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Delphinidin accumulation is associated with abnormal flower development in petunias

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Abstract

The relative floral anthocyanidin contents of 195 commercial petunias with floral colours other than white and yellow were determined using HPLC, and the presence of five anthocyanidins {cyanidin, peonidin, delphinidin, petunidin, and malvidin} was confirmed. Pelargonidin was not detected, and delphinidin was not a major component. Using a principal component analysis of the relative anthocyanidin contents, the petunias were classified into three phenotype-groups accumulating cyanidin, peonidin, or malvidin, {plus petunidin} as the major anthocyanidin. A fourth phenotype was segregated in the progeny obtained by self-pollinating an F_1 hybrid of the malvidin group; this accumulated delphinidin 3-glucoside in a markedly crumpled corolla-limb (delphinidin group). Such inferior floral traits, associated with the accumulation of delphinidin 3-glucoside, are thought to be the driving force that removed the delphinidin group from commercial petunias. A comparison of flowers of the delphinidin group and those of the other groups may provide a useful tool towards a deeper understanding of how anthocyanin biosynthesis relates to normal development of the corolla.

Keywords: *Petunia*; Solanaceae; Anthocyanin biosynthesis; Crumpled flower; Principal component analysis; Flavonoid 3′,5′-hydroxylase; Anthocyanidin 3-glucoside-rhamnosyltransferase

1. Introduction

Cultivars of *Petunia* (hereafter referred to as petunias) have been bred since the early 1830s (Paxton, 1836). The flowers of modern petunias span almost all of the colours that are currently available in floricultural crops. Wiering and de Vlaming (1984) reviewed the features of anthocyanins occurring in petunia flowers. Subsequently, a number of studies have reported additional anthocyanins from selected commercial cultivars (Griesbach et al., 1991; Fukui et al., 1998; Slimestad et al., 1999; Ando et al., 2000; Gonzalez et al., 2001) or selected lines bred in the laboratory (Griesbach, 1996). Another approach to defining the constitution of floral pigments of petunias consists of surveying relative contents of anthocyanidins (Muszynski, 1964) and anthocyanins (Muszynski, 1968) in various commercial petunias. We followed the latter approach, but classified petunias into groups using principal component (PCT) analysis based on the relative content of anthocyanidins. Although we analyzed only anthocyanidins (**1**–**6**), our approach was

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sufficient to establish that commercial petunias do not accumulate large amounts of delphinidin (**4**) in their flowers.

2. Results and discussion

2.1. Distribution of anthocyanidins in commercial petunias

Pelargonidin (**1**), cyanidin (**2**), peonidin (**3**), delphinidin (**4**), petunidin (**5**), and malvidin (**6**) are the most common floral anthocyanidins, and they can be classified into three series by their synthetic pathways (Fig. 1), i.e. pelargonidin-series, cyanidin-series, and delphinidin-series. By comparison with authentic samples using HPLC, no peak corresponding to pelargonidin (**1**) was detected in flowers of the 195 commercial petunias examined in this study (Table 1). This was predictable because the petunia dihydroflavonol 4-reductase (DFR) cannot produce pelargonidin (**1**) using dihydrokaempferol (**7**) as a substrate (Forkmann and Rahnau, 1987). The anthocyanidins identified were cyanidin (**2**), peonidin (**3**), delphinidin (**4**), petunidin (**5**), and malvidin (**6**). Of

