ABSTRACT

Gaus, Jessica Leigh. Genetics and Biochemistry of Flower Color in Stokes Aster [*Stokesia laevis* (J. Hill) Greene]. (Under the direction of Dennis J. Werner.)

The flowers of 9 cultivars, 4 F_1 hybrid plants, and one F_2 hybrid plant of stokes aster [*Stokesia laevis* (J. Hill) Greene] were analyzed using high performance liquid chromatography (HPLC) to determine pigment composition. Flowers that were pale blue ('Omega Skyrocket', 'Blue Danube'), lavender ('Peaches'), or violet ('Honeysong Purple', 'Purple Parasols') each contained a single anthocyanidin, petunidin. Pale magenta flowers ('Maroon', 'Colorwheel') contained a single anthocyanidin, cyanidin. Albescent flowers ('Alba') contained pigment, but the amount was substantially smaller than the amount that was isolated from the other cultivars. We were not able to identify which anthocyanidin(s) was (were) present because the quantity was too small. Flowers of F_1 hybrid progeny from crosses of 'Maroon' (cyanidin) × 'Honeysong' (petunidin), 'Maroon' (cyanidin) × 'Peaches' (petunidin), and 'Peaches' (petunidin) × 'Colorwheel' (cyanidin) contained only petunidin demonstrating that petunidin synthesis is dominant to the synthesis of cyanidin. These biochemical results support a previous genetic study that determined that pale blue/lavender flower color (petunidin) is completely dominant to pale magenta flower color (cyanidin) in the F_1 generation. Pale yellow flowers ('Mary Gregory') did not contain anthocyanidins or carotenoids, but did contain the copigment, luteolin. Thus, it was proposed that yellow flower color in this individual is attributable to the presence of luteolin. Analysis of other cultivars, revealed that all flowers of stokes aster contain luteolin.

Inheritance of flower color was investigated in F_1, F_2, and BC_1 families derived from various combinations of different cultivars. The results of these studies suggest that two genes control pale blue/lavender, albescent, and pale yellow flower color in stokes aster. It was proposed that albescent flower color is controlled by a single gene, designated a*lb*. This gene has two alleles and a system of complete dominance. Based on this classification, plants with the *AlbAlb* or *Albalb* genotype have pale blue/lavender flowers that contain wild type amounts of petunidin and luteolin. Plants with the *albalb* genotype have albescent flowers practically devoid of anthocyanidins and contain reduced amounts of luteolin. The second gene, designated *y*, controls pale yellow flower color. This gene has two alleles and a system of complete dominance where plants with the *YY* or *Yy* genotype have blue/lavender flowers that contain wild type amounts of petunidin and luteolin. Plants with the *yy* genotype are pale yellow and do not contain anthocyanidins, but do contain luteolin. It was proposed that *Y* is the structural gene that encodes for the enzyme, flavanone 3-hydroxylase (F3H). Further analysis suggested that the homozygous recessive form of *Y* is epistatic to *Alb*. As of yet, it is unclear whether the double recessive genotype *albalb yy* is lethal. A third gene, designated *Mag*, was proposed to encode for flavonoid 3',5'-hydroxylase (F3'5'H). A hypothesis was presented to explain how *Mag* controls the synthesis of petunidin versus cyanidin. A theoretical biochemical pathway for flavonoid biosynthesis in stokes aster and suggestions for future research are proposed.

Genetics and Biochemistry of Flower Color in Stokes Aster [*Stokesia laevis* (J. Hill) Greene]

by

Jessica Leigh Gaus

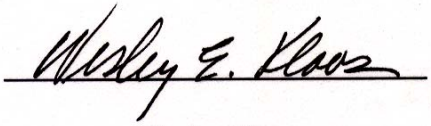
A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science

Horticultural Science

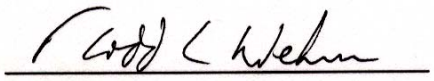
Raleigh, NC

2003

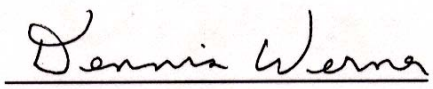
Approved by:



Wesley Kloos



Todd Wehner



Dennis Werner, Chair of Advisory Committee

Biography

Jessica Leigh Gaus was born in Atlanta, Georgia on June 30, 1977 to proud parents Jeff and Joanne Gaus. Jessica moved quite a bit throughout her life stopping in Georgia, Alabama, South Carolina, Canada, and Pennsylvania. Jessica earned her Bachelor of Science degree in Biology at Pennsylvania State University in December of 1999. Following graduation, Jessica worked for PanAmerican Seed Company in Elburn, Illinois as a New Crops Development Intern for one year before enrolling at North Carolina State University in the spring of 2001. Following the completion of her master's degree, Jessica will begin her Ph.D. program at North Carolina State in the Fall of 2003 continuing her work with *Stokesia*.

Table of Contents

	Page
List of Tables	iv
List of Figures	v
Introduction	1
Materials and Methods	4
Plant Material	4
Tissue Collection and Extraction	5
Anthocyanin and Copigment Analysis	5
Acid Hydrolysis and Aglycone Analysis	5
Inheritance Analysis	6
Results and Discussion	8
Anthocyanidin Identification	8
Copigment Identification	9
Inheritance Analysis	11
Blue × Albescent	11
Blue × Pale Yellow	12
Albescent × Pale Yellow	13
Biochemical Pathway	15
The *Alb* gene	15
The *Y* gene	17
The *Mag* gene	19
Gene Expression	20
Breeding for Novel or Improved Flower Colors	20
Conclusions	22
Literature Cited	24

List of Tables

Page

Table 1 Anthocyanidin and copigment composition of flowers from different cultivars, F_1 hybrids, and F_2 hybrids of stokes aster 26

Table 2 Segregation of flower color in F_2 and BC_1 families of stokes aster 27

List of Figures

Page

Figure 1 Flavonoid biosynthetic pathway 28

Figure 2 Anthocyanidin HPLC profiles from samples that contained (A) a standard solution of petunidin, peonidin, and pelargonidin, (B) extract from flowers collected from 'Purple Parasols', and (C) the standard solution of petunidin, peonidin, and pelargonidin mixed with the extract from 'Purple Parasols' 29

Figure 3 Anthocyanidin HPLC profiles from samples that contained (A) extract from flowers collected from 'Colorwheel' and (B) mixture of the extract collected from 'Colorwheel' plus a standard solution of cyanidin 30

Figure 4 Anthocyanidin HPLC profiles from samples that contained (A) extract from the flowers of an F_1 hybrid from a cross of 'Maroon' × 'Honeysong' and (B) extract from the flowers of an F_1 hybrid from a cross of 'Peaches' × 'Colorwheel' 31

Figure 5 Aglycone copigment profiles from samples that contained (A) extract from the flowers collected from ‘Maroon’, (B) a standard solution of luteolin, and (C) extract from the flowers of ‘Maroon’ mixed with the standard solution of luteolin 32

Figure 6 Aglycone copigment profiles from samples that contained (A) extract from the flowers collected from ‘Purple Parasols’, (B) extract from pale yellow flowers taken from a ‘Peaches’ × ‘Mary Gregory’ F_2 plant selected at random, and (C) extract from the flowers of ‘Purple Parasols’ mixed with the extract from the pale yellow flowers taken from a ‘Peaches’ × ‘Mary Gregory’ F_2 plant selected at random 33

Figure 7 Hypothetical biochemical pathway for flavonoid biosynthesis in stokes aster..... 34

Genetics and Biochemistry of Flower Color

in Stokes Aster [*Stokesia laevis* (J. Hill) Greene]

INTRODUCTION

Flavonoids, carotenoids, and betalains are the three classes of pigments that give rise to flower color in plants. Among these, flavonoids are the most abundant class, generating an enormous range of flower colors including pale yellow, ivory, pink, magenta, red, blue, and violet (Courtney-Gutterson, 1994). The presence of flavonoids in a flower is essential to attracting pollinators, and thus influences the reproductive success of an individual plant (Winkel-Shirley, 2001). In addition to ensuring reproductive success, recent evidence has shown that flavonoids also play an important role in other physiological processes including protection from UV-B radiation, microbial infection, and herbivory, as well as aiding in pollen and seed germination (Clegg and Durbin, 2000), (Harborne and Williams, 2000), (Ben-Meir et al., 2002), (Holton and Cornish, 1995), (Bohm, 1998), (Winkel-Shirley, 2001).

Flavonoids are water soluble, secondary metabolites formed by a branch of the phenylpropanoid pathway. The basic 15 carbon skeleton of a flavonoid molecule consists of two aromatic rings connected by an oxygen-containing heterocyclic ring. This 15C skeleton is then modified by hydroxylation, glycosylation, methylation, and/or acylation to form the different types of flavonoids including aurones, flavanones, flavones, isoflavonoids, flavonols, anthocyanins, etc. The biochemistry and enzymology of the biosynthetic pathway that produces flavonoids is well understood in three model species: maize (*Zea mays* L.), snapdragon (*Antirrhinum majus* L.), and petunia (*Petunia ×hybrida*

Vilm.) (Holton and Cornish, 1995). A general pathway for flavonoid biosynthesis is shown in Fig. 1.

Flavonoids are often divided into two groups: the copigments (ex. flavanones, flavones, chalcones, etc.) and the anthocyanins (ex. petunidin, pelargonidin, cyanidin, etc.) (Griesbach and Batdorf, 1995). Anthocyanins accumulate in the vacuoles of epidermal or sub-epidermal cells and produce pink, magenta, red, blue, and violet colored flowers (Ben-Meir et al., 2002). Copigments are also found in the vacuole but they generally produce pale yellow, cream, ivory or colorless flowers when they occur in the absence of anthocyanins (Mol et al., 1989). Although certain copigments (flavones, flavonols) often appear colorless to humans, their strong absorption in the UV range makes them visible to insects.

