The Plant Cell, Vol. 7, 1071–1083, July 1995

Genetics and Biochemistry of Anthocyanin Biosynthesis

Timothy A. Holton[1] and Edwina C. Cornish

Florigene Proprietary Ltd., 16 Gipps Street, Collingwood, Victoria 3066, Australia

INTRODUCTION

Flavonoids represent a large class of secondary plant metabolites, of which anthocyanins are the most conspicuous class, due to the wide range of colors resulting from their synthesis. Anthocyanins are important to many diverse functions within plants. Synthesis of anthocyanins in petals is undoubtedly intended to attract pollinators, whereas anthocyanin synthesis in seeds and fruits may aid in seed dispersal. Anthocyanins and other flavonoids can also be important as feeding deterrents and as protection against damage from UV irradiation. The existence of such a diverse range of functions and types of anthocyanins raises questions about how these compounds are synthesized and how their synthesis is regulated.

The study of the genetics of anthocyanin synthesis began last century with Mendel's work on flower color in peas. Since that time, there have been periods of intensive study into the genetics and biochemistry of pigment production in a number of different species. In the early studies, genetic loci were correlated with easily observable color changes. After the structures of anthocyanins and other flavonoids were determined, it was possible to correlate single genes with particular structural alterations of anthocyanins or with the presence or absence of particular flavonoids. Mutations in anthocyanin genes have been studied for many years because they are easily identified and because they generally have no deleterious effect on plant growth and development. In most cases, mutations affecting different steps of the anthocyanin biosynthesis pathway were isolated and characterized well before their function was identified or the corresponding gene was isolated. More recently, many genes involved in the biosynthesis of anthocyanin pigments have been isolated and characterized using recombinant DNA technologies.

Three species have been particularly important for elucidating the anthocyanin biosynthetic pathway and for isolating genes controlling the biosynthesis of flavonoids: maize (*Zea mays*), snapdragon (*Antirrhinum majus*), and petunia (*Petunia hybrida*). Petunia has more recently become the organism of choice for isolating flavonoid biosynthetic genes and studying their interactions and regulation. At least 35 genes are known to affect flower color in petunia (Wiering and de Vlaming, 1984). Because this field of research has been reviewed fairly extensively in the past (Dooner et al., 1991; van Tunen and Mol, 1991; Gerats and Martin, 1992), in this review we concentrate on the more recent developments in gene isolation and characterization. A review of the genetics of flavonoid biosynthesis in other species was recently covered by Forkmann (1993).

The characterization of genetically defined mutations has enabled the order of many reactions in anthocyanin synthesis and their modification to be elucidated. Some reactions have been postulated only on the basis of genetic studies and have not yet been demonstrated in vitro. Chemico-genetic studies have been very important in determining the enzymatic steps involved in anthocyanin biosynthesis and modification. The generation of transposon-tagged mutations and the subsequent cloning of the transposons provided a relatively straightforward means of isolating many genes from maize (Wienand et al., 1990) and snapdragon (Martin et al., 1991). However, a number of genes in the pathway have not been amenable to transposon tagging.

Anthocyanin biosynthetic genes have been isolated using a range of methodologies, including protein purification, transposon tagging, differential screening, and polymerase chain reaction (PCR) amplification. Functions of isolated anthocyanin genes can be confirmed by restriction fragment length polymorphism (RFLP) mapping, complementation, or expression in heterologous systems. Reverse genetics has also been used recently to identify gene function; this requires a well-defined pathway to correlate phenotype with gene function. Once a gene has been isolated from one species, it is usually a straightforward task to isolate the homologous gene from other species by using the original clone as a molecular probe.

ANTHOCYANIN BIOSYNTHETIC PATHWAY

The anthocyanin biosynthetic pathway is well established (Mol et al., 1989; Forkmann, 1991). A generalized anthocyanin biosynthetic pathway is shown in Figure 1. Although the biosynthetic pathways in maize, snapdragon, and petunia share a majority of common reactions, there are some important differences between the types of anthocyanins produced by each species. One major difference is that petunia does not normally produce pelargonidin pigments, whereas snapdragon and maize are incapable of producing delphinidin

[1]To whom correspondence should be addressed.

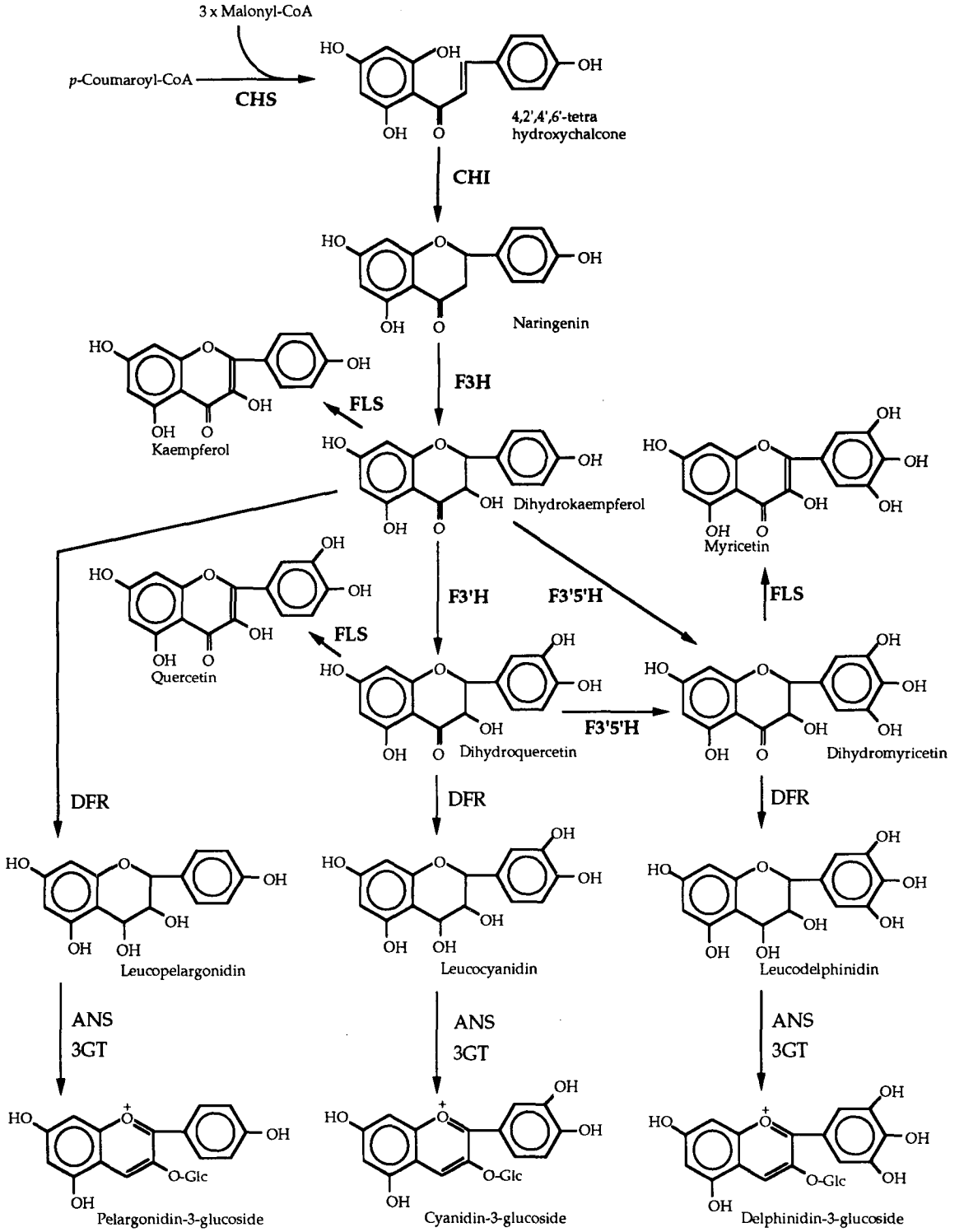

Figure 1. Anthocyanin and Flavonol Biosynthetic Pathway.

pigments. The extent of modification of the anthocyanins also varies among the three species. The reasons for these differences are discussed in the following outline of the enzymes and genes involved in anthocyanin biosynthesis.

The precursors for the synthesis of all flavonoids, including anthocyanins, are malonyl-CoA and *p*-coumaroyl-CoA. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with *p*-coumaroyl-CoA to yield tetrahydroxychalcone. Chalcone isomerase (CHI) then catalyzes the stereospecific isomerization of the yellow-colored tetrahydroxychalcone to the colorless naringenin. Naringenin is converted to dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3H). DHK can subsequently be hydroxylated by flavonoid 3′-hydroxylase (F3′H) to produce dihydroquercetin (DHQ) or by flavonoid 3′,5′-hydroxylase (F3′5′H) to produce dihydromyricetin (DHM). F3′5′H can also convert DHQ to DHM.