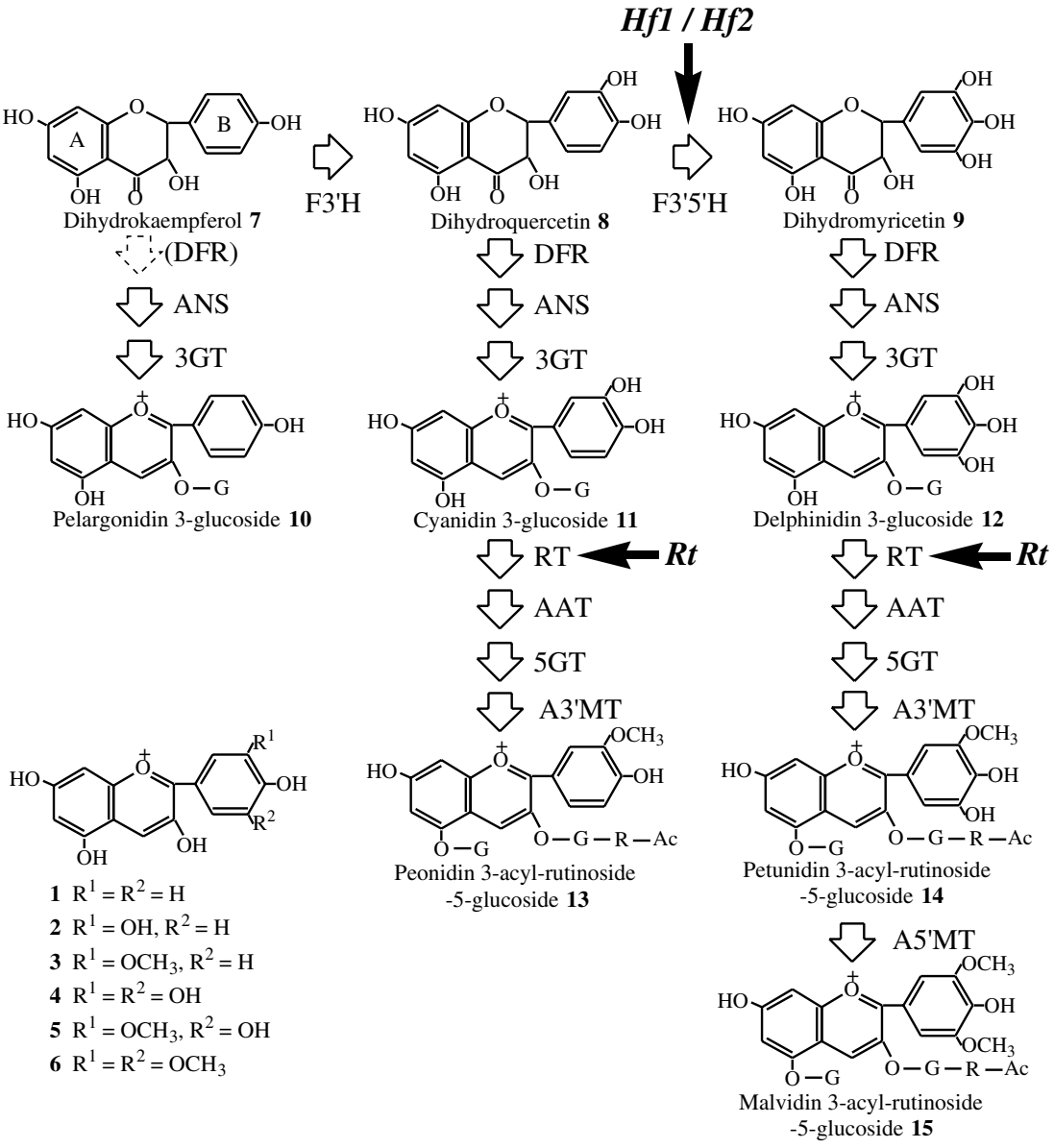

Fig. 1. The dominant anthocyanin biosynthetic pathway in petunia, showing three independent genes (*Hf1*, *Hf2*, and *RT*) encoding two key enzymes controlling the accumulation of floral anthocyanins. F3′H = flavonoid 3′-hydroxylase, F3′5′H = flavonoid 3′,5′-hydroxylase, DFR = dihydroflavonol 4-reductase, ANS = anthocyanidin-synthase, 3GT = UDP-glucose: flavonoid 3-*O*-glucosyltransferase, RT = UDP-rhamnose: anthocyanidin 3-glucoside-rhamnosyltransferase, AAT = aromatic-acyltransferase, 5GT = UDP-glucose: anthocyanin 5-*O*-glucosyltransferase, A3′MT = anthocyanin 3′-methyltransferase, A5′MT = anthocyanin 5′-methyltransferase. F3′5′H also uses dihydrokaempferol (**7**) as a substrate, but this pathway is not illustrated. Petunia DFR does not use dihydrokaempferol (**7**) as a substrate.

Table 1
Commercial petunias used in the present study as classified by the principal component analysis of floral anthocyanidin