Copigments and anthocyanins intermingle in the vacuole and form an anthocyanin-copigment complex. The physical exchange of electrons between the anthocyanin and copigment molecules within this complex generates the actual color that we perceive (Griesbach and Batdorf, 1995). The hue and intensity of this color is dependent on the type and relative amount of anthocyanins and copigments present in this complex (Forkmann, 1991). In general, the higher the concentration of the copigment relative to the anthocyanin, the bluer the flower color will appear (Griesbach, 1997). A high concentration of copigments in the vacuole is important because it improves the stability of anthocyanin molecules at low pHs (Yabuya et al., 2000), (Griesbach, 1997).

Anthocyanidins are anthocyanins stripped of their one or more sugar moieties. Anthocyanidins differ in the number and positioning of the B-ring hydroxyl group and by the degree and methylation of these group(s). Six common anthocyanidins are delphinidin, petunidin, peonidin, malvidin, pelargonidin, and cyanidin (Fig. 1). Generally flower color is

highly correlated with the type of anthocyanidin pigments present in the vacuole. However, abiotic factors such as light, temperature, and water stress can influence flower color by affecting the input that goes into the flavonoid biosynthetic pathway (Ben-Meir et al., 2002). Additionally, biotic factors such as vacuolar pH, the presence of metal ions, co-pigmentation, and cell shape also affect flower color (Griesbach, 1997), (Ben-Meir et al., 2002), (Mol et al., 1989).

The biochemistry and genetics that control flavonoid biosynthesis have been studied throughout the past century, primarily due to a desire to understand the basis of flower color. Since many of the genes that encode for specific enzymes and modifiers of flavonoid biosynthesis have already been identified, it is now possible in certain species to associate different flower colors with differences in a single gene. Genes of the flavonoid biosynthetic pathway can be divided into two different categories: structural and regulatory. Structural genes encode for specific enzymes in the pathway that directly affect the phenotype of the flower by controlling the type and amount of pigment(s) that is(are) produced. Regulatory genes affect flower color more indirectly by encoding for transcriptional or post-transcriptional factors that control the expression of structural genes. Usually regulatory genes influence the type, intensity, and patterning of pigmentation.

Stokes aster is an attractive herbaceous perennial native to the southeastern United States although it is cold hardy to USDA hardiness zone 5. Stokes aster is highly regarded for its versatility in the landscape and its large aster-like flowers that are typically 8 to 12 cm in diameter (Gettys and Werner, 2002). Flowers of stokes aster are characteristically pale blue or lavender, but taxa do exist with violet, pale yellow, pale magenta, or albescent colored flowers. Most cultivars of stokes aster originated from natural populations, gardens,

or nursery production fields, thus no formal documentation exists concerning the pedigrees of these cultivars. Additionally, there is little information available concerning the biochemistry of flower color in this species. The objective of this study was to characterize the genetic and biochemical basis of flower color in stokes aster (*Stokesia laevis*). Based on these investigations, the potential for breeding new cultivars with specific flower colors is discussed and a hypothetical biochemical pathway for flavonoid biosynthesis in stokes aster is presented.

MATERIALS and METHODS

Plant Material

Ten commercial cultivars of stokes aster were used in this study including 2 albescent flowered taxa (‘Alba’, ‘Silver Moon’), 2 pale magenta flowered taxa (‘Maroon, ‘Colorwheel’), 1 pale yellow flowered taxa (‘Mary Gregory’), 2 violet flowered taxa (‘Honeysong Purple’, ‘Purple Parasols’), 1 lavender flowered taxa (‘Peaches’), and 2 pale blue flowered taxa (‘Omega Skyrocket’, ‘Blue Danube’). All plants were maintained as asexually propagated clones with the exception of ‘Omega Skyrocket’, which was both asexually propagated and reproduced sexually from “true to type” seed. All plant materials used in this study were obtained from the breeding collection of D.J. Werner (North Carolina State University, Raleigh, NC) except for the seed generated ‘Omega Skyrocket’ which was obtained from Jelitto Seed Co. (Schwarmstedt, Germany).

Tissue Collection and Extraction

Flower petals (1-3 grams fresh weight) were collected and immediately placed into a freezer (0° C) for storage. Anthocyanidin and copigment compounds were extracted simultaneously with 20 ml 1% HCl in MeOH until the tissue was colorless. The resulting extract was filtered and reduced to dryness at 40° C under reduced pressure. The residue was then dissolved in approximately 10 ml of 100% ethanol, again reduced to dryness at 40° C under reduced pressure, and then dissolved in a minimal volume of 1 % HCl in MeOH.

Anthocyanin and Copigment Analysis

Anthocyanins were characterized using high resolution, high performance liquid chromatography (HPLC) as previously described (Griesbach et al., 1991). Individual anthocyanins were separated on a 7.8 x 300 mm Bondapak C18 column (Waters, Milford, Mass.) using a 30-min linear gradient of 0 to 10% (v/v) acetonitrile in aqueous 15% (v/v) acetic acid, followed by a 10-min linear increase to 20% (v/v) acetonitrile. The column was then held at 20% (v/v) acetonitrile for an additional 10 minutes. Flow rate was 1.0 ml min^{-1} and detection was by visual absorption at 540 nm. Copigments were analyzed in a similar manner as described above with changes in the HPLC elution buffer (1% triethylamine at pH 3.0), gradient (0 to 20% (v/v) linear gradient over 20 minutes and a hold at 20% (v/v) for 20 minutes), and detection wavelength (340 nm).

Acid Hydrolysis and Aglycone Analysis

Following MeOH extraction, 200 uL of each sample was acid hydrolyzed at 100 °C in 3 N HCl for 30 minutes in order to release the anthocyanidin and copigment aglycones

from their sugar moieties. Anthocyanidin aglycones were then separated by HPLC using a 20-min linear gradient of 0 to 15% (v/v) acetonitrile in aqueous 1.5% (v/v) phosphoric acid and 15% (v/v) acetic acid and then held at 15% (v/v) acetonitrile for an additional 20 minutes. Flow rate was 1.0 ml min^{-1} and detection was by visual absorption at 540 nm. Identification of unknown anthocyanidins was determined by co-injecting unknown samples with known anthocyanidin standards previously extracted and purified from other species. Copigment aglycones were analyzed in a similar manner as described above except for the HPLC elution buffer (1% triethylamine at pH 3.0), gradient (0 to 20% (v/v) linear gradient in 20 minutes with a hold at 20% (v/v) for an additional 20 minutes), and detection wavelength (340 nm). The identity of the unknown copigment aglycone was determined by co-injecting unknown samples with known standards previously purified from other species.

Inheritance Analysis

F_1, F_2, and BC_1 families derived from controlled hybridizations of various cultivars were used to investigate the inheritance of flower color. All F_1 hybridizations were made on potted plants grown in the Horticultural Science Greenhouses at North Carolina State University during the summers of 1999, 2000, and 2001. Average daytime temperatures in the greenhouse ranged from 25° to 35° C, and water was provided as needed. Plants were fertilized daily with 100 ppm of Peters 20-20-20 General Purpose fertilizer (The Scotts Co., Marysville, Ohio) using a Dosatron model no. DI 16 11 GPM (Dosatron International, Inc., Clearwater, Fla.). Crosses were made by hand using a camel's hair paintbrush. Flowers were pollinated once daily from the day they opened until they senesced. Paintbrushes were immersed in 70% ethanol and allowed to air-dry between different crosses to prevent

contamination of pollen from previous crosses. Flowers were labeled with jewelry tag that listed the parents and the date. All cultivars used in this study were self-sterile except for 'Alba', so emasculation was unnecessary (Gettys and Werner, 2002). Because F_1 plants were also presumably self-sterile, F_2 populations were obtained by reciprocally intercrossing several full-sibling F_1 plants either by hand or with insects. Plants that were cross-pollinated using insect activity were handled in one of two ways. In most cases, a minimum of 5 and a maximum of 15 full-sibling F_1 plants from a single family were planted in isolation blocks located at the Sandhills Research Station in Jackson Springs, N.C. These isolation blocks were located at least 500 m away from each other in every direction to prevent the possibility of cross-pollination among the blocks. Native insects cross-pollinated the plants within these isolation blocks. Alternatively, full-sibling F_1 plants were placed inside a screened enclosure, which contained hives of captive honeybees (*Apis mellifera*) and bumblebees *(Bombus impatiens*) to facilitate cross-pollination.

Upon maturation, seeds heads were removed and allowed to dry at room temperature in brown paper bags. The seeds were then removed from the chaff by hand and repackaged into labeled manila coin envelopes and stored (3-4° C, 25 % relative humidity). Since evidence has shown that seeds of stokes aster do not require stratification to germinate, (Gettys and Werner, 2002), unstratified seeds were sown in December into 7 in^3 elongated tree tubes (RLC-7 Super "Stubby" Cells, Stuewe and Sons, Inc., Corvallis, Ore.) filled with Fafard No. 4-P potting medium (Conrad Fafard, Inc., Agawam, Mass.). The seeds germinated and grew for 5 months in the greenhouse before they were transplanted directly into the field in plots located either at the Horticultural Field Lab in Raleigh, N.C. or at the Sandhills Research Station in Jackson Springs, N.C. Progeny were visually characterized

for flower color in the summers of 2000, 2001, 2002, and 2003. When segregation was observed, flower data were analyzed using the X^2 goodness of fit test with a confidence level of 0.05. Due to the appropriately large population sizes, the Yates correction factor was not used. Data were pooled for analysis from similar populations if the X^2 test for homogeneity indicated that there was no heterogeneity among the different populations.