At least three enzymes are required for converting the colorless dihydroflavonols (DHK, DHQ, and DHM) to anthocyanins. The first of these enzymatic conversions is the reduction of dihydroflavonols to flavan-3,4-*cis*-diols (leucoanthocyanidins) by dihydroflavonol 4-reductase (DFR). Further oxidation, dehydration, and glycosylation of the different leucoanthocyanidins produce the corresponding brick-red pelargonidin, red cyanidin, and blue delphinidin pigments. Anthocyanidin 3-glucosides may be modified further in many species by glycosylation, methylation, and acylation, as illustrated in Figure 2. There

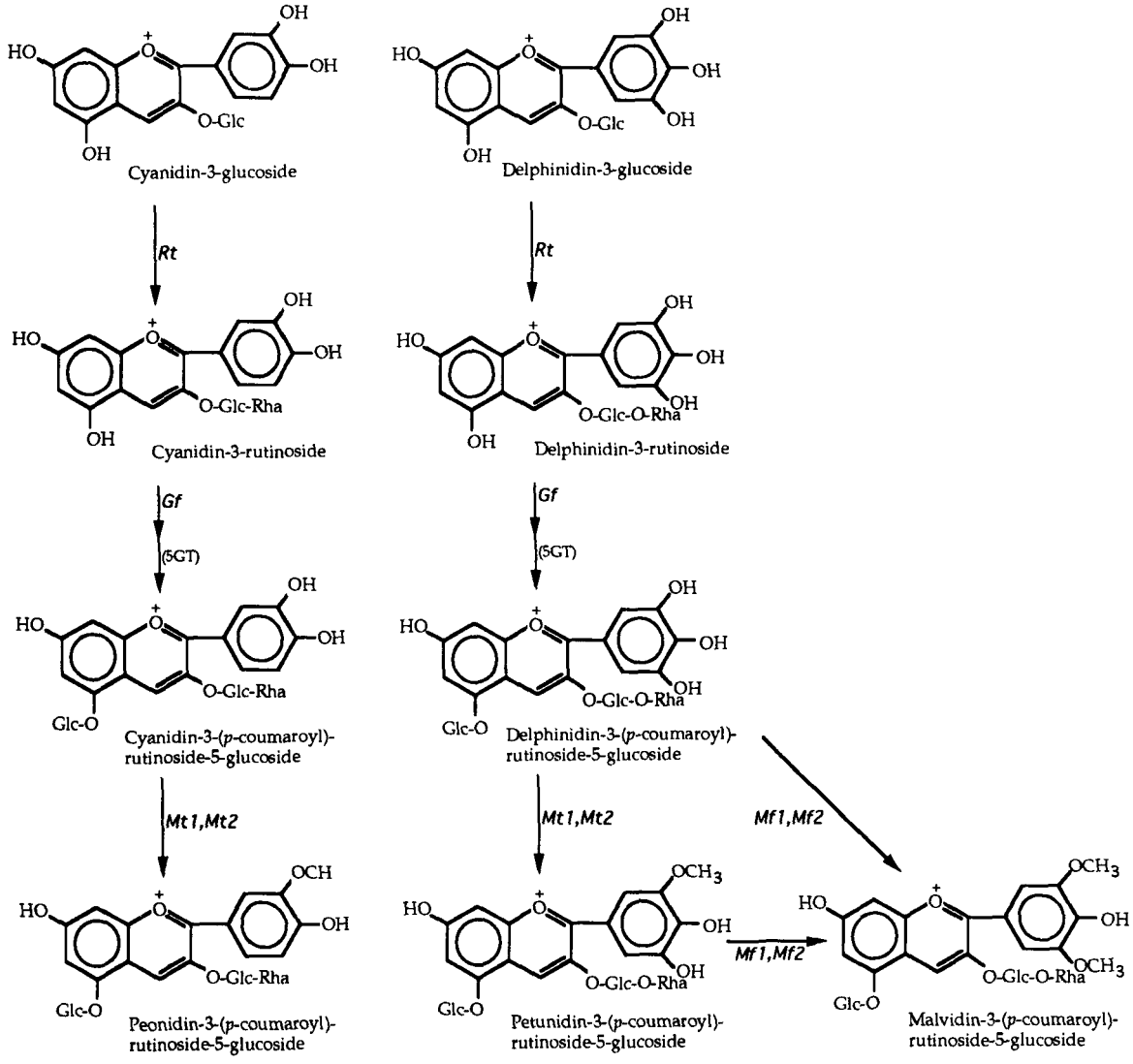

Figure 2. Genetic Control of Anthocyanin Modifications in Petunia.

Genetic loci controlling each enzymatic step of the pathway are shown in italics. No genetic locus encoding the 5GT gene has yet been identified. Glc, glucose; Rha, rhamnose.

are both species and variety differences in the extent of modification and the types of glycosides and acyl groups attached.

STRUCTURAL GENES

Many genes encoding anthocyanin biosynthetic enzymes have been characterized and cloned from maize, snapdragon, and petunia. Table 1 summarizes the genetic loci and structural genes isolated from each of these species.

Chalcone Synthase

The first flavonoid biosynthetic gene isolated was the CHS gene from parsley (Kreuzaler et al., 1983). A combination of differential screening and hybrid-arrested and hybrid-selected translation was used to identify a cDNA clone homologous to CHS mRNA. The parsley CHS clone was also used as a molecular probe to isolate clones of two different CHS genes from petunia (Reif et al., 1985). It was later shown that there are 12 different CHS genes in the petunia genome, but only four of these (*chsA*, *chsB*, *chsG*, and *chsJ*) are known to be expressed (Koes et al., 1989). All four genes are expressed in UV-irradiated seedlings, but only *chsA* and *chsJ* are expressed in floral tissues.

Two genes encode CHS in maize: *c2* is involved in anthocyanin biosynthesis in seed (Dooner, 1983; Wienand et al., 1986), and *whp* controls CHS activity in pollen (Coe et al., 1981). The maize *c2* gene was isolated following transposon tagging using the *En* (*Spm*) transposable element (Wienand et al., 1986). Precursor feeding studies and enzyme activity measurements in different *c2* mutant lines indicated that *c2* might encode CHS. A cDNA clone corresponding to the *c2* locus was sequenced and shown to have a high sequence similarity with the parsley CHS clone. A second CHS gene, *whp*, has subsequently been isolated from maize (Franken et al., 1991).

The *nivea* locus controls CHS enzyme activity in flowers of snapdragon (Spribille and Forkmann, 1982). A CHS gene from snapdragon was isolated by hybridization to the parsley CHS clone (Sommer and Saedler, 1986). The snapdragon genome contains only one CHS gene.

Chalcone Isomerase

The accumulation of chalcone in plant tissues is rare: chalcone is rapidly isomerized by CHI to form naringenin. Even in the absence of CHI, chalcone can spontaneously isomerize to form naringenin, albeit at a slower rate. However, there are some examples of chalcone accumulation in CHI mutants. Corollas of *Callistephus chinensis* (China aster) and *Dianthus caryophyllus* (carnation) are devoid of CHI activity, resulting in the

Table 1. Structural Genes Encoding Anthocyanin Biosynthetic Enzymes

	Maize		Snapdragon		Petunia	
Enzyme	Locus	Clone[a]	Locus	Clone	Locus	Clone
Chalcone synthase (CHS)	*c2*	+	*nivea*	+	NA[b]	+
	whp	+				
Chalone isomerase (CHI)	NA	+	NA	+	*Po*	+
Flavanone 3-hydroxylase (F3H)	NA	–	*incolorata*	+	*An3*	+
Flavonoid 3′-hydroxylase (F3′H)	*Pr*	–	*eosina*	–	*Ht1/Ht2*	+[c]
Flavonoid 3′5′-hydroxylase (F3′5′H)	NA	–	NA	–	*Hf1*	+
					Hf2	+
Dihydroflavonol reductase (DFR)	*A1*	+	*pallida*	+	*An6*	+
Anthocyanidin synthase (ANS)	*A2*	+	*Candica*	+	NA	+
Flavonoid 3-glucosyltransferase (3GT)	*Bz1*	+	NA	+	NA	+
UDP rhamnose:anthocyanidin-3-glucoside rhamnosyltransferase (3RT)	NA	–	NA	–	*Rt*	+
Anthocyanin acyltransferase (AAT)	NA	–	NA	–	*Gf*	–
Anthocyanin 5-*O*-glucosyltransferase (5GT)	NA	–	NA	–	NA	–
Anthocyanin methyltransferase (AMT)	NA	–	NA	–	*Mf1/Mf2*	+[d]
					Mt1/Mt2	
Glutathione *S*-transferase (GST)[e]	*Bz2*	+	NA	–	*An13*	+

[a] +, a cDNA or gene has been cloned; –, a gene has not been cloned.
[b] NA, a genetic locus encoding the structural gene has not been identified.
[c] A clone encoding F3′H activity has been identified, but the gene does not correspond to *Ht1* or *Ht2*.
[d] The genetic locus has yet to be identified.
[e] Enzyme identity is inferred from sequence homology.

production of yellow flowers due to the accumulation of chalcone pigments (Kuhn et al., 1978; Forkmann and Dangelmayr, 1980).