(Cyanidin group: 69 cultivars)				
All Star	Allegro	Astro	Baccara Red	Baccara Salmon
Bridesmaid	Candy Apple	Carnival Coral	Carnival Salmon	Carnival Scarlet
Carpet, Flame	Celebrity Red	Celebrity Salmon	Celebrity Summer Ice	Champion, Red
Cherry Blossom	Cherry Lady	Cloud, Red	Cloud, Salmon	Daddy, Strawberry
Dreams, Red	Falcon Coral	Falcon Red	Falcon Red and White	Falcon Red Morn
Falcon Red Vein	Falcon Salmon	Fantasy Salmon	Frost, Fire	Glitters Select
Harvest Moon	Humming Scarlet Star	Joy, Red	Madness, Coral	Madness, Red
Madness, Simply	Madness, Spring	Madness, Summer	Mauna Loa	Picotee, Red
Polo Red	Polo Salmon	Polynesia	PrimeTime Red	PrimeTime Red Star
PrimeTime Salmon	Recoverer Red	Red Coronet	Sales Salmon	Sales Tahitian
Salmon Bouquet	Salmon Delight	Satin Red	Saturn	Sparkler
Starfire	Supercascade Red	Supercascade Salmon	Supermagic Coral	Supermagic Orange
Supermagic Red	Titan Red	Titan Red and White	Titan Salmon	Ultra Red
Ultra Red Star	Ultra Salmon	Valentine	Viva	
(Malvidin group: 67 cultivars)				
Aoihoshi	Baccara Blue	Blue Lace	Blue Skies	Carnival Purple
Carpet, Plum	Carpet, Velvet	Cascade, Royal	Celebrity Blue	Celebrity Blue Ice
Celebrity Burgundy	Champion, Blue	Champion, Sky Blue	Cloud, Blue	Cloud, Orchid
Daddy, Blue	Daddy, Orchid	Daddy, Sugar	Dreams, Midnight	Falcon Blue
Falcon Burgundy	Falcon Mid Blue	Falcon Plum Vein	Fantasy Violet	Frost, Blue
Frost, Velvet	Grandiflora Burgundy	Joy, Blue	Joy, Purple	Joy, Sky
Joy, Starlight	Madness, Burgundy	Madness, Midnight	Madness, Orchid	Madness, Plum
Madness, Silver	Madness, Sugar	Malibu	Mariner	Nocturne
Pearl Sky Blue	Picotee, Violet	Plum Blue	Plum Purple	Polo Blue
Polo Blue Star	Polo Velvet	PrimeTime Blue	PrimeTime Blue Star	PrimeTime Burgundy
PrimeTime Burgundy Star	PrimeTime Light Blue	PrimeTime Plum	Purple Pirouette	Recoverer Blue
Sales, Lacy	Satin Purple	Supermagic Ruby	Supermagic Sky	Telstar
Titan Blue	Titan Mid Blue	Ultra Blue	Ultra Blue Star	Ultrs Burgundy
Ultra Crimson Star	Ultra Plum			
(Peonidin group: 59 cultivars)				
Baccara Cherry Rose	Baccara Pink	Baccara Rose	Carnival Deep Rose	Carnival Rose
Carpet, Pink	Carpet, Rose	Cascade, Chiffon	Celebrity Raspberry Ice	Celebrity Strawberry Ice
Champion, Rose	Cloud, Pink	Cloud, Rose	Daddy, Peppermint	Daddy, Pink
Daienty Lady	Falcon Pink Vein	Falcon Rose	Falcon Rose & White	Fantasy Rose
Fiesta	Flamenco	Frost, Cherry	Happiness	Humming Rose
Joy, Rose	Joy, Star	Lollipop	Madness, Pink	Madness, Rose
Madness, Sheer	Multiflora Rose	Multiflora Star	Pearl Pink	Picotee, Rose
Pink Profusion	Plum Pink	Polo Pink	PrimeTime Light Pink Veined	PrimeTime Pink
PrimeTime Pink Veined	PrimeTime Rose	PrimeTime Rose Star	Recoverer Pink	Romeo
Sales Pastel Pink	Satin Pink	Starburst	Supercascade Blush Imp	Supercascade Pink
Supercascade Rose	Supermagic Pink	Supermagic Rose	Titan Light Pink	Titan Rose
Ultra Pink	Ultra Peppermint	Ultra Rose	Ultra Rose Star	

the delphinidin-series anthocyanidins {delphinidin (**4**), petunidin (**5**), and malvidin (**6**), Fig. 1}, delphinidin (**4**) itself was a minor component, and no cultivar was found that accumulated delphinidin (**4**) as the most abundant component. Malvidin (**6**) was the most abundant in 60 cultivars, while petunidin (**5**) was most abundant in 7 cultivars. Of the cyanidin-series anthocyanidins {cyanidin (**2**) and peonidin (**3**), Fig. 1}, 69 cultivars accumulated cyanidin (**2**) as the major pigment and 59 accumulated peonidin (**3**).

2.2. Three groups of commercial petunias

When a data matrix comprised of five variables {relative contents of delphinidin (**4**), petunidin (**5**), malvidin (**6**), cyanidin (**2**), and peonidin (**3**)} by 195 cases (cultivars) was subjected to PCT analysis, two PCTs with eigenvalues larger than 1.0 were obtained. These accounted for 78.1% of the total variance in the data matrix (Table 2). The first PCT was highly correlated with the cyanidin (**2**, factor loading = 0.90852) and malvidin (**6**, −0.85184) contents and the second was highly correlated with that of peonidin (**3**, −0.91135), suggesting that these variables effectively separate commercial petunias into groups (Table 3). The scatter diagram for the first and second PCT scores revealed that the cultivars were separated into three distinct groups (Fig. 2). The first group, which included 69 cultivars scattered primarily within the first quadrant in Fig. 2, was characterized by the predominance of cyanidin (**2**, 51.6–95.7%;

Table 2
Result of principal component (PCT) analysis on anthocyanidin contents of commercial petunias

PCT	Eigenvalue	Percentage of variance	Cumulative variance (%)
1	2.07542	41.5	41.5
2	1.83133	36.6	78.1
3	0.71175	14.2	92.4

Table 3
Factor loadings of anthocyanidin contents of commercial petunias to each principal component

Anthocyanidin	Principal component		
	1	2	3
Cyanidin **2**	0.90852	0.26240	−0.25653
Peonidin **3**	−0.00282	−0.91135	0.39845
Delphinidin **4**	0.51923	0.65866	0.34606
Petunidin **5**	−0.50474	0.63493	0.49127
Malvidin **6**	−0.85184	0.30814	−0.35508

mean, 78.2%), and is called the cyanidin group (Table 1); these flowers are various shades of red and salmon. The second group consisted of 67 cultivars in the second quadrant and is called the malvidin group (Table 1), because the predominant anthocyanidin was malvidin (**6**, up to 93.3%; mean, 73.5%) followed by petunidin (**5**, up to 75.4%; mean, 21.9%) with the total {malvidin (**6**) + petunidin (**5**)} ranging from 78.8% to 100% (mean, 95.4%). The flowers of the malvidin group are deep to pale blue, purple, and burgundy. The third group of 59 cultivars was distributed mostly in the third quadrant and is called the peonidin group (Table 1), because they contained peonidin (**3**) predominantly (49.6–91.5%; mean, 76.0%). The flowers of the peonidin group are various shades of pink and rose.