RESULTS and DISCUSSION

Anthocyanidin Identification

Results from HPLC analyses are summarized in Table 1. Anthocyanidin profiles for cultivars with pale blue ('Omega Skyrocket', 'Blue Danube'), lavender ('Peaches'), and violet flowers ('Honeysong Purple', 'Purple Parasols') each contained a single major peak (data not shown). This unknown peak was identified as petunidin using co-elution with three known standards (petunidin, peonidin, and pelargonidin) (Fig. 2). These results suggest that differences in flower color (pale blue, lavender, violet) in these cultivars is not due to the presence of different anthocyanidins, but instead may be attributable to differences in copigmentation, vacuolar pH, glycosylation or acylation patterns, or the presence/absence of metal ions (Griesbach, 1997), (Forkmann, 1991). Anthocyanidin profiles for cultivars with pale magenta flowers ('Maroon', 'Colorwheel') were identical and each contained a single major peak (data not shown). Co-elution with a known standard of cyanidin, confirmed that this peak was cyanidin (Fig. 3). Petunidin was not present in either 'Maroon' or 'Colorwheel'. Flowers of F_1 hybrids of 'Maroon' (cyanidin) × 'Honeysong' (petunidin) and 'Peaches' (petunidin) × 'Colorwheel' (cyanidin), each contained only

petunidin (Fig. 4). These biochemical results support earlier inheritance studies (Gettys and Werner, 2002) that demonstrated that pale blue/lavender/violet flower color (petunidin) is dominant to pale magenta flower color (cyanidin) in the F_1 generation. The anthocyanidin profile for albescent flowers ('Alba') indicated that a trace amount of anthocyanidin pigment(s) was(were) present (data not shown). However, because the quantity of the pigment(s) was so small, further purification will be necessary in order to identify the anthocyanidin pigment(s) was (were) present in this cultivar. Pale yellow flowers ('Mary Gregory') did not contain carotenoids or anthocyanins (data not shown). These results suggest that pale yellow flowers of stokes aster contain flavonoids other than anthocyanins.

Copigment Identification

The aglycone copigment profiles for pale blue, lavender, violet, pale magenta, and albescent flowers each contained a single identical peak (data not shown). This peak was identified as the copigment, luteolin, using co-elution with a known standard of luteolin (Fig. 5) and UV spectral analysis. Luteolin is a common flavone that is usually colorless *in vivo* when it occurs as an aglycone. Even though this copigment is colorless it affects flower color by forming an anthocyanidin-copigment complex. The ratio of anthocyanidin to copigment molecules in this complex influences the hue, intensity, and stability of flower color. This phenomenon may explain why flowers of 'Omega Skyrocket' (pale blue), 'Peaches' (lavender), and 'Honeysong Purple' (violet) are different even though they all contain the same pigments (petunidin and luteolin).

The aglycone copigment profiles for 'Mary Gregory' and a pale yellow flowered individual selected at random from a 'Peaches' × 'Mary Gregory' F_2 family confirmed that

both of these individuals also contain luteolin (Fig. 6). As stated earlier, the aglycone of luteolin stripped of its side groups is usually colorless. However, there are forms of luteolin such as 2'-hydroxyluteolin that are yellow due to the presence of a hydroxyl group at the second position (Forkmann, 1991). Since our samples were acid hydrolyzed prior to identification, we could not determine whether the luteolin in these samples had a hydroxyl group at the 2' position. Therefore, it is possible that the luteolin found in 'Mary Gregory' and the yellow flowered F_2 was actually the yellow form of luteolin, 2'-hydroxyluteolin.

A second explanation for pale yellow flowers involves the phenomenon of intramolecular copigmentation. Intramolecular copigmentation occurs when colorless molecules aggregate together on a surface or at high concentrations and produce yellow coloration. In the case of *Lathyrus chrysanthus* Boiss., *Camellia chrysantha* (Hu) Tuyama, and *Eustoma grandiflorum* (Raf.) Shinn., it has been shown that intramolecular complexes of colorless copigments such as kaempferol and quercetin are capable of producing yellow flower color (Markham et al., 2001). Therefore, it is possible that luteolin which is usually colorless may produce pale yellow flower color in stokes aster if it forms an intramolecular complex.

Based on these examples, it is proposed that yellow coloration in flowers of stokes aster is due to the presence of luteolin. However, it is unclear whether intramolecular copigmentation or hydroxylation play an important role. Further analysis will be necessary to ascertain which form(s) of luteolin are present in order to definitively determine the exact basis for this phenotype.

Inheritance Analysis

Previous work demonstrated that pale blue, lavender, and violet flowers are dominantly inherited in the F_1 generation over pale yellow, pale magenta, and albescent flowers (Gettys and Werner, 2002). In order to better understand the inheritance of flower color, F_2 and BC_1 populations were created from certain blue × albescent, blue × yellow, and albescent × yellow F_1s. These results are summarized in Table 2.

Blue × Albescent. F_2 families derived from 'Alba' (albescent) × 'Blue Danube' (pale blue) and 'Alba' (albescent) × 'Peaches' (lavender) were evaluated in the summers of 2001 and 2002 to determine the mechanism of inheritance that controls the expression of pale blue/lavender versus albescent colored flowers. A chi-square test for homogeneity gave no evidence of heterogeneity between these two populations ($X^2 = 0.02$, $P = 0.88$), so the data were pooled for final analysis. A total of 606 F_2 plants were visually examined and placed into two phenotypic classes based on flower color (458 pale blue/lavender, 148 albescent). The approximate 3 blue/lavender : 1 albescent ratio ($X^2 = 0.11$, $P = 0.74$) observed in these two F_2 families suggests that albescent flower color is recessive and controlled by a single gene. The BC_1 populations of ('Peaches × ('Peaches' × 'Alba')) and (('Peaches' × 'Alba') × 'Alba') segregated 1 lavender : 0 albescent and 1 lavender : 1 albescent ($X^2 = 0.36$, $P = 0.55$) respectively. These ratios support the model that a single gene controls albescent flower color. The BC_1 population of (('Peaches' × 'Alba') × 'Peaches') segregated 103 lavender : 3 albescent. The recovery of albescent flowered progeny in this BC_1 population is inconsistent with our model and is probably the result of self-pollination or an accidental cross-pollination. Although F_1 hybrids of stokes aster, like the one used in this cross, are

presumably self-sterile, self-pollination can occur since it is difficult to emasculate flowers of this species. Despite the fact that the aforementioned BC_1 population produced unexpected results, the results from the other F_1, F_2, and BC_1 populations strongly support the model that a single gene controls blue/lavender versus albescent flower color in stokes aster. We propose that this gene, designated *Alb*, has two alleles and demonstrates complete dominance. Plants with the *AlbAlb* or *Albalb* genotype are blue/lavender flowered and plants with the *albalb* genotype are albescent flowered. Based on the biochemical analysis of these phenotypes presented in this work we can conclude that the wild type form of this gene (*Alb*) allows for the normal synthesis and accumulation of petunidin and luteolin, while the mutant form (*alb*) lowers either the synthesis and/or the accumulation of both of these compounds.

<u>*Blue* × *Pale Yellow*</u>. F_2 progeny derived from two separate populations of 'Peaches' (lavender) × 'Mary Gregory' (pale yellow) were evaluated in the summers of 2001, 2002, and 2003 to ascertain how lavender and pale yellow flowered forms are inherited. A chi-square test for homogeneity gave no evidence of heterogeneity between these two different populations ($X^2 = 0.69$, $P = 0.41$), so the data were pooled. A total of 849 F_2 plants were classified visually for flower color and placed into two phenotypic classes (623 lavender, 226 pale yellow). These frequencies are consistent with the hypothetical 3:1 segregation ratio ($X^2 = 1.19$, $P = 0.28$) suggesting the presence of single gene. We propose that a single gene, designated *Y*, which has two alleles and demonstrates complete dominance, controls pale yellow flower color. Based on this classification, plants with the *YY* or *Y*y genotype are pale blue/lavender flowered (petunidin and luteolin) and plants with the *yy* genotype are

pale yellow flowered (luteolin only). The dominant or wild type state of this gene (*Y*) allows for the normal synthesis of anthocyanidins, in this case petunidin, while the recessive or mutant form of this gene (*y*) completely blocks the production of anthocyanidins.

Albescent × Pale Yellow. F_2 progeny derived from the hybridizations of 'Mary Gregory' (pale yellow) × 'Silver Moon' (albescent), 'Mary Gregory' (pale yellow) × 'Alba' (albescent), and 'Alba' × 'Mary Gregory' (yellow) were evaluated in the summers of 2001 and 2002 to examine the interaction between *Alb* and *Y*. A chi-square test for homogeneity was not significant ($X^2 = 3.71$, $P = 0.45$; $X^2 = 3.70$, $P = 0.45$), for these populations so the data were pooled. A total of 278 F_2 plants were visually examined in the field and placed into three classes (177 pale blue/lavender, 50 albescent, 51 pale yellow). This ratio was tested for goodness of fit to a 9:3:4 and a 9:3:3 ratio. The first hypothesis (9:3:4) is based on the assumption that an epistatic interaction exists between *Alb* and *Y*. Under this hypothesis, we are proposing that the *Y* locus is epistatic to the *Alb* locus, such that all plants with the *yy* genotype have pale yellow flowers regardless of the genotype at the *Alb* locus. The second hypothesis (9:3:3) is based on the assumption that the first hypothesis of recessive epistasis is correct, but the combination of *albalb* and *yy* in the same plant (the double recessive genotype *albalb yy*) is lethal.