A CHI cDNA was first isolated from French bean using antibodies made against the purified enzyme (Mehdy and Lamb, 1987). Similarly, an antiserum raised against CHI enzyme purified from petunia flower buds (van Tunen and Mol, 1987) was used to screen a petunia petal cDNA expression library to isolate CHI clones (van Tunen et al., 1988). *P. hybrida* has two CHI genes (*chiA* and *chiB*), both of which have been isolated and characterized (van Tunen et al., 1988, 1989). The two genes show different patterns of expression: *chiA* is expressed in all floral tissues and in UV-irradiated seedlings, whereas *chiB* is expressed in immature anthers only (van Tunen et al., 1988).

In petunia, the *Po* gene controls the expression of CHI in anthers. In *po/po* plants, tetrahydroxychalcone accumulates in pollen, which is therefore yellow or green. RFLP analysis showed a 100% linkage between *chiA* and *Po* (van Tunen et al., 1991), suggesting that *Po* corresponds to *chiA*. Transformation of a *po/po* line with a functional *chiA* gene resulted in complementation of the mutation, as visualized by a shift in pollen color from yellow to white. It has been proposed that the *po* mutation is a mutation in the regulatory region of *chiA* that abolishes transcription in anthers but not in corollas (van Tunen, 1990).

Snapdragon and maize CHI genes were isolated by homology with previously cloned CHI genes (Martin et al., 1991; Grotewold and Peterson, 1994). RFLP mapping data indicated that some maize lines contain three loci with CHI-homologous sequences. This may explain the observation that to date, no CHI mutants of maize have been identified.

Flavanone 3-Hydroxylase

A combination of differential screening and genetic mapping was used to isolate a cDNA clone corresponding to the *incolorata* locus of snapdragon, which is known to encode F3H (Martin et al., 1991). The petunia enzyme, which is encoded by the *An3* locus (Froemel et al., 1985), has been purified to homogeneity and characterized as a typical 2-oxoglutarate–dependent dioxygenase (Britsch and Grisebach, 1986; Britsch, 1990). A cDNA encoding F3H was isolated from petals of petunia (Britsch et al., 1992). The function of the clone was verified by comparison of the encoded amino acid sequence with sequences obtained from the purified plant enzyme and by prokaryotic expression, which yielded an enzymatically active hydroxylase. The snapdragon and petunia sequences share high sequence similarity (Britsch et al., 1993).

Using the petunia F3H cDNA clone as a heterologous probe, the corresponding cDNAs have been cloned from carnation, China aster, and stock (Britsch et al., 1993). The cDNAs of China aster and carnation were verified as F3H clones by in vitro expression in a reticulocyte system.

Flavonoid 3′-Hydroxylase

In flower extracts of defined genotypes of petunia, an enzyme activity was demonstrated that catalyzes the hydroxylation of naringenin and dihydrokaempferol in the 3′ position (Stotz et al., 1985). Two genetic loci, *Ht1* and *Ht2*, control F3′H activity in flowers of petunia. *Ht1* acts in the limb and tube of the corolla, whereas *Ht2* acts only in the corolla tube (Wiering, 1974). The *Ht1* and *Ht2* genes control 3′-hydroxylation of anthocyanins and flavonols. A cytochrome P-450 cDNA clone encoding F3′H activity has been isolated from petunia, but the corresponding gene is not linked to either *Ht1* or *Ht2* (Y. Tanaka and T.A. Holton, unpublished results).

Flavonoid 3′-hydroxylation in maize is controlled by the *Pr* locus (Coe et al., 1988). The aleurone of *Pr* plants is purple due to the accumulation of mostly cyanidin glucoside, whereas the aleurone of *pr* plants is red due to accumulation of mostly pelargonidin glucoside. Larson and Bussard (1986) detected the presence of F3′H activity in microsomal preparations obtained from maize seedlings. Kaempferol (a flavonol), naringenin (a flavanone), and apigenin (a flavone) all serve as substrates for the hydroxylase. Inhibitor studies suggested that the F3′H enzyme belongs to the cytochrome P-450 superfamily.

In snapdragon, the gene *eosina* (*eos*) controls the B-ring hydroxylation of flavonoids in the 3′ position (Forkmann and Stotz, 1981). Recessive genotypes (*eos/eos*) are known to produce apigenin, kaempferol, and pelargonidin in the flowers, whereas the corresponding 3′,4′-hydroxylated compounds are found in *Eos* plants.

Flavonoid 3′,5′-Hydroxylase

Two genetic loci, *Hf1* and *Hf2*, control F3′5′H activity in flowers of petunia (Wiering, 1974). *Hf1* acts in the corolla, stigma, and pollen, whereas *Hf2* acts only in the corolla limb. The F3′5′H enzyme belongs to the cytochrome P-450 superfamily (Holton et al., 1993a). More than 200 different P-450 sequences are available, predominantly from mammals (Nelson et al., 1993). All P-450 sequences share a number of small regions of sequence similarity. This sequence conservation was used to design degenerate oligonucleotide primers to isolate P-450 sequences from petunia via PCR amplification (Holton et al., 1993a). Clones of two different P-450 genes were shown to encode F3′5′H activity by expression of full-length cDNA clones in yeast. RFLP mapping and complementation of mutant petunia lines showed that these two genes correspond to the *Hf1* and *Hf2* genetic loci.

Dihydroflavonol 4-Reductase

DFR genes have been isolated from maize and snapdragon by transposon tagging. Recessive mutations at the *a1* gene of maize lead to a colorless aleurone layer. The *a1* gene was

tagged with the transposable element *Spm/En* (O'Reilly et al., 1985) and was shown to encode DFR following in vitro translation of an *a1* cDNA clone (Reddy et al., 1987).

Mutations at the *pallida* (*pal*) locus of snapdragon block anthocyanin biosynthesis, giving rise to uncolored or partially colored flowers. It was concluded that the *pal* gene encodes DFR from precursor feeding experiments in which anthocyanin synthesis was observed when flowers of *pal* mutants were infused with leucocyanidin but not when they were supplied with DHQ (Almeida et al., 1989). The *pal* locus was cloned using a transposable element (*Tam3*) probe to isolate an unstable insertion allele (Martin et al., 1985). Further evidence for the identity of the *pal* product came from sequence analysis, which showed amino acid homology between *pal* and the *a1* gene of maize.

A snapdragon DFR clone was used to isolate a homologous gene from petunia (Beld et al., 1989). Petunia contains three different DFR genes (*dfrA*, *dfrB*, and *dfrC*), but only the *dfrA* gene is transcribed in floral tissues. The *dfrA* gene was shown to correspond to the *An6* locus using RFLP mapping and complementation (Huits et al., 1994). The petunia DFR enzyme preferentially converts DHM to leucodelphinidin; DHQ is a poor substrate, and DHK is not converted to leucopelargonidin (Forkmann and Ruhnau, 1987). The distinct substrate specificity explains the preferential accumulation of delphinidin derivatives and the lack of pelargonidin pigments in petunia (Gerats et al., 1982).

Anthocyanidin Synthase

The enzymatic steps catalyzing the conversion of leucoanthocyanidins to colored anthocyanidins are not well characterized but involve an oxidation and a dehydration step (Heller and Forkmann, 1988). In maize, mutation of the *A2* gene blocks the enzymatic conversion of leucoanthocyanidins to anthocyanidins (Reddy and Coe, 1962). The snapdragon gene *Candica* (*Candi*) is also considered to be involved in the conversion of leucoanthocyanidins to anthocyanidins (Bartlett, 1989). Both the *A2* and the *Candi* gene have been cloned (Menssen et al., 1990; Martin et al., 1991). The deduced amino acid sequence of the product of a petunia gene, *ant17* (Weiss et al., 1993), is 48% identical to the *A2*-encoded sequence of maize and 73% identical to the *Candi*-encoded sequence of snapdragon. Because of the significant similarities between these three proteins, it is likely that they have the same enzymatic activity (anthocyanidin synthase, or ANS). The ANS proteins also share some sequence similarity with 2-oxoglutarate–dependent dioxygenases, which include such enzymes as F3H, flavonol synthase (FLS), and 1-aminocyclopropane-1-carboxylate (ACC) oxidase.