As shown in Table 3, the second PCT had slightly higher, positive correlations with the delphinidin (**4**, 0.65866) and petunidin (**5**, 0.63493) contents. This feature is seen in Fig. 2: four cultivars accumulating delphinidin (**4**) as the second major anthocyanidin ('Celebrity Salmon', 'Spring Madness', 'Summer Madness', and 'Mauna Loa') are outliers within the cyanidin group (broken line inside the cyanidin group, Fig. 2); four cultivars with petunidin (**5**) as the most abundant pigment ('Fantasy Violet', 'Silver Madness', 'PrimeTime Blue Star', and 'Satin Purple') form a subgroup within the malvidin group (broken line inside the malvidin group, Fig. 2).

As seen in Table 1, the epithet of a commercial petunia often has two parts: a series name special to each seed company plus the colour name. The exception is the Plum series ('Plum Blue', 'Plum Blue Star', and 'Plum Pink') in which "Plum" is the series name. Colour names exclusive to the cyanidin group are "Red" (24 cultivars), "Salmon" (14), "Coral" (4), "Scarlet" (2), "Fire" (1), "Flame" (1), and "Orange" (1) (47 cultivars in total, 68.1% of the cyanidin group). "Blue" (25 cultivars), "Burgundy" (7), "Plum" (5), "Purple" (5), "Velvet" (3), "Orchid" (3), "Midnight" (2), "Sky" (2), "Violet" (2), and "Crimson" (1) are exclusive to the malvidin group (55 cultivars in total, 82.1% of the malvidin group). "Rose" (22 cultivars) and "Pink" (20) are exclusive to the peonidin group (42 cultivars in total, 71.2%). When 'Aoihoshi', meaning "Blue Star" in Japanese, a member of the malvidin group, is added, it becomes possible to classify 145 cultivars (74.3%) into one of the three groups correctly solely using the cultivar's epithet.

Muszynski (1964) analyzed the anthocyanidin composition of 24 commercial petunias and assigned them to six classes based on the main pigment: (1) malvidin (**6**), (2) petunidin (**5**) plus malvidin (**6**), (3) malvidin (**6**) plus petunidin (**5**), (4) petunidin (**5**), (5) peonidin (**3**), and (6) cyaniding (**2**). However, all of his values (% composition calculated from spot areas on TLC plates) are within the ranges of our malvidin (11 cultivars he assigned to the malvidin, malvidin plus petunidin, petunidin plus malvidin, or petunidin classes), peonidin (4 cultivars from his peonidin class), or cyanidin (9 cultivars from his cyanidin class) groups. He analyzed the same ('Nocturne', malvidin group, Table 1) and possibly the same ('Red Satin'/'Satin Red', cyanidin group) cultivars and classified to malvidin and cyanidin classes, respectively. Although neither survey included all the petunias available in the respective eras, the principal floral anthocyanidins of the commercial petunias that we studied do not seem different from those of petunias traded in the 1960s.

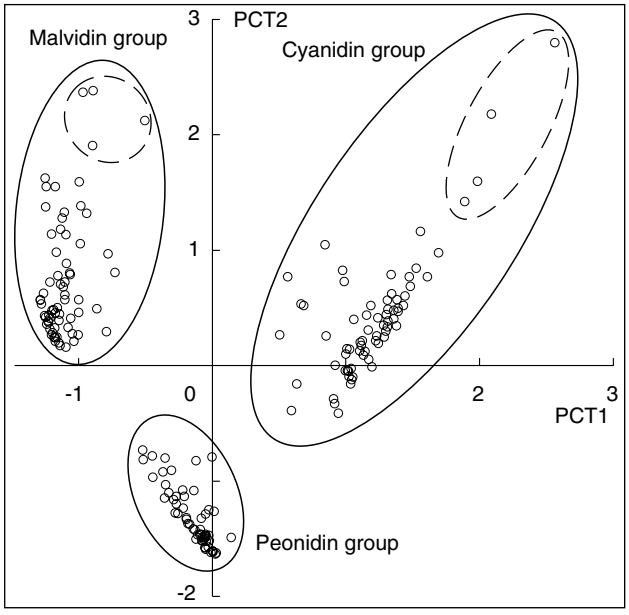

Fig. 2. Scatter diagram of 195 commercial petunias in the first (PCT1) and second (PCT2) principal component planes. The subgroups enclosed in a broken line have slightly different compositions (see text).

2.3. Flavonoid-3′,5′-hydroxylase and anthocyanidin-3-glucoside-rhamnosyltransferase

Although we analyzed only anthocyanidins, the actual pigments that accumulate in floral tissues are their glycosides, i.e. anthocyanins, which are synthesized via the pathway shown in Fig. 1. Petunia is a major experimental model in molecular biology, and much information has been collected on this topic (Holton and Cornish, 1995; Tanaka et al., 1998; Winkel-Shirley, 2001). All wild petunias accumulate delphinidin-series anthocyanidins in the flower (Ando et al., 1999), and two key enzymes are thought to control the class of anthocyanidin that accumulates in the flowers of various commercial petunias: flavonoid-3′,5′-hydroxylase (F3′5′H) and anthocyanidin-3-glucoside-rhamnosyltransferase (RT) (Cornu and Maizonnier, 1983; Holton and Cornish, 1995). F3′5′H controls the flux into the cyanidin and delphinidin branches of anthocyanin biosynthesis (Fig. 1). A loss-of-function mutation for the gene encoding F3′5′H leads to the accumulation of cyanidin-series anthocyanidins in the flowers of petunias.