The observed ratio of 177:51:50 did not fit the 9:3:4 ratio within the 5% confidence interval ($X^2 = 7.73$, $P = 0.02$), but did fit the 9:3:3 ratio ($X^2 = 1.63$, $P = 0.44$) within these limits. Based on these X^2 values it appears that the hypothesis of recessive epistasis plus lethality (9:3:3) is more appropriate than the hypothesis of recessive epistasis (9:3:4). However, since we have no reason to believe that the double recessive genotype *albalb yy* is

lethal, we propose that the hypothesis of recessive epistasis between *Alb* and *Y* without lethality is more plausible. Furthermore, we are proposing that the deficit in the expected number of yellow flowered plants (50 observed versus 71 expected) is probably due to yellow flowered plants having lower germination or reduced field survival.

In order to conclude if either of these hypotheses are correct we will need to determine the genotype of the 50 pale yellow flowered plants recovered from these populations. If it found that the double recessive genotype (*albalb yy*) does exist and it is pale yellow flowered the hypothesis of epistasis is more appropriate. Alternatively, if none of the pale yellow flowered progeny have the *albalb yy* genotype, then the hypothesis of epistasis plus lethality is more appropriate. In order to determine the genotype(s) of the pale yellow flowered progeny we will have to cross all of the progeny to an albescent flowered tester (*albalb YY*). Depending on how the progeny from these crosses segregate it should be possible to determine the unknown genotype(s) with respect to both loci (*Alb* and *Y*). A plant with the *AlbAlb yy* genotype should produce all blue flowered progeny when crossed to an albescent flowered plant, where as a plant with the *Albalb yy* genotype should segregate equally for blue and albescent flowered progeny. A plant with the double recessive genotype, *albalb yy*, should segregate equally for pale yellow and albescent flowered progeny. Based on the results of the aforementioned testcross we should be able to confirm whether or not the double recessive genotype, *albalb yy*, is or is not lethal and whether or not it is pale yellow flowered.

BC_1 populations of ('Alba' × ('Alba' × 'Mary Gregory')) and ('Mary Gregory' × ('Alba' × 'Mary Gregory')) closely matched the expected segregation ratios of 1 blue: 1 albescent ($X^2 = 0.18$, $P = 0.67$) and 1 blue: 1 pale yellow ($X^2 = 0.04$, $P = 0.84$) respectively.

The 82:58:2 ratio of the BC_1 population of ((‘Alba’ × ‘Mary Gregory’) × ‘Alba’) did not fit the expected 1 blue: 1 albescent: 0 pale yellow segregation ratio. The increased number of blue flowered progeny and the decreased number of albescent flowered progeny coupled with the unexpected recovery of 2 pale yellow flowered progeny suggested that the female plant in this cross (‘Alba’ × ‘Mary Gregory’) is nominally self-fertile. This assumption was confirmed in previous work (Gettys and Werner, 2002) where it was determined that an ‘Alba’ × ‘Mary Gregory’ F_1 hybrid plant is capable of producing an average of 4.2 selfed seeds per inflorescence. Depending on the total number of seeds collected from each flower, this percentage of possible selfs could significantly influence segregation ratios in populations where an ‘Alba’ × ‘Mary Gregory’ F_1 hybrid plant was used as the female. Based on these findings, we excluded any populations that used an ‘Alba’ × ‘Mary Gregory’ F_1 hybrid plant as the female parent.

Biochemical Pathway

The Alb gene. Results from the genetic and HPLC analyses suggest that there are at least two, possibly three, major genes that control flower color in stokes aster. *Alb*, allows for the wild type synthesis and accumulation of anthocyanidins (petunidin) and copigments (luteolin), while the mutant form, *alb*, either reduces the synthesis and/or prevents the accumulation of both of these compounds. In nature, many plant species have mutants in which anthocyanin biosynthesis is reduced or completely blocked. A plant that is completely devoid of anthocyanins (acyanic) usually has a mutation in a structural gene that encodes for a necessary enzyme in the biosynthetic pathway, like chalcone synthase (CHS)

or flavanone 3-hydroxylase (F3H). Plants that have a reduced level of pigmentation as compared to the wild type usually have a mutation that affects a regulatory gene (Griesbach and Klein, 1993). Thus, based on our results we are tentatively concluding that *Alb* is a regulatory gene that affects anthocyanin biosynthesis in stokes aster (Fig. 7). One explanation for the reduced pigmentation in the albescent flowers of stokes aster involves the presence of a regulatory gene (*Alb*) that controls the transcription of other genes in the anthocyanin biosynthetic pathway. In *alb* plants, the transcription of these other genes does not occur normally so anthocyanin biosynthesis is reduced. Another explanation may be that *Alb* encodes for a post-transcriptional factor that is responsible for maintaining the stability of a particular mRNA. If the absence of this factor in an *alb* plant causes early degradation of this mRNA, it is conceivable that protein synthesis will be reduced, which will then cause a reduction in anthocyanin biosynthesis. A third explanation suggests that *Alb* encodes for a post-translational factor that prevents premature degradation of an enzyme produced by the pathway. Thus, *alb* plants contain less anthocyanins than *Alb* plants because this enzyme is degraded prematurely in the absence of this post-translational factor. A fourth explanation is that *alb* is the functionally active form that encodes for a regulatory protein that limits anthocyanin biosynthesis by physically interfering with the promoter of another gene in the pathway and thus preventing normal transcription of this gene. Although all of these explanations are plausible it would be impossible to confirm or deny any of them based only on the results of the present work.

We have confirmed that *alb* plants contain less than half of the normal amount of luteolin and almost none of the normal amount of anthocyanidins usually found in a wild type plant. From these observations one could assume that *alb* probably affects genes in the

pathway prior to the synthesis of luteolin (Fig. 7). A possible explanation for why luteolin synthesis is only slightly reduced instead of being virtually eliminated may have to do with the enzyme kinetics of flavone synthase (FNS) and flavanone 3-hydroxylase (F3H) (Fig. 7). Because we are assuming that *alb* affects steps prior to luteolin synthesis, it is reasonable to anticipate that the concentration of eriodictyol is probably lower in an *alb* plant. Presumably, FNS may be more active than F3H when the concentration of eriodictyol is lower than normal. Therefore, the FNS-mediated conversion of eriodictyol to luteolin would occur faster in an *alb* plant than the F3H-mediated conversion of eriodictyol to dihydroquercetin. The differences in the kinetics of these two enzymes, therefore creates a situation where more eriodictyol is used to produce luteolin versus anthocyanidins in cases where eriodictyol is in low supply.

The Y gene. In *Y* plants, the synthesis of anthocyanidins (petunidin) and copigments (luteolin) is normal, however, in *y* plants the synthesis of luteolin appears normal but the synthesis of anthocyanidins (petunidin) is completely eliminated. The HPLC aglycone copigment profile from a *y* plant indicates that luteolin is the only copigment aglycone present (Fig. 5). Other copigments such as dihydroquercetin and dihydromyricetin were not detected in this sample. This suggests that flavonoid biosynthesis is blocked prior to the synthesis of dihydroquercetin and dihydromyricetin in *y* plants (Fig. 7). Based on these findings we are proposing a hypothesis that *Y* is the structural gene that encodes for the enzyme, flavanone 3-hydroxylase (F3H). F3H catalyzes the stereospecific hydroxylation of flavanones (eriodictyol) to form dihydroflavonols (dihydroquercetin). In stokes aster, we propose that, *y* encodes for a non-functional form of F3H, which results in a block in the

pathway at this step. Based on this hypothesis, plants that carry this mutation in the homozygous state, *yy*, do not have the ability to produce anthocyanidins in the flowers. Interestingly, when vegetative material (leaves, scapes, crown) was examined from *y* plants, no anthocyanin pigmentation was observed even though these same tissues contained anthocyanin pigmentation in *Y* plants. Based on these observations, we are proposing that the expression of this gene occurs universally throughout the entire plant and is not just limited to the floral tissues. Thus, we are concluding that *y* plants do not make anthocyanins in the flowers or in the vegetative tissues.

Because it was difficult to extract luteolin from flowers that did not contain anthocyanins, we could not accurately quantify the amount of luteolin present in flowers of a *y* plant. Without this information we could not compare the quantity of luteolin found in anthocyanin-less (*y*) flowers to the quantity of luteolin found in anthocyanin-containing (*Y*) flowers. This comparison would have allowed us to determine if *y* plants synthesize more luteolin because of the hypothesized breakdown in the pathway at F3H or if the amount of luteolin synthesis remains unchanged.

Although many genes associated with the flavonoid biosynthetic pathway such as chalcone synthase (CHS) and chalcone isomerase (CHI) have multiple copies, research has determined that F3H is a single-copy gene in *Petunia ×hybrida*, *Dianthus caryophyllus*, *Callistephus chinesis*, *Rosa hybrida*, *Matthiola incana*, and *Arabidopsis thaliana* (Ben-Meir et al., 2002), (Bohm, 1998), (Dedio et al., 1995). Thus, it is quite reasonable to assume that a single gene such as, *Y*, encodes for F3H in stokes aster.

Zuker et al., (2002) were able to artificially eliminate the synthesis of flavanone 3-hydroxylase (F3H) in *Dianthus caryophyllus* by introducing an antisense copy of *F3h*. The

resulting anti-f3h plants were yellow flowered and accumulated only 2-4% of the normal amount of anthocyanins. The flowers of these transgenic plants were pale yellow due to the unmasking of the yellow colored compound, isosalipurposide (ISP), which is already present in most carnations. ISP is formed from chalcone prior to the step where F3H converts flavanones to dihydroflavonols. In this example, yellow flower color is not due to the creation of a new compound, but is instead due to the ISP that is already present normally. This paper provides an example similar to the example in stokes aster where pale yellow flowers are produced when the absence of F3H blocks normal anthocyanin biosynthesis.