Anthocyanin Glycosyltransferases

The maize *Bz1* gene, encoding UDP glucose:flavonoid 3-*O*-glucosyltransferase (3GT), was isolated by transposon tagging with *Ac* (Fedoroff et al., 1984; Dooner et al., 1985). A putative 3GT clone, pJAM338, was isolated from snapdragon using the maize gene as a probe (Martin et al., 1991). The snapdragon clone was confirmed to encode 3GT by expression in lisianthus (Schwinn et al., 1993). A 3GT gene has recently been isolated from petunia (Y. Tanaka, personal communication).

In petunia and snapdragon as well as in many other species, anthocyanidin 3-glucosides can be modified further by rhamnosylation to produce anthocyanidin 3-rutinosides. A combination of differential screening and genetic mapping was used to isolate clones of UDP rhamnose:anthocyanidin-3-glucoside rhamnosyltransferase (3RT) from petunia (Brugliera et al., 1994; Kroon et al., 1994). The 3RT gene corresponds to the *Rt* locus in petunia. The 3RT gene product shares a low level of sequence homology with 3GT and other glycosyltransferases.

Anthocyanins found in flowers of petunia are glucosylated at the 3 position (Figure 2). Depending on genetic background, anthocyanins may also possess a glucose group at the 5 position. Glucosylation of anthocyanins at the 5 position was thought to be controlled by the gene *Gf*, based on the lack of 5-glucosylated anthocyanins in *Gf* mutants (Wiering and de Vlaming, 1973). However, further analysis demonstrated that anthocyanin 5-*O*-glucosyltransferase (5GT) activity correlates with the *An1* gene, not the *Gf* gene (Jonsson et al., 1984). *An1* does not encode 5GT but is a regulatory gene controlling expression of many anthocyanin structural genes, including 5GT. *Gf* is therefore likely to encode an anthocyanin acyltransferase (AAT) enzyme. Because the 5GT enzyme from petunia requires anthocyanidin-3-(*p*-coumaroyl)rutinosides as substrates (Jonsson et al., 1984), mutation of an AAT gene results in the accumulation of anthocyanidin 3-rutinosides.

Anthocyanin Methyltransferases

In petunia, the genetic loci *Mt1* and *Mt2* control methylation at the 3′ position; *Mf1* and *Mf2* control methylation at the 3′ and 3′,5′ positions (Wiering, 1974; Jonsson et al., 1983). Quattrocchio et al. (1993) reported on the cloning of an anthocyanin-*O*-methyltransferase gene from petunia but did not identify its corresponding genetic locus.

Other Structural Genes

Recessive mutations of the *Bronze2* (*Bz2*) gene of maize result in bronze pigmentation of the aleurone layer and modify purple plant color to reddish brown (Nueffer et al., 1968). *Bz2* sequences were cloned by transposon tagging (Theres et al., 1987) and by combining the techniques of transposon tagging and differential hybridization (McLaughlin and Walbot, 1987). Intertissue complementation studies (Reddy and Coe, 1962; McCormick and Coe, 1977) provided evidence that the product of the *Bz2* gene is involved in one of the last steps of anthocyanin synthesis. The polypeptide encoded by *Bz2* shares homology with various stress-related proteins (Schmitz

and Theres, 1992), including glutathione *S*-transferase (GST). A gene corresponding to the *An13* locus of petunia has recently been isolated (Quattrocchio et al., 1993). The *An13* gene product also shares homology with GSTs, but it has not been shown why this enzyme is required for anthocyanin synthesis. It may be that conjugation to glutathione is required for transport of anthocyanins into the vacuole.

Competition between Flavonoid-Metabolizing Enzymes

Dihydroflavonols are the precursors of both anthocyanins and flavonols. In most plants, both anthocyanins and flavonols are synthesized within the same cell and usually accumulate in the same subcellular location. The dihydroflavonols are substrates for DFR, FLS, F3′H, and F3′5′H. Therefore, the potential exists for competition between each of these enzymes for common substrates. Lack of specificity for dihydroflavonols and flavanones of the flavonoid modification enzymes permits a grid-type pathway, or multiple paths to the same intermediate (Stafford, 1990).

If both F3′H and F3′5′H enzymes are present in petunia, predominantly 3′,5′-hydroxylated anthocyanins are produced, presumably due to the substrate specificity of enzymes involved in the conversion of dihydroflavonols to anthocyanins (Beld et al., 1989). Mutant petunia lines that have no F3′5′H activity but possess F3′H and FLS activity accumulate flavonols at the expense of anthocyanins derived from cyanidin (Gerats, 1985). High levels of cyanidin derivatives accumulate only in petunia lines that possess F3′H activity but lack F3′5′H and FLS activity. However, in the presence of F3′H, F3′5′H, and FLS activities, 4′- and 3′,4′-hydroxylated flavonols (kaempferol and quercetin, respectively) accumulate. The accumulation of specific flavonoids is dependent both on the enzymes produced and on their relative substrate specificities. Stafford (1991) proposed that during biosynthesis, the flavonoid biosynthetic enzymes are organized into an ordered sequence as an aggregate or linear multienzyme complex, but no data support this idea.

Most of the genes necessary for the synthesis of anthocyanin pigments have now been isolated from a number of species. These genes can be used to help study the interactions among individual enzymes in the pathway as well as to characterize further each of the enzymes in isolation. When individual cloned genes are expressed in a heterologous host, they are generally functional. Petunia provides an ideal model system for testing competition and interactions among flavonoid-modifying enzymes because of the availability of many defined mutants and a simple transformation system.

REGULATORY GENES

Genetics

Regulatory genes that control expression of the structural genes of the anthocyanin biosynthetic pathway have been identified in many plants. These genes influence the intensity and pattern of anthocyanin biosynthesis and generally control expression of many different structural genes. Evidence for this regulation can be obtained by either enzyme assays or mRNA assays of structural gene activity. Table 2 summarizes the regulatory genes described in maize, snapdragon, and petunia.

The *R* gene family determines the timing, distribution, and amount of anthocyanin pigmentation in maize. This family comprises a set of regulatory genes, consisting of the *R* locus (which includes *S*, *Lc*, and *Sn*) and the *B* locus (Dooner et al., 1991). Each gene determines pigmentation of different parts of the

Table 2. Regulatory Genes of Anthocyanin Biosynthesis

Species	Locus	Cloned[a]	Genes Regulated[b]	Gene Cloning Reference
Maize	*R*	+	CHS, DFR, 3GT	Dellaporta et al. (1988)
	R(S)	+	CHS, DFR, 3GT	Perrot and Cone (1989)
	R(Sn)	+	CHS, DFR	Tonelli et al. (1991)
	R(Lc)	+	CHS, DFR	Ludwig et al. (1989)
	B	+	DFR, 3GT	Chandler et al. (1989)
	C1	+	CHS, DFR, 3GT	Cone et al. (1986); Paz-Ares et al. (1986, 1987)
	Pl	+	CHS, DFR, 3GT	Cone and Burr (1989)
	Vp1	+	*C1*	McCarty et al. (1989)
Snapdragon	*Delila*	+	F3H, DFR, ANS, 3GT	Goodrich et al. (1992)
	Eluta	–	F3H, DFR, ANS, 3GT	
	Rosea	–	F3H, DFR, ANS, 3GT	
Petunia	*An1*	–	*chsJ*, DFR, ANS, 3GT, 3RT, AMT, F3′5′H, GST	
	An2	+	*chsJ*, DFR, ANS, 3GT, 3RT, AMT, GST	Quattrocchio (1994)
	An4	+[c]	*chsJ*, DFR, ANS, 3GT, 3RT, AMT, GST	Quattrocchio (1994)
	An11	–	*chsJ*, DFR, ANS, 3RT, AMT, GST	

[a] +, gene or cDNA has been cloned; –, gene has not been cloned.

[b] See Table 1 for full names of enzymes.

[c] Expression patterns and RFLP analysis suggest that the clone *jaf13* corresponds to *An4*, but this has not been confirmed.

plant. Accumulation of anthocyanins in competent tissues also requires the presence of either *C1* (in the seed) or *Pl* (in the plant tissue) (Coe et al., 1988). *Viviparous-1* (*Vp1*) controls the anthocyanin pathway in the developing maize seed primarily through regulation of the *C1* gene (Hattori et al., 1992). However, the anthocyanin-less phenotype of the *vp1* mutant is also associated with a general failure of seed maturation, resulting in viviparous development of the embryo.