RT transforms anthocyanidin 3-glucoside into the 3-rhamnosylglucoside (3-rutinoside). RT itself does not influence the anthocyanidin class, but allows the synthetic pathway to continue further and synthesize methylated anthocyanidins, such as peonidin (**3**), petunidin (**5**), and malvidin (**6**) (Fig. 1). Petunias with red flowers (red petunias), which we include in the cyanidin group (Table 1), accumulate a simple anthocyanin, cyanidin 3-glucoside, as the major floral pigment (Muszynski, 1968; Schram et al., 1983; Griesbach et al., 1991; Ando et al., 2000). This strongly implies that a loss-of-function mutation for the gene encoding RT is responsible for separating the cyanidin and peonidin groups (Fig. 1). In other words, it is responsible for separating cultivars that accumulate non-methylated anthocyanidins {cyanidin (**2**) and delphinidin (**4**)} from those that accumulate methylated ones {peonidin (**3**), petunidin (**5**), and malvidin (**6**)}.

Based on this evidence, we have long speculated that commercial petunias could be separated into four groups: the malvidin (active F3′5′H and RT), delphinidin (active F3′5′H and inactive RT), peonidin (inactive F3′5′H and active RT), and cyanidin (inactive F3′5′H and RT) groups. However, this is not true as the delphinidin group does not exist.

2.4. Segregation of the delphinidin group in the progeny of '*Falcon Burgundy*'

To obtain individuals with different major anthocyanidins in the flower, seven cultivars of the Falcon series representing the cyanidin ('Falcon Coral', 'Falcon Red', and 'Falcon Salmon'), malvidin ('Falcon Blue', 'Falcon Burgundy', and 'Falcon Mid Blue'), and peonidin ('Falcon Rose') groups were self-pollinated, and ca. 50 individuals of the progeny of each were raised from the seeds obtained. Of the seven cultivars, individuals having delphinidin (**4**) as the major floral anthocyanidin were segregated in only 'Falcon Burgundy' progeny.

Table 4
Result of principal component (PCT) analysis on anthocyanidin contents of progenies of 'Falcon Burgundy'

PCT	Eigenvalue	Percentage of variance	Cumulative variance (%)
1	2.22544	44.5	44.5
2	1.42124	28.4	72.9
3	0.94679	18.9	91.9

A data matrix consisting of 5 variables (relative content of the five anthocyanidins detected) by 46 cases (individuals) obtained from the progeny of 'Falcon Burgundy' was subjected to PCT analysis. Three PCTs with larger eigenvalues were obtained and these accounted for 91.9% of the total variance in the data matrix (Table 4). The first PCT was highly correlated with the malvidin (**6**, factor loading = 0.81149), petunidin (**5**, 0.81610), and peonidin (**3**, −0.73872) contents, and the second and third PCTs were highly correlated with the delphinidin (**4**, 0.90519) and cyanidin (**2**, 0.75004) contents, respectively (Table 5). As shown in Fig. 3, the scatter diagram for the first and second PCT scores shows a fourth group, the delphinidin group {flowers, dull pink to pale brown; major floral anthocyanidin, delphinidin (**4**), 54.8–87.7%, mean, 66.4%} in addition to the cyanidin {red; cyanidin (**2**), 80.7–95.2%, mean, 89.9%}, malvidin {various shades of purple to blue; malvidin (**6**) + petunidin (**5**), 70.8–96.7%, mean, 89.2%}, and peonidin {various shades of rose to pink; peonidin (**3**), 62.3–83.6%, mean, 75.9%} groups. The segregation ratio of individuals in the malvidin:delphinidin:peonidin:cyanidin groups was 29:5:9:3.

The corolla-limb of the delphinidin group was markedly crumpled and much reduced in size. Although such floral morphology is never observed in commercial cultivars, breeders for seed companies are aware that plants with crumpled flowers often emerge, especially when crossing red individuals with those of different colours (Dr. Masao Bessho, Sakata Seed Co. and Dr. Michael

Table 5
Factor loadings of anthocyanidin contents of progenies of 'Falcon Burgundy'

Anthocyanidin	Principal component		
	1	2	3
Cyanidin **2**	−0.59596	0.27035	0.75004
Peonidin **3**	−0.73872	−0.43257	−0.48420
Delphinidin **4**	−0.00331	0.90519	−0.34539
Petunidin **5**	0.81610	0.30523	−0.05631
Malvidin **6**	0.81149	−0.49851	0.16528