The Mag gene. As determined by this work, most flowers of stokes aster contain petunidin, but mutants containing cyanidin do exist. In all cases, petunidin and cyanidin occurred independently of each other and were never detected in the same plant (Table 1). The proposed biochemical pathway (Fig. 7) indicates that petunidin containing flowers make the precursor, dihydroquercetin, in order to synthesize petunidin. As shown by the pathway, dihydroquercetin is also an essential precursor for the synthesis of cyanidin. Since petunidin and cyanidin were never detected in the same flower, one could surmise that dihydromyricetin is the preferred substrate of dihydroflavonol reductase (DFR) even if dihydroquercetin is also present. This hypothesis would explain why most flowers of stokes aster contain petunidin instead of cyanidin. Based on this hypothesis, one could assume that cyanidin synthesis only occurs when dihydromyricetin is not present. Therefore, cyanidin would be synthesized instead of petunidin in cases where flavonoid 3',5'-hydroxylase (F3'5'H), is nonfunctional and can not convert dihydroquercetin to dihydromyricetin. Based on these assumptions we are proposing that a third gene, designated *Mag*, exists in stokes

aster that encodes for F3'5'H and is capable of controlling the synthesis of petunidin versus cyanidin. This hypothesis is strictly hypothetical and requires future investigation, but it could explain why some flowers of stokes aster contain petunidin and other flowers contain cyanidin.

Gene expression

In most plants, genes that control the "early" steps in the flavonoid biosynthetic pathway are expressed throughout flower development, while genes that encode "later" steps are turned on just prior to anthesis. This phenomenon also appears to occur in stokes aster. Visual observations revealed that unopened petals of yellow flowers are yellow (luteolin) and unopened petals of blue, lavender, violet, or pale magenta flowers are creamy white (luteolin + other colorless flavonoids). Then approximately one day prior to anthesis, the creamy white petals turned to their respective color (blue, lavender, purple, or pale magenta) while the yellow petals remained yellow. These observations suggest that "early" genes in stokes aster like chalcone synthase, chalcone isomerase, flavanone 3'-hydroxylase, flavonoid synthase, flavonoid 3'-hydroxylase, and flavonoid 3'5'-hydroxylase are constantly expressed throughout flower development and "late" genes like dihydroflavonol 4-reduductase are not expressed until the last day before anthesis.

Breeding for Novel or Improved Flower Colors

There are several different approaches to breeding for different and improved flower colors in stokes aster, however, there are limitations due to the narrow diversity of the available germplasm. In order to create red flowers of stokes aster a mutation must be

identified or created that shifts the biochemical pathway towards the synthesis of pelargonidin (orange-red pigment) instead of petunidin or cyanidin (Fig. 1). For example, cultivars of 'Maroon' and 'Colorwheel' only contain cyanidin. However, if naringenin was used to produce dihydrokaempferol instead of eriodictyol, and this dihydrokaempferol was used to produce pelargonidin, it is conceivable that orange-red flowers could be produced (Fig.1). Another way to breed for red flowers does not affect anthocyanin biosynthesis but instead manipulates the pH in the vacuole. In general, cyanidin appears redder at an acidic pH, and bluer at a basic pH. Therefore, if a pH mutant was identified with a lower than average pH it could be used to alter the vacuolar pH in 'Maroon' and 'Colorwheel' to make them appear redder. Another way to develop a cultivar of stokes aster with red flowers would involve the screening for accessions with reduced copigmentation. When anthocyanins are complexed with copigments, they appear bluer than anthocyanins that are uncomplexed (Griesbach, 1984). In preliminary studies, cyanidin containing flowers of stokes aster had a higher ratio of copigments (luteolin) to anthocyanidins (cyanidin) than was found in the petunidin containing cultivars. This increased ratio of copigments to anthocyanidins may explain why pale magenta flowers of stokes aster appear to have a bluish tint that makes the color appear "muddy". In order to improve the hue and intensity of these flowers and make them appear red, the concentration of luteolin has to be reduced but not eliminated, since the total absence of luteolin may make the cyanidin molecules unstable. Alternatively, the concentration of cyanidin has to be increased without a subsequent increase in luteolin. One could envision that if the efficiency of flavone synthase (FNS) were reduced by a mutation, more precursor would be used to make cyanidin instead of luteolin. To date, no such mutation has been identified.

Breeding for a pure white flowers of stokes aster may be difficult. In other species, most pure white or nearly white flowers are the result of mutations in structural genes like (CHS, CHI, F3H, and DFR) that block the synthesis of anthocyanins. However, in stokes aster, a mutation like this would have to occur prior to the synthesis of luteolin, or the resulting flowers will be pale yellow instead of pure white. Thus, development of cultivars with pure white flowers may require that one screen for plants having a mutation in the genes that encode for CHS, CHI, or F3'H.

CONCLUSIONS

HPLC analysis of several cultivars, F_1, and F_2 hybrids of stokes aster revealed that petunidin and cyanidin were the two primary anthocyanidin pigments responsible for flower color. These two pigments occurred independently of each other in all cases at varying concentrations. Pale blue, lavender, and violet flowers contained a single anthocyanidin, petunidin. Pale magenta flowers contained only cyanidin. Albescent flowers did contain anthocyanins at a substantially reduced concentration, but it is unclear which anthocyanidin(s) were present. Luteolin was the primary copigment in all cultivars.

Our inheritance studies suggested that two genes control pale blue/lavender, albescent, and pale yellow flower color in stokes aster. It was proposed that a single gene, designated *Alb*, controls albescent flower color and pale yellow flower color is controlled by a second gene, designated *Y*. It was also proposed that *Y* is the structural gene that encodes for the enzyme, flavanone 3-hydroxylase (F3H). We proposed that the homozygous recessive form of *Y* is epistatic to *Alb*, but it is still unclear whether the double recessive

(*albalb yy*) is lethal, so more testing will be necessary. A third gene, designated *Mag*, was proposed to encode for flavonoid 3',5'-hydroxylase (F3'5'H).

LITERATURE CITED

Ben-Meir, H., A. Zuker, D. Weiss, and A. Vainstein. 2002. Molecular control of floral pigmentation: Anthocyanins, p. 253-272. In: A. Vainstein (ed.). Breeding for ornamentals: Classical and molecular approaches. Kluwer Academic Publishers, Netherlands.

Bohm, B.A. 1998. Introduction to flavonoids. Harwood Academic, Vancouver, B.C.

Clegg, T.G. and M.L. Durbin. 2000. Flower color variation: A model for the experimental study of evolution. PNAS 97(13):7016-7023.

Courtney-Gutterson, N. 1994. The biologist's palette: genetic engineering of anthocyanin biosynthesis and flower color. p.93-124. In: B.E. Ellis et al. (eds.) Genetic engineering of plant secondary metabolism. Plenum Press, New York.

Dedio, J., H. Saedler, and G. Forkmann. 1995. Molecular cloning of the flavanone 3B-hybroxylase gene (FHT) from carnation (*Dianthus caryophyllus*) and analysis of stable and unstable FHT mutants. Theor. Appl. Genet. 90:611-617.

Forkmann, G. 1991. Flavonoids as flower pigments: The formation of the natural spectrum and its extension by genetic engineering. Plant Breeding 106:1-26.

Gettys, L.A. and D.J. Werner. 2002. Stokes Aster. HortTechnology 12(1):138-142.

Griesbach, R.J. 1984. Effects of carotenoids-anthocyanin combinations on flower color. J. of Hered. 75:145-147.

Griesbach, R.J. 1997. The biochemical basis for the blue flower color mutations in *Doritis pulcherrima* and *Phalaenopsis violacea*. Lindleyana 12(2):64-71.

Griesbach, R.J. and S. Asen. 1990. Characterization of the flavonol glycosides in *Petunia*. Plant Sci. 70:49-56.

Griesbach, R.J., S. Asen, and B.A. Leonnarat. 1991. *Petunia hybrida* anthocyanins acylated with caffeic acid. Phytochemistry 30(5):1729-1731.

Griesbach, R.J. and T.M. Klein. 1993. In situ genetic complementation of a flower color mutant in *Doritis pulcherrima* (Orchidaceae). Lindleyana 8(4):223-226.

Griesbach, R.J. and L. Batdorf. 1995. Flower pigments within *Hemerocallis fulva* L. fm. *Fulva*, fm. *Rosea*, and fm. *Disticha*. HortScience 30(2)353:354.

Harborne, J.B., and C.A. Williams. 2000. Advances in flavonoid research since 1992. Phytochemistry 55:481-504.

Holton, T.A. and E.C. Cornish. 1995. Genetics and biochemistry of anthocyanin biosynthesis. The Plant Cell 7:1071-1083.

Markham, K.R., K.S. Gould, and K.G. Ryan. 2001. Cytoplasmic accumulation of flavonoids in flower petals and its relevance to yellow flower colouration. Phytochemistry 58:403-413.

Mol, J.N.M., A.R. Stuitje, and A. van der Krol. 1989. Genetic manipulation of floral pigmentation genes. Plant Mol. Biol. 13:287-294.

Winkel-Shirley, B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126:485-493.

Yabuya, T., M. Saito, T. Iwashina, and M. Yamaguchi. 2000. Stability of flower colors due to anthocyanin-flavone copigmentation in Japanese garden iris, *Iris ensata* Thunb. Euphytica 115:1-5.

Zuker, A., T. Tzfira, H. Ben-Meir, M. Ovadis, E. Shklarman, H. Itzhaki, G. Forkmann, S. Martens, I. Neta-Sharir, D. Weiss, and A. Vainstein. 2002. Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Molecular Breeding 9:33-41.

Table 1. Anthocyanidin and copigment composition of flowers from different cultivars, F_1 hybrids, and F_2 hybrids of stokes aster.