In snapdragon flowers, three anthocyanin regulatory genes—*Delila* (*Del*), *Eluta*, and *Rosea*—have been identified. The first two steps, CHS and CHI, show minimal regulation, but subsequent steps have an absolute requirement for the *Del* gene product and show quantitative regulation by *Eluta* and *Rosea*. Mutation of the *Del* gene reduces the level of anthocyanin biosynthetic gene transcripts of DFR (Almeida et al., 1989), F3H, *Candi*, and 3GT (Martin et al., 1991) in the flower tube. This suggests that the *Del* gene product acts by transcriptional activation of these structural genes. The *Del* gene product also acts as a repressor of CHS gene expression in the flower lobe mesophyll (Jackson et al., 1992). Both *Eluta* and *Rosea* mutants have decreased expression of F3H, DFR, *Candi*, and 3GT (Bartlett, 1989).

In petunia, mutations at four loci, *An1*, *An2*, *An4*, and *An11*, have similar regulatory effects on transcription of at least six structural anthocyanin biosynthetic genes, including DFR, ANS, *An13*, 3RT, anthocyanin methyltransferase (AMT), and *chsJ* (Quattrocchio et al., 1993; Huits et al., 1994). *An1* and *An11* are required for transcription of this set of structural anthocyanin biosynthetic genes in all pigmented tissues of the plant. In addition, *An2* controls the transcription of these anthocyanin genes in the flower limb and *An4* controls expression of the same set of genes in the anthers. Enzymatic activity of 3GT is controlled by *An1*, *An2*, and *An4* (Tabak et al., 1981); *An1* also controls F3′5′H activity associated with *Hf1* (Gerats, 1985). Therefore, it appears that the petunia regulatory genes control expression of essentially all of the anthocyanin genes after F3H, excluding F3′H.

Cloning and Characterization of Regulatory Genes

Transposon tagging has proven very useful in isolating anthocyanin regulatory genes for which no prior information existed concerning the nature of the gene products. Other regulatory genes have been isolated based on their homology to previously characterized regulatory genes.

The proposed duplicated function of the *R*, *Sn*, *Lc*, and *B* loci is reflected in their sequence similarity. The *R* gene family encodes proteins with homology with the basic helix-loop-helix motif in the *myc* transcription activator (Consonni et al., 1993). *C1* and *Pl* were also shown to be homologous (Cone and Burr, 1989). The *C1* and *Pl* gene products share sequence similarity with the DNA binding domains from the *myb* oncogenes. All of the regulatory genes have been shown to affect the expression of multiple anthocyanin structural genes, as detailed in Table 2. *B* and *C1* fusions with the yeast GAL4 DNA binding and transcriptional activation domains were found to interact when synthesized and assayed in yeast (Goff et al., 1992). These studies suggest that the regulation of the maize anthocyanin pathway involves a direct interaction between members of two distinct classes of transcriptional activators.

The *Del* gene from snapdragon was isolated by transposon tagging using the *Tam2* transposable element. *Del* shares extensive sequence similarity with the *R* gene family of maize (Goodrich et al., 1992), indicating that a homologous regulator controls anthocyanin synthesis in both plants.

A gene fragment of *An2* from petunia was cloned by tagging with a *Tph1* transposon, and a full-length cDNA clone was isolated (Quattrocchio, 1994). The encoded amino acid sequence of *An2* shows the highest similarity with the proteins encoded by the maize *C1* and *Pl* genes. The *An2* gene is expressed in the corolla and tube but not in the anthers. A PCR approach was used to isolate a petunia homolog (*jaf13*) of the maize *R* genes (Quattrocchio, 1994). The encoded amino acid sequence of *jaf13* shares extensive homology with the products of *R* and *Del*. Expression patterns and RFLP analysis suggest that *jaf13* corresponds to the *An4* gene.

Transformation of tobacco and Arabidopsis with a maize *Lc* gene leads to increased pigmentation in several tissues (Lloyd et al., 1992), indicating that *Lc* can activate anthocyanin biosynthesis genes from species other than maize. Transformation of tomato with *Del* leads to increased anthocyanin synthesis in flowers and vegetative tissues, whereas transformation of tobacco increased anthocyanin biosynthesis in flowers only (Mooney et al., 1995). However, in Arabidopsis, *Del* has no strong phenotypic effect. Although there are some minor differences, the control of anthocyanin biosynthesis appears to be mediated by similar factors in different species.

APPLICATIONS: MOLECULAR FLOWER BREEDING

Recent advances in molecular biology, especially in gene isolation and gene transfer between species, have made it possible to alter flower color in a highly directed fashion. Genetic engineering techniques have been used in two ways to change flower color: (1) introduction of genes encoding novel enzyme activities, and (2) inactivation of endogenous genes. Figure 3 shows some of the flower color changes that have been genetically engineered in petunia.

The generation of transgenic petunia plants with pelargonidin-producing flowers was the first example of the creation of a novel flower color by genetic engineering. In general, DFR catalyzes the conversion of DHK, DHQ, and DHM to the respective leucoanthocyanidins. However, petunia DFR cannot convert DHK to leucopelargonidin, which explains the natural lack of pelargonidin pigments in petunia (Forkmann and Ruhnau, 1987). Transformation of a DHK-accumulating petunia line with a DFR gene (*A1*) from maize, which is able to convert DHK to leucopelargonidin, enables the production of pelargonidin by the flowers and leads to a change in flower color from pale

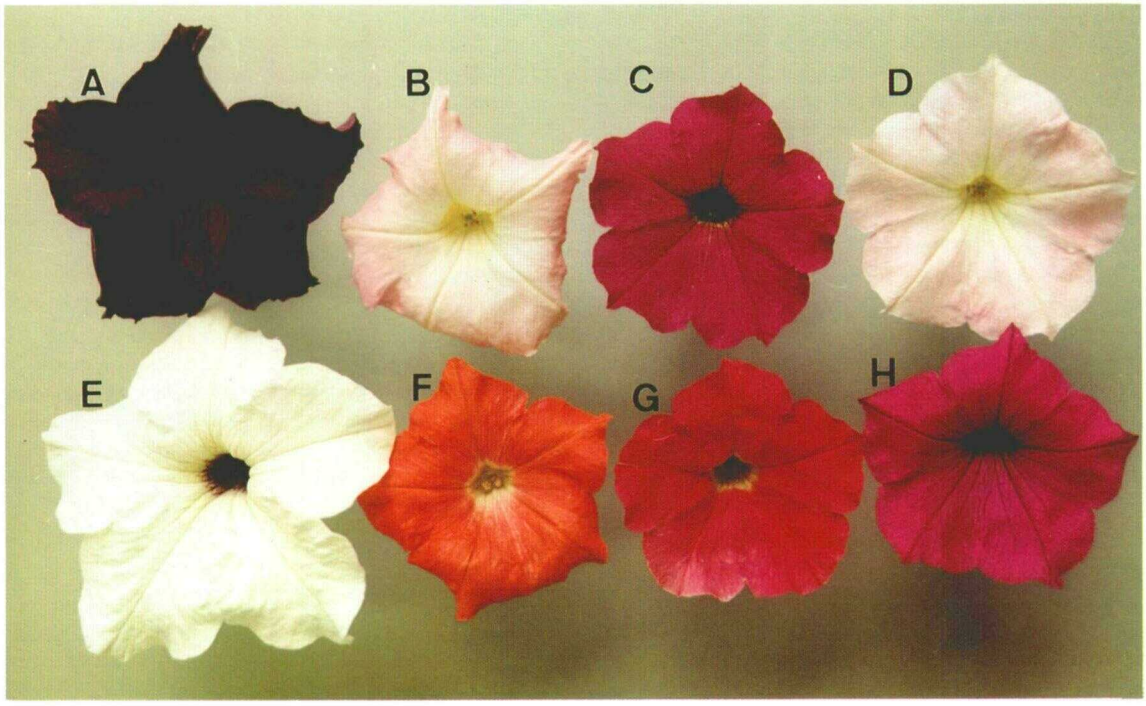

Figure 3. Modification of Petunia Flower Color via Genetic Engineering.

Flowers **(A)** to **(D)** are from nontransgenic plants; flowers **(E)** to **(H)** are from transgenic ones. The genotypes (where known) are shown in parentheses.
(A) cv Old Glory Blue.
(B) Skr4 × SW63 (*hf1hf1*, *hf2hf2*, *ht1ht1*, *ht2ht2*).
(C) VR (*Hf1hf1*, *Hf2hf2*, *Ht1ht1*, *Flfl*).
(D) Skr4 × SW63 (*hf1hf1*, *hf2hf2*, *ht1ht1*, *ht2ht2*).
(E) cv Old Glory Blue transformed with an antisense CHS gene.
(F) Skr4 × SW63 transformed with a rose DFR gene.
(G) VR transformed with a gene that suppresses both F3′5′H and FLS activity.
(H) Skr4 × SW63 transformed with a sense petunia F3′5′H gene.

pink to brick red (Meyer et al., 1987). A similar change in flower color has been achieved by transformation of petunia with DFR genes from gerbera (Helariutta et al., 1993) and rose (Tanaka et al., 1995; Figure 3).