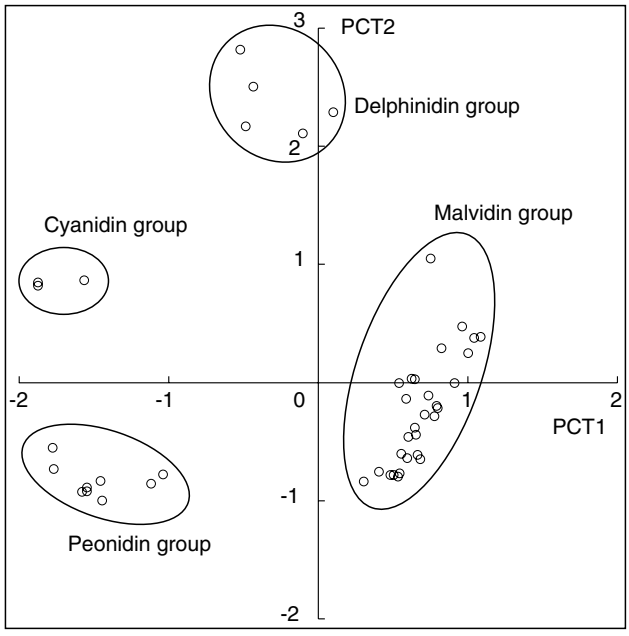

Fig. 3. Scatter diagram of 46 progeny obtained by self-pollination of 'Falcon Burgundy' in the first (PCT1) and second (PCT2) principal component planes.

S. Uchneat, PanAmerican Seed Co., pers. commun.). These are always discarded. The individuals in the delphinidin group were fertile and produced a capsule filled with viable seeds when cross- or self-pollinated.

'Falcon Burgundy' is a member of the malvidin group (Table 1), and is thought to synthesize malvidin (**6**) using both F3′5′H and RT. The progeny of 'Falcon Burgundy' are expected to segregate into four different groups only when the parent is heterozygous for the alleles encoding both F3′5′H and RT as described in the Section 2.6.

2.5. Identification of delphinidin and cyanidin glycosides

The main floral anthocyanin in the delphinidin group that segregated from 'Falcon Burgundy' was identical to delphinidin 3-glucoside (**12**) in its ultraviolet–visible light absorption (UV–Vis), TLC, and HPLC profiles. The results for the main anthocyanin and delphinidin 3-glucoside (**12**) were as follows: UV–Vis (λ_{max} 540, 289 nm, E_{acyl}/E_{max} 20%, E_{440}/E_{max} 18%, $AlCl_3$ shift +), TLC (R_f-values BAW 0.17, BuHCl 0.03, 1% HCl 0.06, AHW 0.11), and HPLC (R_t 14.2 min). These features are distinct from those of other non-acylated delphinidins found in petunias, such as delphinidin 3-rutinoside {UV–Vis (λ_{max} 543, 277 nm, E_{acyl}/E_{max} 15%, E_{440}/E_{max} 18%, $AlCl_3$ shift +), TLC (R_f-values BAW 0.19, BuHCl 0.05, 1% HCl 0.19, AHW 0.30), HPLC (R_t 15.5 min)}, and delphinidin 3-rutinoside-5-glucoside {UV–Vis (λ_{max} 540, 276 nm, E_{acyl}/E_{max} 15%, E_{440}/E_{max} 11%, $AlCl_3$ shift +), TLC (R_f-values BAW 0.14, BuHCl 0.02, 1% HCl 0.28, AHW 0.47), HPLC (R_t 10.4 min)}. The HPLC profiles of anthocyanins in the flowers of individuals from the cyanidin group that segregated from 'Falcon Burgundy' resembled those of red petunias, as reported previously (Ando et al., 2000), and the major pigment was determined to be cyanidin 3-glucoside (**11**) based on co-chromatography with authentic samples. The UV–Vis, TLC, and HPLC profiles of the main anthocyanin and cyanidin 3-glucoside (**11**) are as follows: UV–Vis (λ_{max} 528, 282 nm, E_{acyl}/E_{max} 19%, E_{440}/E_{max} 27%, $AlCl_3$ shift +), TLC (R_f-values BAW 0.30, BuHCl 0.10, 1% HCl 0.13, AHW 0.26), and HPLC (R_t 16.7 min). These features are distinct from those of other non-acylated cyanidins found in petunias, such as cyanidin 3-rutinoside {UV–Vis (λ_{max} 531, 282 nm, E_{acyl}/E_{max} 18%, E_{440}/E_{max} 22%, $AlCl_3$ shift +), TLC (R_f-values BAW 0.30, BuHCl 0.11, 1% HCl 0.25, AHW 0.40), HPLC (R_t 18.1 min)} and cyanidin 3-sophoroside {UV–Vis (λ_{max} 528, 281 nm, E_{acyl}/E_{max} 15%, E_{440}/E_{max} 24%, AlCl3 shift +), TLC (R_f-values BAW 0.27, BuHCl 0.09, 1% HCl 0.64, AHW 0.67), HPLC (R_t 14.2 min)}.