Taxa	Flower color	Anthocyanidin[z]		Copigment[z]
		Petunidin	Cyanidin	Luteolin
Omega Skyrocket	pale blue	+	–	+
Blue Danube	pale blue	+	–	not tested
Peaches	lavender	+	–	+
Honeysong Purple	violet	+	–	+
Purple Parasols	violet	+	–	+
Alba	albescent	trace ?	trace ?	+
Mary Gregory	pale yellow	–	–	+
Colorwheel	pale magenta	–	+	+
Maroon	pale magenta	–	+	+
Maroon × Peaches F_1	violet	+	–	not tested
Maroon × Honeysong Purple F_1	violet	+	–	not tested
Mary Gregory × Omega Skyrocket F_1	violet	+	–	+
Peaches × Colorwheel F_1	lavender	+	–	not tested
Peaches × Mary Gregory F_2	pale yellow	not tested	not tested	+

[z]+ = present; – = absent;

Table 2. Segregation of flower color in F_2 and BC_1 families of stokes aster

Blue × Albescent Populations	Flower Color Ratio blue[z] : albescent Observed	Expected	X^2	P
F_2 (Alba × Blue Danube)	322:105	3:1	0.04	0.84
F_2 (Alba × Peaches)	136:43	3:1	0.09	0.76
Total	458:148	3:1	0.11	0.74
BC_1 (Peaches × (Peaches × Alba))	37:0	1:0	all blue[z]	
BC_1 ((Peaches × Alba) × Alba)	37:32	1:1	0.36	0.55
BC_1 ((Peaches × Alba) × Peaches)	103:3	1:0	all blue[z]	

Blue × Yellow Populations	Flower Color Ratio blue[z] : pale yellow Observed	Expected	X^2	P
F_2 (Peaches × Mary Gregory)	76:23	3:1	0.16	0.68
F_2 (Peaches × Mary Gregory)	547:203	3:1	1.71	0.19
Total	623:226	3:1	1.19	0.28

Yellow × Albescent Populations	Flower Color Ratio blue[z]:albescent:pale yellow Observed	Expected	X^2	P
F_2 (Mary Gregory × Silver Moon)	15:7:6	9:3:4	0.76	0.68
F_2 (Mary Gregory × Alba)	40:7:14	9:3:4	2.77	0.25
F_2 (Alba × Mary Gregory)	122:36:31	9:3:4	7.91	0.02
Total	177:50:51	9:3:4	7.73	0.02
F_2 (Mary Gregory × Silver Moon)	15:7:6	9:3:3	0.57	0.75
F_2 (Mary Gregory × Alba)	40:7:14	9:3:3	2.80	0.25
F_2 (Alba × Mary Gregory)	122:36:31	9:3:3	1.96	0.38
Total	177:50:51	9:3:3	1.63	0.44
BC_1 (Alba × (Alba × Mary Gregory))	105:99:0	1:1:0	0.176[y]	0.67
BC_1 ((Alba × Mary Gregory) × Alba)	82:58:2	1:1:0		
BC_1 (Mary Gregory × (Alba × Mary Gregory))	11:0:12	1:0:1	0.043[y]	0.83
BC_1 ((Alba × Mary Gregory) × Mary Gregory)	59:2:38	1:0:1		

[z]blue flower color includes pale blue, lavender, and violet colored flowers

[y]X^2 values only include the phenotypic classes that have observed individuals

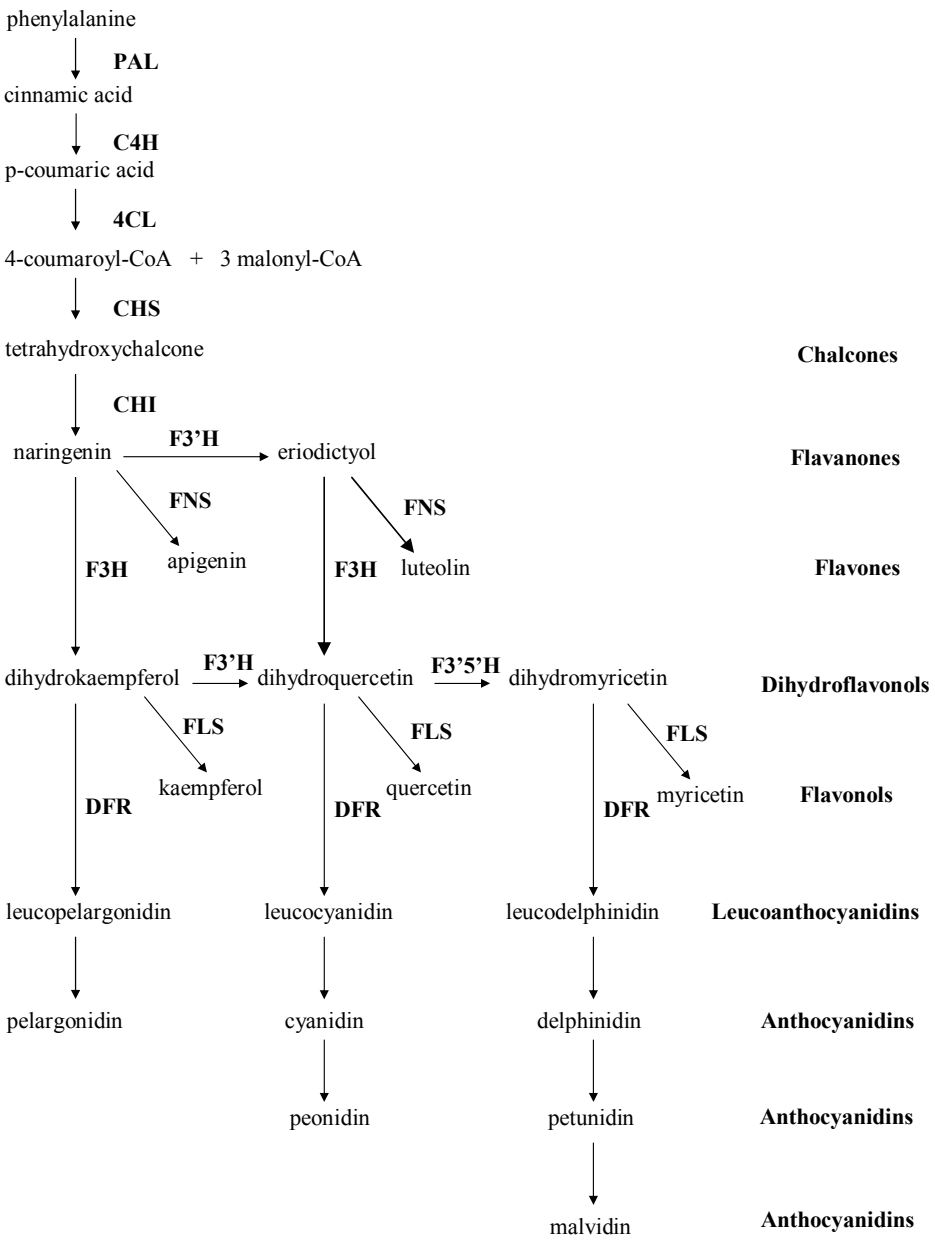


Figure 1. Flavonoid biosynthetic pathway (modified from Ben-Meir et al., 2002; Winkel-Shirley, 2001).

Abbreviations: PAL, phenylalanine ammonia-lysase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase.

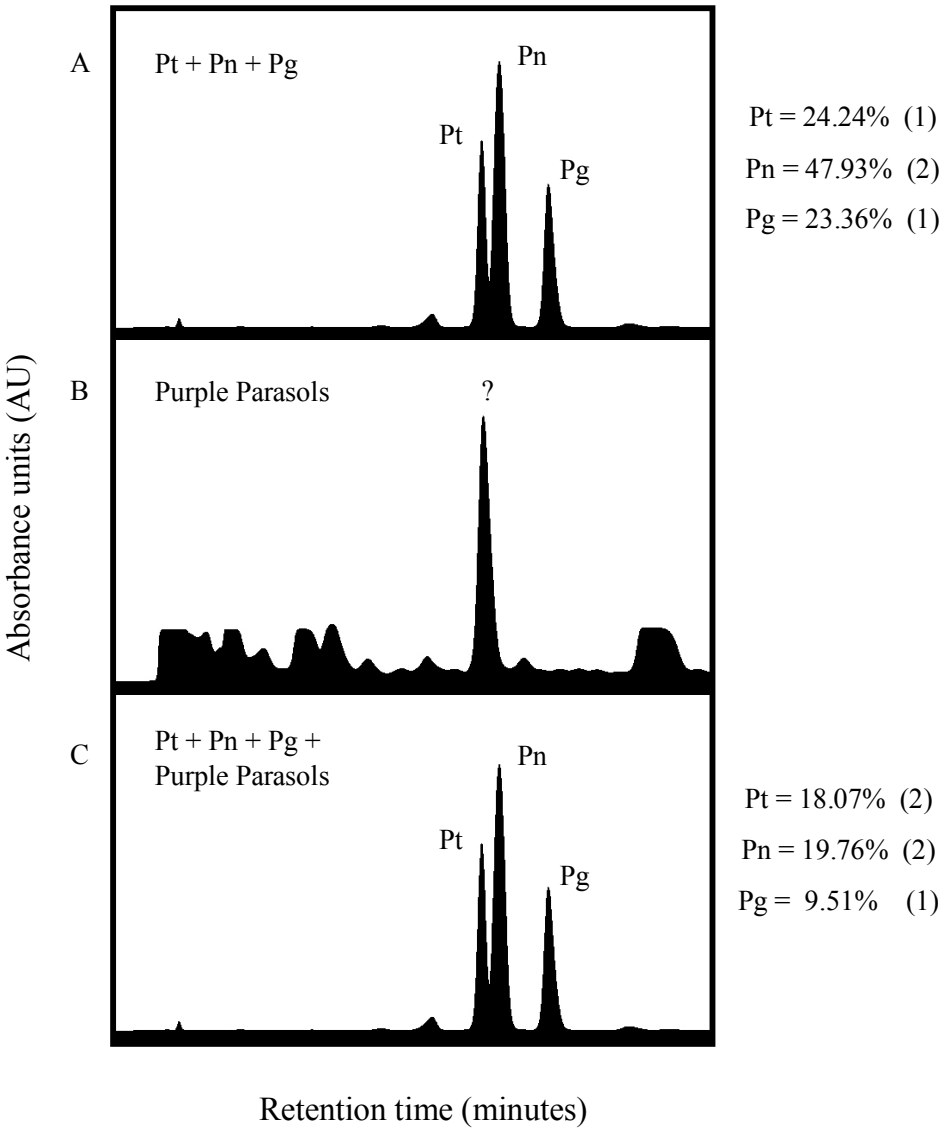


Figure 2. Anthocyanidin HPLC profiles from samples that contained (A) a standard solution of petunidin, peonidin, and pelargonidin, (B) extract from flowers collected from 'Purple Parasols', and (C) the standard solution of petunidin, peonidin, and pelargonidin mixed with the extract from 'Purple Parasols'. Peak areas measured as a percentage of the total are given to the right of the figure for important peaks along with their approximate ratios.