CHS activity can be reduced or eliminated in plants by transformation with an antisense version of the CHS gene (van der Krol et al., 1988). Antisense suppression of gene expression has been shown to occur in a range of organisms. Experiments performed at DNA Plant Technology Corporation (Oakland, CA) (Napoli et al., 1990) led to the discovery of a phenomenon known as sense suppression or cosuppression. Cosuppression can occur when extra "sense" copies of a gene are introduced into an organism. Therefore, both antisense and cosuppression technologies can be used to change flower color by reducing or eliminating expression of genes affecting the synthesis of flower pigments. Antisense or cosuppression of CHS genes has also been achieved in petunia, gerbera (Elomaa et al., 1993), chrysanthemum (Courtney-Gutterson et al., 1994), and rose (Elomaa and Holton, 1994) to produce flowers with reduced pigmentation. Increased anthocyanin accumulation in flowers has been achieved by cosuppression of an FLS gene in tobacco (Holton et al., 1993b).

FUTURE RESEARCH

Isolation of Flavonoid Modification Genes

In petunia lines with a mutation at the regulatory *An1* locus, the transcription of several anthocyanin biosynthesis genes is strongly reduced. Kroon et al. (1994) used a differential screening strategy to isolate cDNA clones of genes regulated by the *An1* gene. The differential clones represent seven distinct classes; the largest cDNA clone from each class was designated *difA*, *difC*, *difE*, *difF*, *difG*, *difH*, and *difI*. Expression of the genes corresponding to each of these classes displays a similar spatial, temporal, and genetic control. Further

characterization of these clones indicates that *difA* encodes ANS, *difD* encodes CHS, *difE* encodes an AMT, *difF* encodes an anthocyanidin glucosyltransferase, *difG* encodes 3RT, and *difI* encodes a GST (R.E. Koes, unpublished results). The remaining classes may represent as-yet-unidentified clones of anthocyanin biosynthetic genes.

The availability of genetically defined mutants and genetic maps enables the identification of gene function in two ways—by genetic complementation and gene suppression. Such methods can be used even when the gene product cannot be assayed directly. The first of these methods relies on transforming a genetically defined mutant with the gene of interest and relies on expression of the introduced gene to overcome the effects of a genetic lesion. The second approach utilizes the ability of an introduced gene to suppress expression of an endogenous gene homolog. The effect on phenotype may then be used to predict the function of the gene product (reverse genetics). Gene mapping, complementation, and reverse genetics have played an important part in identifying the DFR (Beld et al., 1989; Huits et al., 1994), F3′5′H (Holton et al., 1993a), FLS (Holton et al., 1993b), and 3RT (Brugliera et al., 1994) genes from petunia. Application of similar methods may enable identification of function of the petunia *dif* genes. The development of a straightforward method for cloning genes tagged by the multicopy *Tph1* transposon (Souer et al., 1995) should facilitate the cloning of other petunia genes involved in anthocyanin synthesis.

It should now be possible to isolate many novel flavonoid modification genes from a variety of species without needing to purify an enzyme to homogeneity or to generate and characterize mutant lines. If the class of enzyme catalyzing a particular reaction is known, then the corresponding gene can be isolated by PCR amplification using degenerate oligonucleotides specific for that class of enzyme. Function of a cloned gene can then be confirmed through expression in a heterologous system or through transient expression following particle bombardment in genetically defined lines. These gene isolation/identification methods should be applicable to cloning of genes encoding such enzymes as flavone synthase, anthocyanidin glycosyltransferases, and methyltransferases. The advantage of PCR-based methods is that genes can be rapidly isolated from species in which little or no genetic information is available.

Production of Novel Flower and Fruit Colors

The availability of many cloned anthocyanin biosynthesis genes and the development of transformation protocols make it possible to create novel flower colors in a number of commercially important species. Knowledge of the genotype and substrate specificity of the endogenous flavonoid enzymes is important for developing strategies for modifying pigment production in a target plant. Color changes can be created by manipulating levels of a particular pigment or by synthesizing novel pigments. It is possible that controlled expression of the appropriate regulatory genes may lead to useful increases in anthocyanin synthesis in important cut-flower species. It should also be possible to modify fruit color using similar strategies. Many anthocyanin biosynthetic genes have been isolated from grape (Sparvoli et al., 1994) and apple (Davies, 1993; Podivinsky et al., 1993).

Blue-flowering varieties are missing from a number of important ornamental plants, including carnations, chrysanthemums, and roses. None of these plants is capable of producing blue delphinidin pigments, presumably due to the absence of a gene encoding F3′5′H. Transformation of these species with an F3′5′H gene should overcome this limitation and allow the production of delphinidin derivatives, thereby increasing the possibility of producing blue flowers. Other genes that may be required for creation of blue flowers have also been recently isolated (Holton and Tanaka, 1994).

REFERENCES

Almeida, J., Carpenter, R., Robbins, T.P., Martin, C., and Coen, E.S. (1989). Genetic interactions underlying flower color patterns in *Antirrhinum majus*. Genes Dev. **3,** 1758–1767.

Bartlett, N.J.R. (1989). The Genetic Control of Anthocyanin Biosynthesis in *Antirrhinum majus*. PhD Dissertation (Norwich, England: University of East Anglia).

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

Britsch, L. (1990). Purification of flavanone 3β-hydroxylase from *Petunia hybrida*: Antibody preparation and characterization of a chemicogenetically defined mutant. Arch. Biochem. Biophys. **276,** 348–354.

Britsch, L., and Grisebach, H. (1986). Purification and characterization of (2S)-flavanone 3-hydroxylase from *Petunia hybrida*. Eur. J. Biochem. **156,** 569–577.

Britsch, L., Ruhnau-Brich, B., and Forkmann, G. (1992). Molecular cloning, sequence analysis, and *in vitro* expression of flavanone 3β-hydroxylase from *Petunia hybrida*. J. Biol. Chem. **267,** 5380–5387.

Britsch, L., Dedio, J., Saedler, H., and Forkmann, G. (1993). Molecular characterization of flavanone 3β-hydroxylases. Consensus sequence, comparison with related enzymes and the role of conserved histidine residues. Eur. J. Biochem. **217,** 745–754.

Brugliera, F., Holton, T.A., Stevenson, T.W., Farcy, E., Lu, C.-Y., and Cornish, E.C. (1994). Isolation and characterization of a cDNA clone corresponding to the *Rt* locus of *Petunia hybrida*. Plant J. **5,** 81–92.

Chandler, V.L., Radicella, J.P., Robbins, T.P., Chen, J., and Turks, D. (1989). Two regulatory genes of the maize anthocyanin pathway are homologous: Isolation of *B* utilizing *R* genomic sequences. Plant Cell **1,** 1175–1183.

Coe, E.H., McCormick, S., and Modena, S.A. (1981). White pollen in maize. J. Hered. **72,** 318–320.

Coe, E.H., Hoisington, D.A., and Neuffer, M.G. (1988). The genetics of corn. In Corn and Corn Improvement, G.F. Sprague and J. Dudley, eds (Madison, WI: American Society of Agronomy), pp. 81–258.

Cone, K.C., and Burr, B. (1989). Molecular and genetic analyses of light requirement for anthocyanin synthesis in maize. In The Genetics

of Flavonoids, D.E. Styles, G.A. Gavazzi, and M.L. Racchi, eds (Milan: Edizioni Unicopli), pp. 143–146.

Cone, K.C., Burr, F.A., and Burr, B. (1986). Molecular analysis of the maize anthocyanin regulatory locus *C1*. Proc. Natl Acad. Sci. USA **83,** 9631–9635.

Consonni, G., Geuna, F., Gavazzi, G., and Tonelli, C. (1993). Molecular homology among members of the *R* gene family in maize. Plant J. **3,** 335–346.

Courtney-Gutterson, N., Napoli, C., Lemieux, C., Morgan, A., Firoozabady, E., and Robinson, K. (1994). Modification of flower color in Florist's Chrysanthemum: Production of a white-flowering variety through molecular genetics. Bio/Technology **12,** 268–271.

Davies, K.M. (1993). A cDNA clone for flavanone 3-hydroxylase from *Malus*. Plant Physiol. **103,** 291.

Dellaporta, S.L., Greenblatt, I.M., Kermicle, J.L., Hicks, J.B., and Wessler, S.R. (1988). Molecular cloning of the *R-nj* gene by transposon tagging with *Ac*. In Chromosome Structure and Function: Impact of New Concepts, J.P. Gustafson and R. Appels, eds (New York: Plenum Press), pp. 263–282.

Dooner, H.K. (1983). Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Mol. Gen. Genet. **189,** 136–141.

Dooner, H.K., Weck, E., Adams, S., Ralston, E., Favreau, M., and English, J. (1985). A molecular genetic analysis of insertions in the *bronze* locus in maize. Mol. Gen. Genet. **200,** 240–246.

Dooner, H.K., Robbins, T.P., and Jorgensen, R.A. (1991). Genetic and developmental control of anthocyanin biosynthesis. Annu. Rev. Genet. **25,** 173–199.

Elomaa, P., and Holton, T. (1994). Modification of flower colour using genetic engineering. Biotechnol. Genet. Eng. Rev. **12,** 63–88.

Elomaa, P., Honkanen, J., Puska, R., Seppänen, P., Helariutta, Y., Mehto, M., Kotilainen, M., Nevalainen, L., and Terri, T.H. (1993). *Agrobacterium*-mediated transfer of antisense chalcone synthase cDNA to *Gerbera hybrida* inhibits flower pigmentation. Bio/Technology **11,** 508–511.

Fedoroff, N.V., Furtek, D.B., and Nelson, O.E. (1984). Cloning of the *bronze* locus in maize by a simple and generalizable procedure using the transposable element *Activator*. Proc. Natl. Acad. Sci. USA **81,** 3825–3829.

Forkmann, G. (1991). Flavonoids as flower pigments: The formation of the natural spectrum and its extension by genetic engineering. Plant Breed. **106,** 1–26.

Forkmann, G. (1993). Genetics of flavonoids. In The Flavonoids: Advances in Research since 1986, J.B. Harborne, ed (London: Chapman and Hall), pp. 538–564.

Forkmann, G., and Dangelmayr, B. (1980). Genetic control of chalcone isomerase activity in flowers of *Dianthus caryophyllus*. Biochem. Genet. **18,** 519–527.

Forkmann, G., and Ruhnau, B. (1987). Distinct substrate specificity of dihydroflavonol 4-reductase from flowers of *Petunia hybrida*. Z. Naturforsch. **42C,** 1146–1148.

Forkmann, G., and Stotz, G. (1981). Genetic control of flavanone 3-hydroxylase activity and flavonoid 3′-hydroxylase activity in *Antirrhinum majus* (Snapdragon). Z. Naturforsch. **36C,** 411–416.

Franken, P., Niesbach-Klosgen, U., Weydemann, U., Marechal-Drouard, L., Saedler, H., and Wienand, U. (1991). The duplicated chalcone synthase genes *C2* and *Whp* (white pollen) of *Zea mays* are independently regulated: Evidence for translational control of *Whp* expression by the anthocyanin intensifying gene *in*. EMBO J. **10,** 2605–2612.

Froemel, S., de Vlaming, P., Stotz, G., Wiering, H., Forkmann, G., and Schram, A.W. (1985). Genetic and biochemical studies on the conversion of flavanones to dihydroflavonols in flowers of *Petunia hybrida*. Theor. Appl. Genet. **70,** 561–568.

Gerats, A.G.M. (1985). Mutable Systems: Their Influence on Flavonoid Synthesis in *Petunia hybrida*. PhD Dissertation (Amsterdam: Vrije Universiteit).

Gerats, A.G.M., and Martin, C. (1992). Flavonoid synthesis in *Petunia hybrida*: Genetics and molecular biology of flower colour. In Recent Advances in Phytochemistry, Vol. 26, H.A. Stafford and R.K. Ibrahim, eds (New York: Plenum Press), pp. 165–199.

Gerats, A.G.M., de Vlaming, P., Doodeman, M., Al, B., and Schram, A.W. (1982). Genetic control of the conversion of dihydroflavonols into flavonols and anthocyanins in flowers of *Petunia hybrida*. Planta **155,** 364–368.

Goff, S.A., Cone, K.C., and Chandler, V.L. (1992). Functional analysis of the transcriptional activator encoded by the maize *B* gene: Evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev. **6,** 864–875.

Goodrich, J., Carpenter, R., and Coen, E.S. (1992). A common gene regulates pigmentation pattern in diverse plant species. Cell **68,** 955–964.

Grotewold, E., and Peterson, T. (1994). Isolation and characterization of a maize gene encoding chalcone flavonone isomerase. Mol. Gen. Genet. **242,** 1–8.

Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L.C., McCarty, D.R., and Vasil, I.K. (1992). The *Viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. Genes Dev. **6,** 609–618.

Helariutta, Y., Elomaa, P., Kotilainen, M., Seppänen, P., and Teeri, T.H. (1993). Cloning of cDNA coding for dihydroflavonol-4-reductase (DFR) and characterization of *dfr* expression in the corollas of *Gerbera hybrida* var. Regina (Compositae). Plant Mol. Biol. **22,** 183–193.

Heller, W. and Forkmann, G. (1988). Biosynthesis. In The Flavonoids, J.B. Harborne, ed (London: Chapman and Hall), pp. 399–425.

Holton, T.A., and Tanaka, Y. (1994). Blue roses—A pigment of our imagination? Trends Biotechnol. **12,** 40–42.

Holton, T.A., Brugliera, F., Lester, D.R., Tanaka, Y., Hyland, C., Menting, J.G.T., Lu, C.-Y., Farcy, E., Stevenson, T.W., and Cornish, E.C. (1993a). Cloning and expression of cytochrome P450 genes controlling flower colour. Nature **366,** 276–279.

Holton, T.A., Brugliera, F., and Tanaka, Y. (1993b). Cloning and expression of flavonol synthase from *Petunia hybrida*. Plant J. **4,** 1003–1010.

Huits, H.S.M., Gerats, A.G.M., Kreike, M.M., Mol, J.N.M., and Koes, R. (1994). Genetic control of dihydroflavonol 4-reductase gene expression in *Petunia hybrida*. Plant J. **6,** 295–310.

Jackson, D., Roberts, K., and Martin, C. (1992). Temporal and spatial control of expression of anthocyanin biosynthetic genes in developing flowers of *Antirrhinum majus*. Plant J. **2,** 425–434.

Jonsson, L.M.V., de Vlaming, P., Wiering, H., Aarsman, M.E.G., and Schram, A.W. (1983). Genetic control of anthocyanin-O-methyltransferase activity in flowers of *Petunia hybrida*. Theor. Appl. Genet. **66,** 349–355.

Jonsson, L.M.V., Aarsman, M.E.G, van Diepen, J., de Vlaming, P., Smit, N., and Schram, A.W. (1984). Properties and genetic control

of anthocyanin 5-O-glucosyltransferase in flowers of *Petunia hybrida*. Planta **160,** 341–347.

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

Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D.N., and Hahlbrock, K. (1983). UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. Proc. Natl. Acad. Sci. USA **80,** 2591–2593.

Kroon, J., Souer, E., de Graaff, A., Xue, Y., Mol, J., and Koes, R. (1994). Cloning and structural analysis of the anthocyanin pigmentation locus *Rt* of *Petunia hybrida*: Characterization of insertion sequences in two mutant alleles. Plant J. **5,** 69–80.

Kuhn, B., Forkmann, G., and Seyffert, W. (1978). Genetic control of chalcone flavanone isomerase activity in *Callistephus chinensis*. Planta **138,** 199–203.

Larson, R.L., and Bussard, J.B. (1986). Microsomal flavonoid 3′-monooxygenase from maize seedlings. Plant Physiol. **80,** 483–486.

Lloyd, A.M., Walbot, V., and Davis, R.W. (1992). *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *C1*. Science **258,** 1773–1775.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., and Wessler, S.R. (1989). *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the *myc*-homology region. Proc. Natl. Acad. Sci. USA **86,** 7092–7096.

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

Martin, C., Prescott, A., Mackay, S., Bartlett, J., and Vrijlandt, E. (1991). Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J. **1,** 37–49.

McCarty, D.R., Carson, C.B., Stinard, P.S., and Robertson, D.S. (1989). Molecular analysis of *viviparous-1*: An abscisic acid–insensitive mutant of maize. Plant Cell **1,** 523–532.

McCormick, S., and Coe, E.H. (1977). Gene action sequence and regulation of anthocyanin biosynthesis in corn: *In vivo* complementation. Genetics **86,** 42.

McLaughlin, M., and Walbot, V. (1987). Cloning of a mutable *bz2* allele of maize by transposon tagging and differential hybridization. Genetics **117,** 771–776.

Mehdy, M.C., and Lamb, C.J. (1987). Chalcone isomerase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. EMBO J. **6,** 1527–1533.

Menssen, A., Höhmann, S., Martin, W., Schanle, P.S., Peterson, P.A., Saedler, H., and Gierl, A. (1990). The En/Spm transposable element of *Zea mays* contains splice sites at the termini generating a novel intron from a *dSpm* element in the *A2* gene. EMBO J. **9,** 3051–3058.

Meyer, P., Heidmann, I., Forkmann, G., and Saedler, H. (1987). A new petunia flower colour generated by transformation of a mutant with a maize gene. Nature **330,** 677–678.

Mol., J., Stuitje, A., Gerats, A., van der Krol, A., and Jorgensen, R. (1989). Saying it with genes: Molecular flower breeding. Trends Biotechnol. **7,** 148–153.

Mooney, M., Desnos, T., Harrison, K., Jones, J., Carpenter, R., and Coen, E. (1995). Altered regulation of tomato and tobacco pigmentation genes caused by the *delila* gene of *Antirrhinum*. Plant J. **7,** 333–339.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. Plant Cell **2,** 279–289.

Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K., and Nebert, D.W. (1993). The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol. **12,** 1–51.

Neuffer, M.G., Jones, L., and Zuber, M.S. (1968). The Mutants of Maize. (Madison, WI: Crop Science Society of America).

O'Reilly, C., Shepherd, N.S., Pereira, A., Schwarz-Sommer, Z., Bertram, I., Robertson, D.S., Peterson, P.A., and Saedler, H. (1985). Molecular cloning of the *a1* locus in *Zea mays* using the transposable elements *En* and *Mu1*. EMBO J. **4,** 877–882.

Paz-Ares, J., Wienand, U., Peterson, P.A., and Saedler, H. (1986). Molecular cloning of the *c* locus of *Zea mays*: A locus regulating the anthocyanin pathway. EMBO J. **5,** 829–833.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A., and Saedler, H. (1987). The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. EMBO J. **6,** 3553–3558.

Perrot, G.H., and Cone, K.C. (1989). Nucleotide sequence of the maize *R-S* gene. Nucleic Acids Res. **17,** 8003.

Podivinsky, E., Bradley, J.M., and Davies, K.M. (1993). Sequence announcements. Plant Mol. Biol. **21,** 737–738.

Quattrocchio, F.M. (1994). Regulatory Genes Controlling Flower Pigmentation in *Petunia hybrida*. PhD Dissertation (Amsterdam: Vrije Universiteit).

Quattrocchio, F., Wing, J.F., Leppen, H.T.C., Mol, J.N.M., and Koes, R. (1993). Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell **5,** 1497–1512.

Reddy, G.M., and Coe, E.H. (1962). Inter-tissue complementation: A simple technique for direct analysis of gene action sequence. Science **138,** 149–150.

Reddy, A.R., Britsch, L., Salamini, F., Saedler, H., and Rohde, W. (1987). The *A1* (Anthocyanin-1) locus in *Zea mays* encodes dihydroquercetin reductase. Plant Sci. **52,** 7–13.

Reif, H.J., Niesbach, U., Deumling, B., and Saedler, H. (1985). Cloning and analysis of two genes for chalcone synthase from *Petunia hybrida*. Mol. Gen. Genet. **199,** 208–215.

Schmitz, G., and Theres, K. (1992). Structural and functional analysis of the *Bz2* locus of *Zea mays*: Characterization of overlapping transcripts. Mol. Gen. Genet. **233,** 269–277.

Schwinn, K.E., Davies, K.M., Miller, R.M., Deroles, S.C., and Bradley, J.M. (1993). Floral flavonoids and their modification in lisianthus. Phytochem. Soc. North America Newslett. **33,** 17 (abstr.).

Sommer, H., and Saedler, H. (1986). Structure of the chalcone synthase gene of *Antirrhinum majus*. Mol. Gen. Genet. **202,** 429–434.

Souer, E., Quattrocchio, F., de Vetten, N., Mol, J., and Koes, R. (1995). A general method to isolate genes tagged by a high copy number transposable element. Plant J. **7,** 677–685.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G., and Tonelli, C. (1994). Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.). Plant Mol. Biol. **24,** 743–755.

Spribille, R., and Forkmann, G. (1982). Genetic control of chalcone synthase activity in flowers of *Antirrhinum majus.* Phytochemistry **21,** 2231–2234.

Stafford, H. (1990). Flavonoid Metabolism. (Boca Ration, FL: CRC Press).

Stafford, H. (1991). Flavonoid evolution: An enzymic approach. Plant Physiol. **96,** 680–685.

Stotz, G., de Vlaming, P., Wiering, H., Schram, A.W., and Forkmann, G. (1985). Genetic and biochemical studies on flavonoid 3'-hydroxylation in flowers of *Petunia hybrida.* Theor. Appl. Genet. **70,** 300–305.

Tabak, A.J.H., Schram, A.W., and Ennink, G.J.H. (1981). Modification of the B-ring during flavonoid synthesis in *Petunia hybrida*: Effect of the hydroxylation gene *Hf1* on dihydroflavonol intermediates. Planta **153,** 462–465.

Tanaka, Y., Fukui, Y., Fukuchi-Mizutani, M., Holton, T., Higgins, E., and Kusumi, T. (1995). Molecular cloning and characterization of *Rosa hybrida* dihydroflavonol 4-reductase gene. Plant Cell Physiol., in press.

Theres, N., Scheele, T., and Starlinger, P. (1987). Cloning of the *Bz2* locus of *Zea mays* using the transposable element *Ds* as a gene tag. Mol. Gen. Genet. **209,** 193–197.

Tonelli, C., Consonni, G., Dolfini, S.F., Dellaporta, S.L., Viotti, A., and Gavazzi, G. (1991). Genetic and molecular analysis of *Sn*, a light-inducible, tissue specific regulatory gene in maize. Mol. Gen. Genet. **225,** 401–410.

van der Krol, A.R., Lenting, P.E., Veenstra, J., van der Meer, I.M., Koes, R.E., Gerats, A.G.M., Mol, J.N.M., and Stuitje, A.R. (1988). An antisense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature **333,** 866–869.

van Tunen, A.J. (1990). Regulation of Flavonoid Biosynthesis in *Petunia*: Isolation, Molecular Analysis and Manipulation of the Two Chalcone Flavanone Isomerase Genes. PhD Dissertation (Amsterdam: Vrije Universiteit).

van Tunen, A.J., and Mol, J.N.M. (1987). A novel purification procedure for chalcone flavanone isomerase from *Petunia hybrida* and the use of its antibodies to characterize the *Po* mutation. Arch. Biochem. Biophys. **257,** 85–91.

van Tunen, A.J., and Mol, J.N.M. (1991). Control of flavonoid synthesis and manipulation of flower colour. In Plant Biotechnology, Vol. 2, D. Grierson, ed (Glasgow: Blackie), pp. 94–130.

van Tunen, A.J., Koes, R.E., Spelt, C.E., van der Krol, A.R., Stuitje, A.R., and Mol, J.N.M. (1988). Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: Coordinate, light-regulated and differential expression of flavonoid genes. EMBO J. **7,** 1257–1263.

van Tunen, A.J., Hartman, S.A., Mur, L.A., and Mol, J.N.M. (1989). Regulation of chalcone isomerase (CHI) gene expression in *Petunia hybrida*: The use of alternative promoters in corolla, anthers and pollen. Plant Mol. Biol. **12,** 539–551.

van Tunen, A.J., Mur, L.A., Recourt, K., Gerats, A.G.M., and Mol, J.N.M. (1991). Regulation and manipulation of flavonoid gene expression in anthers of petunia: The molecular basis of the *Po* mutation. Plant Cell **3,** 39–48.

Weiss, D., van der Luit, A.H., Kroon, J.T.M., Mol, J.N.M., and Kooter, J.M. (1993). The petunia homologue of the *Antirrhinum majus candi* and *Zea mays A2* flavonoid genes; homology to flavanone 3-hydroxylase and ethylene forming enzyme. Plant Mol. Biol. **22,** 893–897.

Wienand, U., Weydemann, U., Niesbach-Klösgen, U., Peterson, P.A., and Saedler, H. (1986). Molecular cloning of the *C2* locus of *Zea mays*, the gene coding for chalcone synthase. Mol. Gen. Genet. **203,** 202–207.

Wienand, U., Paz-Ares, J., and Saedler, H. (1990). Cloning and molecular analysis of anthocyanin genes in *Zea mays.* In Genetic Engineering of Crop Plants, G.W. Lycett and D. Grierson, eds (London: Butterworths), pp. 207–214.

Wiering, H. (1974). Genetics of flower colour in *Petunia hybrida* hort. Genen. Phaenen. **17,** 117–134.

Wiering, H., and de Vlaming, P. (1973). Glycosylation and methylation patterns of anthocyanins in *Petunia hybrida*. I. The gene *Gf*. Genen. Phaenen. **16,** 35–50.

Wiering, H., and de Vlaming, P. (1984). Genetics of flower and pollen colours. In Petunia, K.C. Sink, ed (Berlin: Springer- Verlag), pp. 49–75.