2.6. Conclusion

Based on the above evidence, we hypothesize that the accumulation of delphinidin 3-glucoside (**12**) in flowers is associated with a specific floral character, a dull-coloured extremely crumpled corolla-limb, which impairs its ornamental value. Such inferior floral traits may be the driving force that has led breeders to remove the delphinidin group from commercial petunias. F3′5′H is governed by dominant alleles at two loci, called *Hf1* and *Hf2*, while RT is governed by a dominant allele at the *Rt* locus (Cornu and Maizonnier, 1983; Wiering and de Vlaming, 1984). Of the petunia genes known to control floral morphology, the phenotype caused by the *Cr* (crumpled) locus resembles the flower of the delphinidin group. However, this locus is on chromosome II and is considered independent of the *Hf1* (chromosome I), *Hf2* (V), and *Rt* (VI) loci (Cornu and Maizonnier, 1983), so it is unlikely that *Cr* is the cause of the abnormal floral morphology seen in the delphinidin group.

The progeny of 'Falcon Burgundy' should segregate into only four phenotypes when the parent is heterozygous for *Rt* allele and the *Hf1* or *Hf2* alleles. Since *Hf2* is hypostatic to *Hf1*, i.e., phenotypes caused by *Hf2* are not detectable with the presence of dominant *Hf1* (Cornu and Maizonnier, 1983; Wiering and de Vlaming, 1984), possible genotypes of this cultivar include (1) *Rt rt/Hf1 hf1/Hf2 hf2*, (2) *Rt rt/Hf1 hf1/hf2 hf2*, and (3) *Rt rt/hf1 hf1/Hf2 hf2*. When the actual segregation ratio (the ratio of individuals in the malvidin:delphinidin:peonidin:cyanidin groups = 29:5:9:3) was tested against the expected ratio (9:3:3:1) of the second and third hypothetical genotypes, the null hypothesis (the actual ratio equals the expected ratio) could not

be discarded statistically (chi squared = 1.9277). Consequently, the possible genotypes of the four groups under consideration are the malvidin group: *Hf1*-/*Rt*- or *hf1 hf1*/*Hf2*-/*Rt*-, the delphinidin group: *Hf1*-/*rt rt* or *hf1 hf1*/*Hf2*-/*rt rt*, the peonidin group: *hf1 hf1*/*hf2 hf2*/*Rt*-, and the cyaniding group: *hf1 hf1*/*hf2 hf2*/*rt rt*.

Genotype *rt rt* is not the direct cause of the crumpled corolla-limb of the delphinidin group since the cyanidin group has the *rt rt* genotype. Moreover, genotype *Hf1*- or *hf1 hf1*/*Hf2*- of the delphinidin group is also not the direct cause, since the malvidin group has the same genotype. The distinct floral morphology, a crumpled corolla-limb with a dull colour, should be expressed only when genotype *rt rt* is combined with genotype *Hf1*- or *hf1 hf1*/*Hf2*-.

Loss of function of aromatic-acyltransferase (AAT), UDP-glucose: anthocyanin 5-*O*-glucosyltransferase (5GT) and anthocyanin 3′-methyltransferase (A3′MT) may also cause the accumulation of cyanidin (**2**) or delphinidin (**4**) in a flower, although it is unlikely that these enzymes are responsible for the abnormal floral character of the delphinidin group, since loss of function of these enzymes should cause accumulation of anthocyanins other than anthocyanidin 3-glucoside (cf. Fig. 1).

Wiering and de Vlaming (1984) stated that if the pigment is present as crystals the colour becomes lighter with a concomitant graying of the hue and that delphinidin 3-glucoside (**12**) is often in this form. Neither they nor Cornu and Maizonnier (1983) remarked on the morphology of petunias accumulating delphinidin 3-glucoside (**12**) in the flower. They may have selected lines in which the association between the accumulation of delphinidin 3-glucoside (**12**) in the flower and a crumpled corolla-limb was absent. If so, a gene close to the *Rt*, *Hf1*, or *Hf2* loci should be considered.

The relation observed here between delphinidin (**4**) accumulation and crumpled corolla may present an interesting subject how anthocyanin biosynthesis relates to normal development of the corolla. Although we have a strong circumstantial evidence that these phenomena are related, we still need to clarify the mechanism that causes crumpled corolla.

A previous paper (Ando et al., 1999) revealed that three natural species of *Petunia* accumulate delphinidin (**4**) in the flower: *Petunia exserta* Stehmann, *P. reitzii* L. B. Sm. & Downs, and *P. saxicola* L. B. Sm. & Downs. Contrary to general understanding that delphinidin (**4**) causes blue colour, the floral colour is classified as red (RHS colour chart, R53C) in *P. exserta* or reddish purple (RP61C) in *P. reitzii* and *P. saxicola.* These species never have a crumpled corolla-limb. Interestingly, their major floral anthocyanin is not delphinidin 3-glucoside (**12**), but delphinidin 3-rutinoside (*P. exserta*), delphinidin 3-rutinoside-5-glucoside (*P. reitzii* and *P. saxicola*) (Ando et al., 1999), or acylated delphinidin 3-rutinoside-5-glucoside (selected individuals of *P. reitzii*) (Tatsuzawa et al., 2000). Therefore, they likely use RT to convert the 3-glucoside moiety into a 3-rhamnosylglucoside (3-rutinoside). From a horticultural perspective, these *Petunia* species should be used as a genetic resource to create novel floral colours due to the accumulation of delphinidin (**4**) in the flower, and to establish the fourth group, the delphinidin group, in commercial petunias.

