and Iida S, 1999b. Identification of the *magenta* mutations for flower pigmentation in the Japanese and common morning glories [abstract]. *Plant Cell Physiol* 40(suppl):S124.
- Rausher MD and Fry JD, 1993. Effects of a locus affecting floral pigmentation in *Ipomoea purpurea* on female fitness components. *Genetics* 134:1237–1247.
- Saito N, Tatsuzawa F, Yoda K, Yokoi M, Kasahara K, Iida S, Shigihara A, and Honda T, 1995. Acylated cyanidin glycosides in the violet-blue flowers of *Ipomoea purpurea*. *Phytochemistry* 40:1283–1289.
- Saito N, Tatsuzawa F, Yokoi M, Kasahara K, Iida S, Shigihara A, and Honda T, 1996. Acylated pelargonidin glycosides in red-purple flowers of *Ipomoea purpurea*. *Phytochemistry* 43:1365–1370.
- Schoen DJ, Giannasi DE, Ennos RA, and Clegg MT, 1984. Stem color and pleiotropy of genes determining flower color in the common morning glory. *J Hered* 75:113–116.
- Schoenbohm C, Martens S, Eder C, Forkmann G, and Weisshaar B, 2000. Identification of the *Arabidopsis thaliana flavonoid 3'-hydroxylase* gene and functional expression of the encoded P450 enzyme. *Biol Chem* 381: 749–753.
- Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, and Goodman HM, 1995. Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J* 8:659–671.
- Subramaniam B and Rausher MD, 2000. Balancing selection on a floral polymorphism. *Evolution* 54:691–695.
- Tajima F, 1989. Statistical method for testing the neutral mutation hypothesis in DNA polymorphism. *Genetics* 123:585–595.
- Tiffin P, Miller RE, and Rausher MD, 1998. Control of expression patterns of anthocyanin structural genes by two loci in the common morning glory. *Genes Genet Syst* 73:105–110.
- van der Meer IM, Stam M, Tunen AJ, Mol JNM, and Stuitje AR, 1992. Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. *Plant Cell* 4:253–262.

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