Abbreviations: Pt, petunidin; Pn, peonidin; Pg, pelgargonidin.

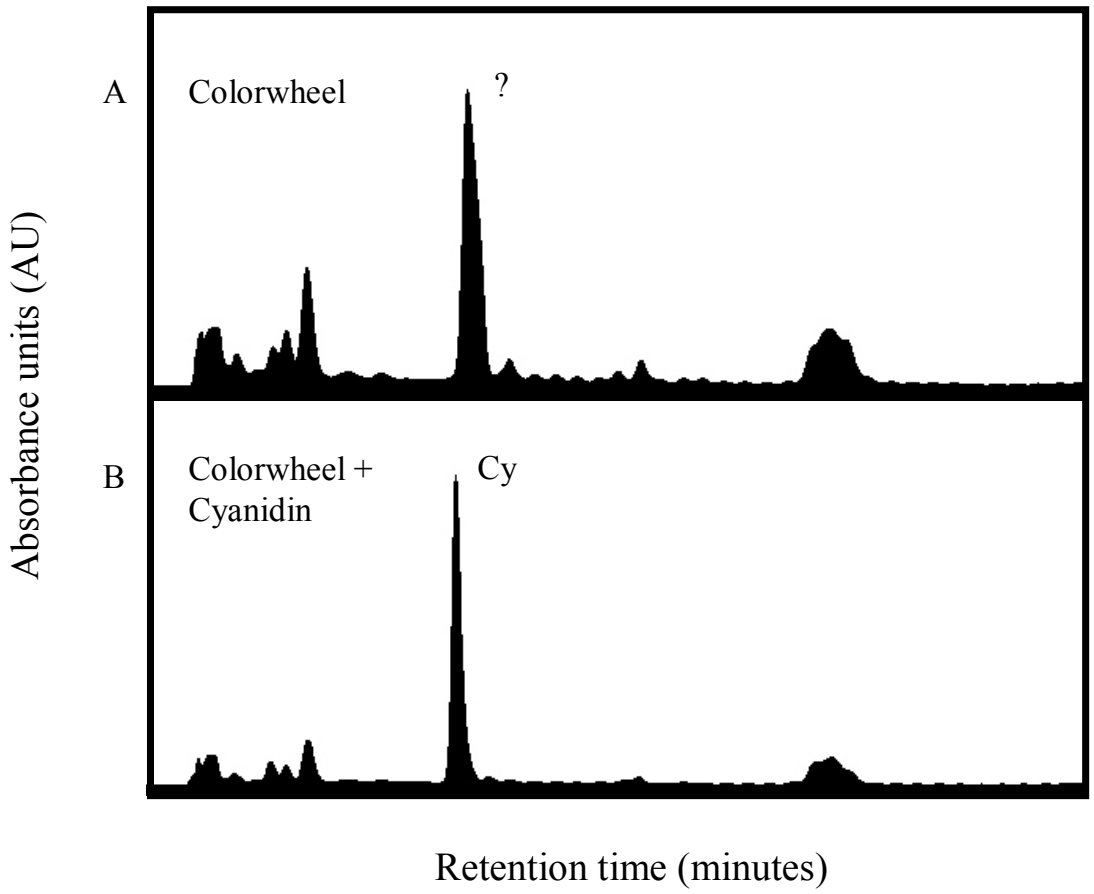


Figure 3. Anthocyanidin HPLC profiles from samples that contained (A) extract from flowers collected from 'Colorwheel' and (B) mixture of the extract collected from 'Colorwheel' plus a standard solution of cyanidin.

Abbreviations: Cy, cyanidin.

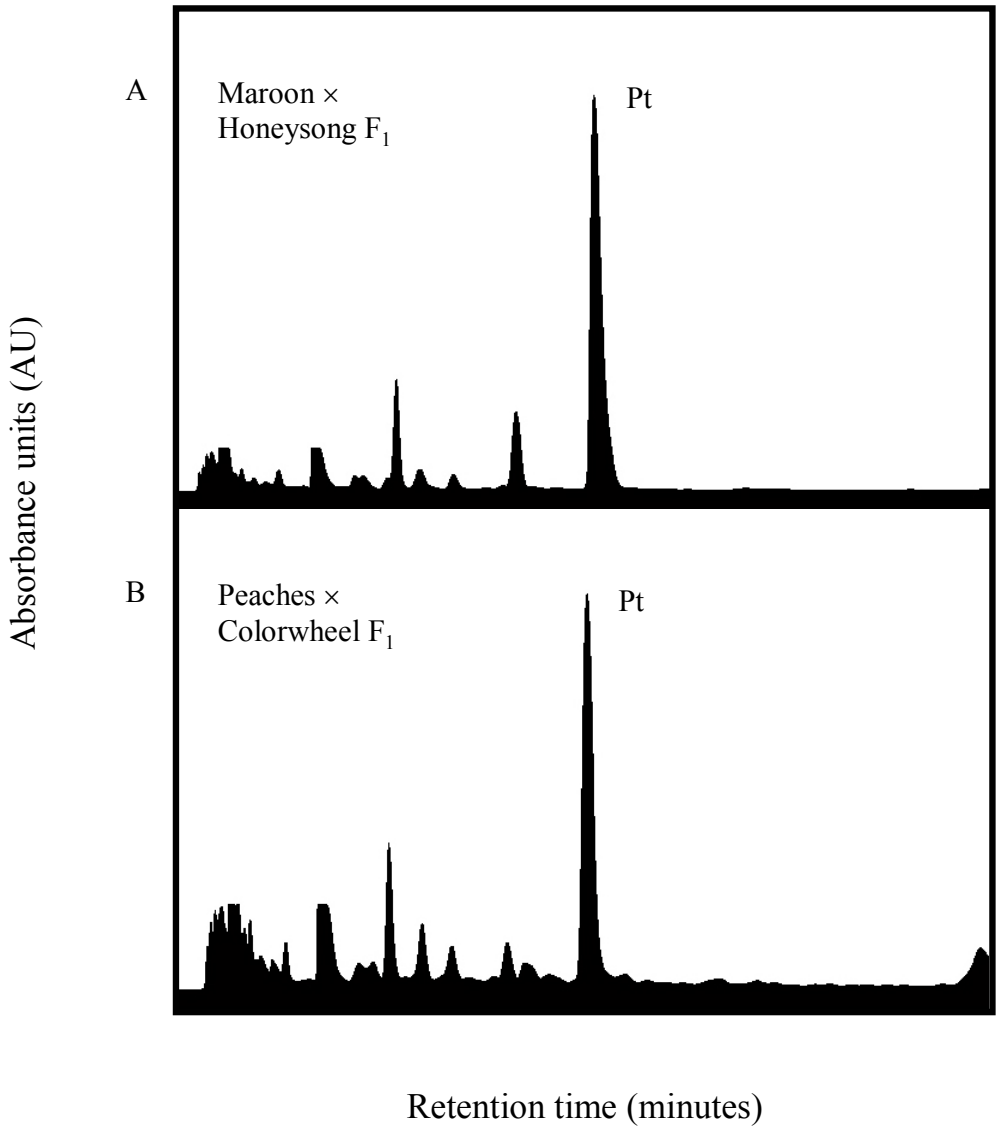


Figure 4. Anthocyanidin HPLC profiles from samples that contained (A) extract from the flowers of an F_1 hybrid from a cross of 'Maroon' × 'Honeysong' and (B) extract from the flowers of an F_1 hybrid from a cross of 'Peaches' × 'Colorwheel'.

Abbreviations: Pt, petunidin.