3. Experimental

3.1. Plant materials

As shown in Table 1, seeds of 195 cultivars of commercial petunia (*Petunia*×*hybrida* Vilm., Solanaceae) were obtained in 1990 from Bodger Seeds Ltd. (Celebrity and Satin series), Goldsmith Seeds Inc. (Cloud, Fantasy, Frost, Joy, Plum, PrimeTime and Ultra series, and others), Harris Seeds Inc. (Pearl and Sales series), Novartis Seeds Inc. (Polo series), PanAmerican Seed Co. (Carpet, Cascade, Daddy, Dreams, Madness, Supercascade, and Supermagic series, and others), Sakata Seed Co. {Baccara (=Merlin), Champion, Falcon, Picotee, Recoverer, and Titan series, and others}, and Takii Seed Co. (Carnival and Humming series, and others). No trailing-type petunia, which became available after 1990 (Rice, 1997), was examined in this study. Plants were raised from seeds in January and grown in a greenhouse following standard practices for garden petunias. The corolla-limb was collected and dried in a forced-air chamber at 40 °C for a few days and the dried materials were kept in a refrigerator for up to one year until the pigment was analyzed.

3.2. Analysis of anthocyanidins

Following the methods of Lu et al. (1991), the pigments were extracted from 100 mg of dried corolla-limb with 2 ml of 1.5% (v/v) aqueous phosphoric acid for 30 min at a room temperature. After filtration, an equal volume of 20% hydrochloric acid was added and the sample was hydrolyzed in a boiling water-bath for 1 h. After cooling, the volume was made up to 4 ml with distilled water, and the reaction mixture was extracted with 300-μl iso-amyl alcohol. After diluting the sample with methanol, and filtering it through a Millipore filter (0.22 μm), a 10-μl sample was subjected to HPLC (Shimadzu LC-6A) equipped with an integrator (Chromatopack, Shimadzu C-R6A) for calculating the peak area. HPLC was conducted using a Waters C18 column (250×4.6 mm) at 40 °C with a flow rate of 1 ml min^{-1}, and was monitored at 530 nm. The solvent system used was a linear gradient of solvent A (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent B (1.5% H_3PO_4 in H_2O) from 25% to 100% run for 60 min.

The anthocyanidin class was determined by HPLC co-chromatography with samples purified from flowers of *Pelargonium zonale* (L.) L'Hérit (pelargonidin), *Rosa* 'Karl Red' (cyanidin), *Paeonia lactifolia* Pall. (peonidin), *Delphinium elatum* L. 'Blue Bird' (delphinidin), and *Vicia angustifolia* L. var. *segetalis* (Thuill.) Koch (petunidin and malvidin).

3.3. Principal component analysis

To separate the cultivars into groups and analyze the factors responsible for the separation, HPLC data were subjected to a principal component (PCT) analysis using the FACTOR procedure of the SPSS statistics program (SPSS, Inc.) at the Institute of Media Technology of Chiba University. The contents of the five anthocyanidins {cyanidin (**2**), peonidin (**3**), delphinidin (**4**), petunidin (**5**), and malvidin (**6**)} expressed as the percentage of the peak area without considering extinction coefficients were used as an input matrix to obtain the factor matrix. Rotation of the factor matrix was not used.

3.4. Segregation of the delphinidin group

To obtain individuals with different major anthocyanidins in the flower, seven cultivars of the Falcon series representing the three groups identified in the PCT analysis ('Falcon Blue', 'Falcon Burgundy', 'Falcon Coral', 'Falcon Mid Blue', 'Falcon Red', 'Falcon Rose', and 'Falcon Salmon') were self-pollinated, and ca. 50 individuals of the progeny of each were raised from the seeds obtained.

3.5. Identification of anthocyanins

The anthocyanins were extracted from the flowers of the segregated individuals that accumulated delphinidin (**4**) or cyanidin (**2**) as the most abundant pigment. The dried corolla-limb was extracted with MAW (MeOH:HOAc:H_2O = 9:1:10) for 2 h. The major anthocyanin in the crude extract was purified by two-dimensional TLC using BAW (*n*-BuOH:HOAc:H_2O = 4:1:2) and AHW (HOAc:HCl:H_2O = 15:3:82) as the solvents. The purified anthocyanins were identified using TLC, HPLC, and UV–Vis spectrometry in comparisons with authentic samples of non-acylated delphinidins and cyanidins from the genus *Petunia*: delphinidin 3-rutinoside, delphinidin 3-glucoside (**12**), cyanidin 3-rutinoside (from the flower of *Petunia exserta*), delphinidin 3-rutinoside-5-glucoside (from *P. reitzii*) (Ando et al., 1999), cyanidin 3-glucoside (**11**), and cyanidin-3-sophoroside (from red petunias, Ando et al., 2000). The solvents used for TLC were BAW, BuHCl (*n*-BuOH:2N HCl = 1:1 upper phase), 1% HCl, and AHW. UV–Vis profiles were obtained for a 0.1% HCl–MeOH solution of the purified anthocyanin. The features of the 5-OH of anthocyanin and hydroxycinnamic acid binding were judged from the values of E_{440}/E_{max} (%) and E_{acyl}/E_{max} (%) (Harborne, 1958). The conditions for analytical HPLC were the same as for anthocyanidin (see Section 3.2).

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