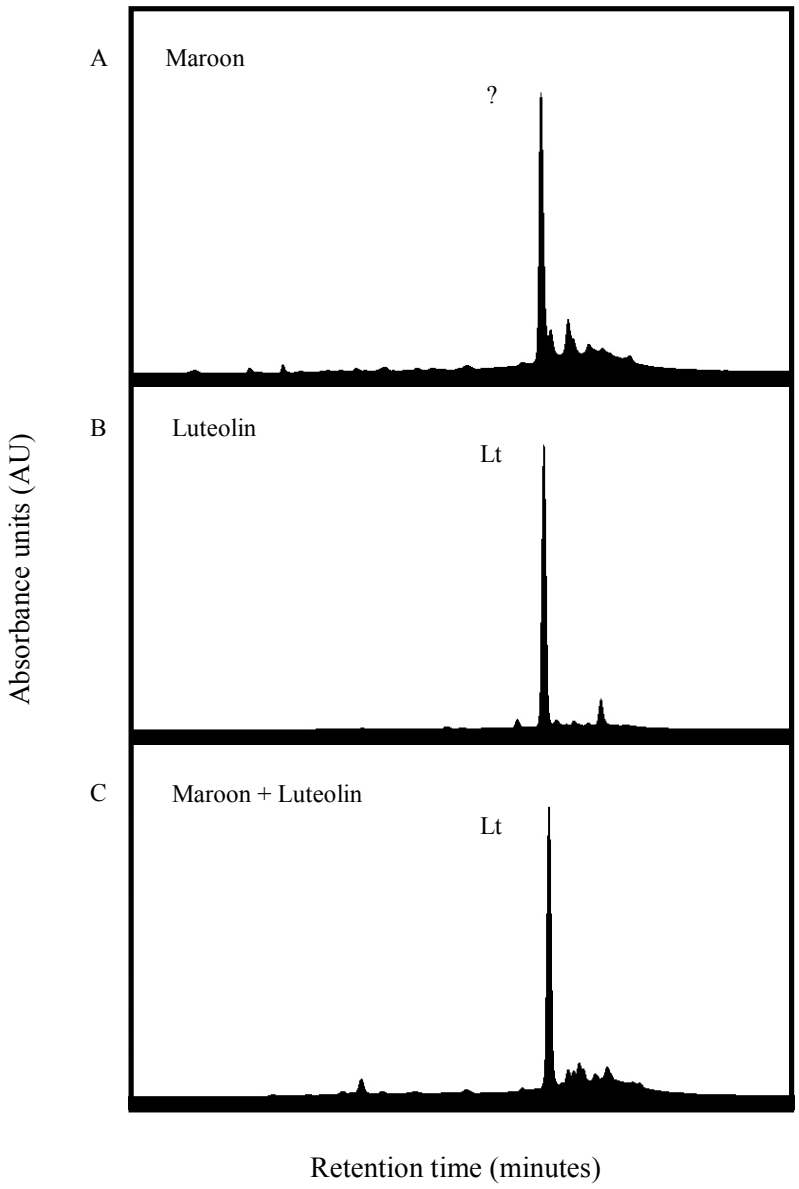


Figure 5. Aglycone copigment profiles from samples that contained (A) extract from the flowers collected from 'Maroon', (B) a standard solution of luteolin, and (C) extract from the flowers of 'Maroon' mixed with the standard solution of luteolin.

Abbreviations: Lt, luteolin.

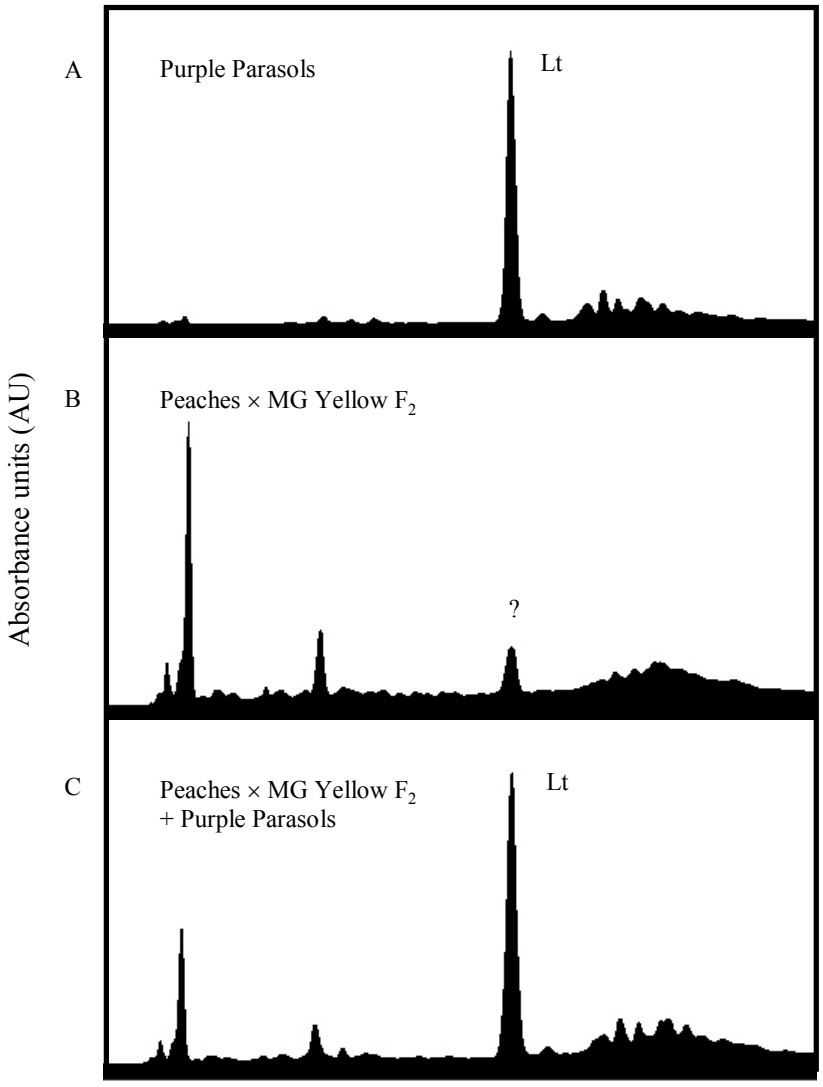


Figure 6. Aglycone copigment profiles from samples that contained (A) extract from the flowers collected from 'Purple Parasols', (B) extract from pale yellow flowers taken from a 'Peaches' × 'Mary Gregory' F_2 plant selected at random, and (C) extract from the flowers of 'Purple Parasols' mixed with the extract from the pale yellow flowers taken from a 'Peaches' × 'Mary Gregory' F_2 plant selected at random.

Abbreviations: Lt, luteolin.

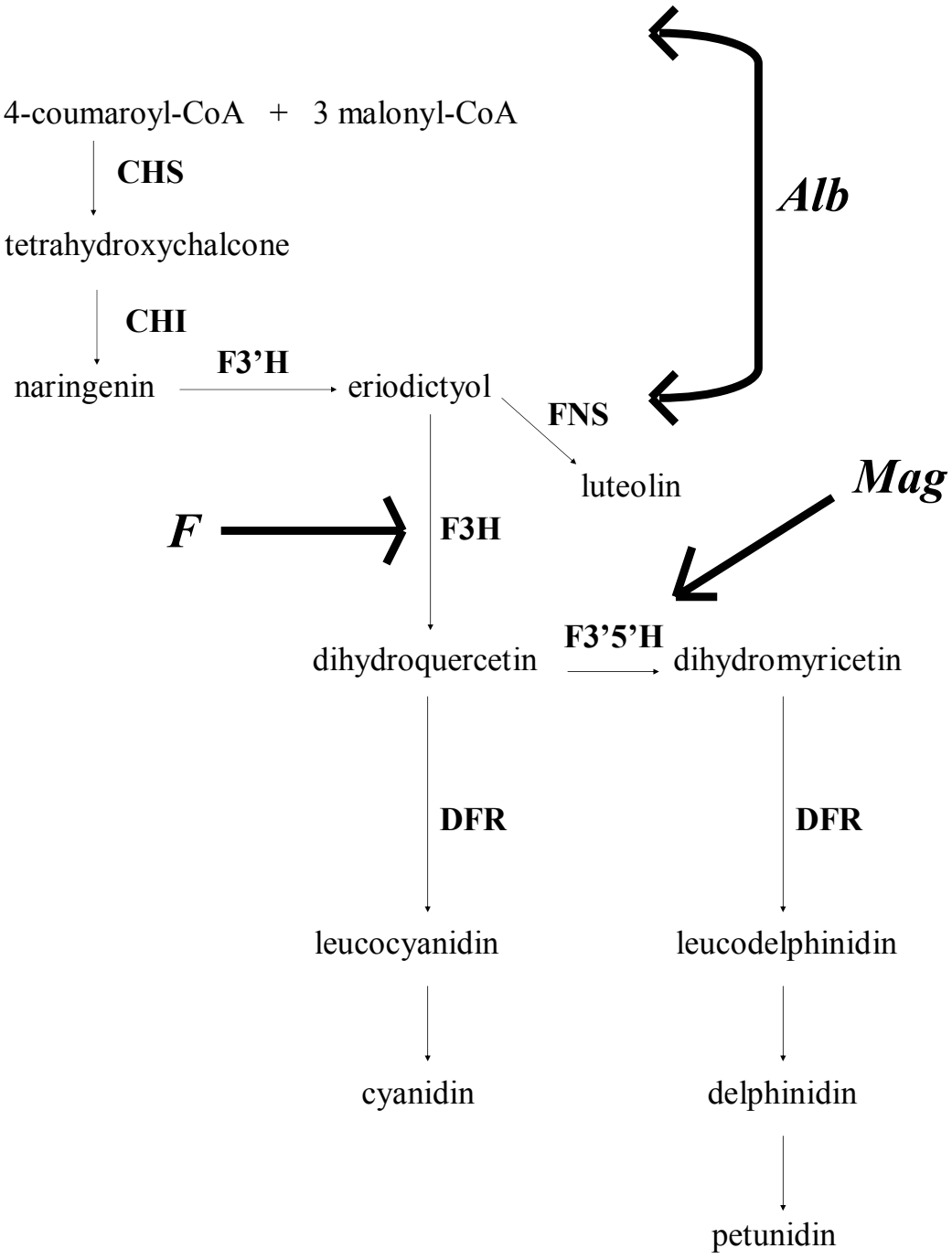


Figure 7. Hypothetical biochemical pathway for flavonoid biosynthesis in stokes aster.

Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase.