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A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavonoid 3′,5′-hydroxylase gene

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Abstract

Recently marketed genetically modified violet carnations cv. Moondust and Moonshadow (*Dianthus caryophyllus*) produce a delphinidin type anthocyanin that native carnations cannot produce and this was achieved by heterologous flavonoid 3′,5′-hydroxylase gene expression. Since wild type carnations lack a flavonoid 3′,5′-hydroxylase gene, they cannot produce delphinidin, and instead accumulate pelargonidin or cyanidin type anthocyanins, such as pelargonidin or cyanidin 3,5-diglucoside-6″-*O*-4, 6‴-*O*-1-cyclic-malyl diester. On the other hand, the anthocyanins in the transgenic flowers were revealed to be delphinidin 3,5-diglucoside-6″-*O*-4, 6‴-*O*-1-cyclic-malyl diester (main pigment), delphinidin 3,5-diglucoside-6″-malyl ester, and delphinidin 3,5-diglucoside-6″,6‴- dimalyl ester. These are delphinidin derivatives analogous to the natural carnation anthocyanins. This observation indicates that carnation anthocyanin biosynthetic enzymes are versatile enough to modify delphinidin. Additionally, the petals contained flavonol and flavone glycosides. Three of them were identified by spectroscopic methods to be kaempferol 3-(6‴-rhamnosyl-2‴-glucosyl-glucoside), kaempferol 3-(6‴-rhamnosyl-2‴-(6-malyl-glucosyl)-glucoside), and apigenin 6-*C*-glucosyl-7-*O*-glucoside-6‴-malyl ester. Among these flavonoids, the apigenin derivative exhibited the strongest co-pigment effect. When two equivalents of the apigenin derivative were added to 1 mM of the main pigment (delphinidin 3,5-diglucoside-6″-*O*-4,6‴-*O*-1-cyclic-malyl diester) dissolved in pH 5.0 buffer solution, the λ_{max} shifted to a wavelength 28 nm longer. The vacuolar pH of the Moonshadow flower was estimated to be around 5.5 by measuring the pH of petal. We conclude that the following reasons account for the bluish hue of the transgenic carnation flowers: (1) accumulation of the delphinidin type anthocyanins as a result of flavonoid 3′,5′-hydroxylase gene expression, (2) the presence of the flavone derivative strong co-pigment, and (3) an estimated relatively high vacuolar pH of 5.5.

Keywords: *Dianthus caryophyllus*; Caryophyllaceae; Transgenic plant; Anthocyanin; Co-pigment; Flavonoid 3′,5′-hydroxylase

1. Introduction

Flower colour is mainly determined by the structures of anthocyanins, co-pigments and the vacuolar pH of petals (Tanaka et al.,1998; Mol et.al., 1999; Goto and Kondo, 1991; Goto, 1987; Yoshida, 1995, Yabuya et al., 1997). For example, blue or violet flowers have never occurred in roses (Eugster and Fisher, 1991), chrysanthemums and carnations because they do not accumulate delphinidin-type anthocyanins. This, in turn, is due to the absence of flavonoid 3′,5′-hydroxylase (F3′,5′H) (Mol et al., 1999; Kusumi et al., 1992). Recently, violet flower carnation varieties such as Florigene Moondust™ (FMD) and Florigene Moonshadow™ (FMS) (Fig. 1) have been successfully created by expressing heterologous F3′,5′H (Mol et al., 1999; Tanaka et al., 1998). Wild type carnation petals contain the 3-malyl-glucosides of pelargonidin and cyanidin (Terahara et al.,1986; Terahara and Yamaguchi, 1986), the 3,5-diglucoside-6″, 6‴-malyl diesters of pelargonidin and cyanidin, and the 3,5-diglucoside-6″-malyl esters of pelargonidin and cyanidin. Interestingly, carnation petals contain pelargonidin and cyanidin type ''cyclic'' anthocyanins (Bloor, 1997; Nakayama et al., 2000). In this paper, we elucidate the reasons why the carnation

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Fig. 1. Photograph of FMS (left) and FMD (right).

flower colour shifted to blue by studying the structures of their novel anthocyanins and flavonoids, identifying a strong co-pigment effect of a flavone, and estimation of the vacuolar pH.

2. Results and discussion

2.1. Structural determination of anthocyanins

HR-FABMS analysis of anthocyanins **1**, **2** and **3** from FMS exhibited molecular ions at m/z 743.1689 $[M]^+$ (Err: +1.8 mmu) and 725.1585 $[M]^+$ (Err: +2.0 mmu) and 859.1755 $[M]^+$ (Err: −2.6 mmu), which were in accordance with the masses calc. for $C_{31}H_{35}O_{21}$, $C_{31}H_{33}O_{20}$, and $C_{35}H_{39}O_{25}$, respectively. The respective λ_{max} of **1**, **2** and **3** are 525, 529, and 525 nm in 50% MeCN in H_2O with 0.5% TFA. After acid hydrolysis, HPLC analysis of **1**, **2** and **3** indicated the presence of delphinidin (R_t: 4.0 min λ_{max}: 538 nm), which was identified by comparison with authentic delphinidin. We show full assignment data for the ^1H NMR and ^{13}C NMR signals of **1**, **2** and **3** using $^1H\{^{13}C\}$–HSQC, $^1H\{^{13}C\}$–HMBC, TOCSY, and DQF–COSY in Table 1. ^1H NMR and ^{13}C NMR spectroscopy data indicated the presence of two glucosyl residues and a malyl moiety in **1** and **2**, and two glucosyl residues and two malyl moieties in **3**. In the case of compound **1**, analysis of the $^1H\{^{13}C\}$–HMBC revealed that the hydroxyl groups at the C-3 and C-5 positions in the A ring of the delphinidin nucleus were bound with Glc-1 and Glc-2, respectively. In addition, the signals of methylene protons at the H-6 position in Glc-1 (δ 4.35 and 4.34) correlated with the ^{13}C signal of the C-4 in the malate (δ 172.91), as shown in Fig. 2. Compound **2** was 18 mass units smaller than **1**, and the ^1H NMR spectrum was almost the same as that of **1** except for the chemical shift of the signals of the methylene protons at the H-6 position in Glc-2. This suggested that **2** was esterified by the dehydration of two hydroxyl groups at the C-6 in Glc-1 and the C-6 in Glc-2 with two carboxyl groups of a malic acid. This was supported by detailed experimental results of $^1H\{^{13}C\}$–HMBC. On the other hand, **3** had two malyl moieties in the molecule, on the $^1H\{^{13}C\}$–HMBC analysis, cross-peaks between H-6 of Glc-1 (δ 4.35 and 4.42) and C-4 of Mal-1 (δ 172.04) and between H-6 of Glc-2 (δ 4.32 and 4.52) and C-1 of Mal-2 (δ 174.9) were observed. From the NMR and

Table 1
^{1}H and ^{13}C NMR spectral data of **1**,**2** and **3**

	1			**2**			**3**		
	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)
Delphinidin									
2	–		165.08	–		164.2	–		164.76
3	–		146.87	–		146.0	–		146.27
4	8.83	*s*	134.43	8.75	*s*	131.6	8.86	*s*	133.90
5	–		157.39	–		156.0	–		156.49
6	6.96	*d* 2	105.96	6.90	*d* 2.0	104.2	6.97	*d* 2	105.48
7	–		170.24	–		169.1	–		169.22
8	6.99	*d* 2	98.06	7.00	*d* 2.0	97.1	7.01	*d* 2	97.27
9	–		157.74	–		156.8	–		157.00
10	–		113.66	–		112.4	–		113.24
1′	–		120.56	–		119.8	–		119.84
2′	7.71	*s*	113.47	7.74	*s*	112.9	7.75	*s*	112.99
3′	–		148.44	–		147.7	–		147.65
4′	–		146.51	–		145.8	–		145.77
5′	–		148.44	–		147.7	–		147.65
6′	7.71	*s*	113.47	7.74	*s*	112.9	7.75	*s*	112.99
Glc-1 (C-3 of delphinidin)									
1	5.50	*d* 8.0	102.73	5.6	*d* 7.8	100.5	5.44	*d* 7.5	102.29
2	3.77	*dd* 9, 8	75.12	3.79	*dd* 9, 8	74.4	3.77	*dd* 9, 8	74.03
3	3.62	*t* 9	78.84	3.62	*t* 9	78.0	3.6	*t* 9	77.76
4	3.45	*dd* 10, 9	72.44	3.38	*t* 9	71.5	3.46	*t* 9	71.53
5	3.90	*ddd* 10, 7, 3	76.40	3.93	*dd* 9, 2	76.0	3.86	*ddd* 9, 7, 2	75.63
6a	4.35	*dd* 12, 3	65.89	4.35	*dd* 12, 9	65.5	4.35	*dd* 12, 7	64.96
6b	4.34	*dd* 12, 7		4.31	*dd* 12, 2		4.42	*dd* 12, 2	
Glc-2 (C-5 of delphinidin)									
1	5.19	*d* 7.8	103.42	5.36	*d* 7.6	100.8	5.19	*d* 8	102.44
2	3.71	*dd* 9, 8	75.56	3.68	*dd* 9, 8	74.5	3.743	*dd* 9, 8	74.65
3	3.57	*t* 9	79.56	3.6	*t* 9	78.0	3.57	*t* 9	77.96
4	3.49	*t* 9	71.78	3.78	*t* 9	70.0	3.48	*t* 9	71.29
5	3.59	*ddd* 9, 6, 2	78.66	3.74	*dd* 9, 3	76.1	3.8	*ddd* 9, 7, 2	76.02
6a	3.96	*dd* 12, 2	63.02	5.05	*dd* 12, 3	61.9	4.32	*dd* 12, 7	64.96
6b	3.77	*dd* 12, 6		4.28	*d* 12		4.52	*dd* 12, 2	
Malic acid Mal–1 (C-6 of Glc-1)									
1	–		175.87	–		174.4	–		175.1
2	4.38	*dd* 7, 5	69.05	4.43	*dd* 10, 2.6	69.5	4.36	*dd* 8, 4	68.26
3a	2.78	*dd* 16, 6.7	40.64	2.81	*dd* 17, 10	44.2	2.73	*d* 8	39.87
3b	2.68	*dd* 16, 5	40.64	2.52	*dd* 17, 2.6	44.2	2.73	*d* 4	
4	–		172.91	–		171.5	–		172.04
Malic acid Mal-2 (C-6 of Glc-2)									
1									174.9
2							4.55	*dd* 8, 4	68.61
3a							2.90	*dd* 16, 4	39.97
3b							2.78	*dd* 16, 8	
4									171.92

Solvent: 10% TFA-*d* /CD_3OD.

HR-FABMS results, the C-6 position of Glc-1 was esterified to one malate by the C-4 carbonyl, and Glc-2 was esterified by the C-1 of another malate. Therefore, **1** and **2** were deduced to be delphinidin 3 -(6″-*O*-4-malyl-glucosyl)-5-glucoside and delphinidin 3,5-diglucoside-6″-*O*-4, 6‴-*O*-1-cyclic-malyl diester, respectively. Compound **2** had a unique cyclic structure linked with a malyl moiety between both C-6 in Glc-1 and Glc-2. Such modifications of anthocyanins have been found only in carnations (Bloor, 1997; Nakayama et al., 2000). Compound **3** was deduced to be delphinidin 3-(6″-*O*-4-malyl-glucosyl)-5-(6‴-*O*-1-malyl-glucoside). This is the first determination of this type of modification in carnations. The proportion of **1**, **2** and **3** was 33.1, 46.8 and 20.1%, respectively, on the basis of HPLC analysis of the extract.

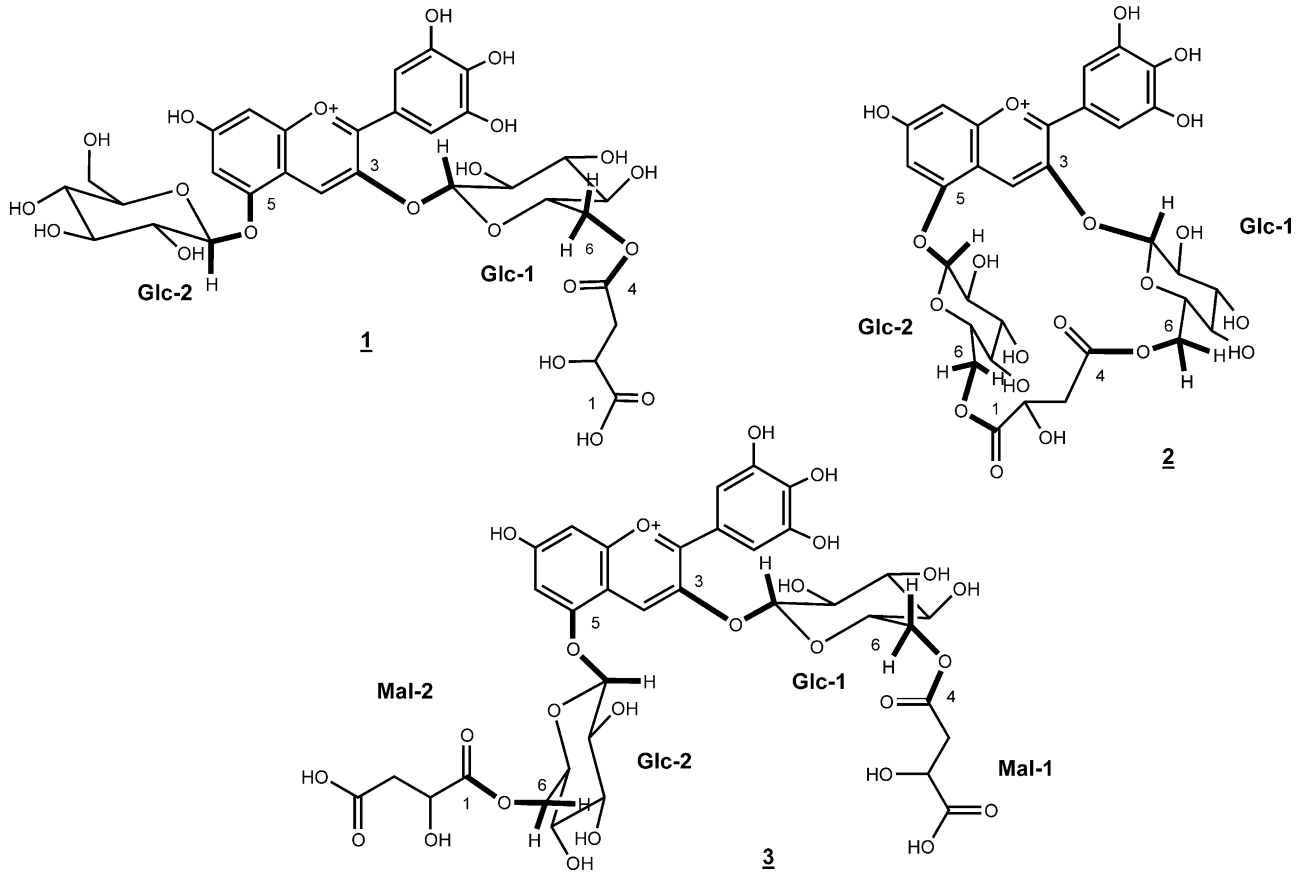

Fig. 2. Structure of anthocyanins, **1**, **2** and **3**. $^{1}H\{^{13}C\}$–HMBC connections are indicated by thick lines.

The presence of a malyl group in carnation anthocyanins indicates that carnation petals contain anthocyanin malyltransferase. However, neither the enzyme nor the gene encoding the enzyme has been identified yet. Furthermore, the mechanism for the formation of cyclic anthocyanins has not been studied, although the anthocyanin biosynthetic pathway itself has been well characterized (Mol et al., 1999) and some anthocyanin acyltransferases have been characterized and a few genes encoding them isolated (Mol et al., 1999). The results obtained here suggest that carnation anthocyanin biosynthetic enzymes (anthocyanidin synthase, anthocyanidin 3-glucosyltransferase, anthocyanin 5-glucosyltransferase, and anthocyanin malyltransferase) can recognize delphinidin derivatives that native carnations do not contain.

2.2. Structural determination of co-pigments

The mass spectra of **4**, **5**, and **6** showed molecular ions $[M+H]^{+}$ with *m*/*z* 757, 711, and 873 and $[M-H]^{-}$ with *m*/*z* 755, 709, and 871, corresponding to masses calc. for $C_{33}H_{40}O_{20}$ (756), $C_{31}H_{34}O_{19}$ (710), and $C_{37}H_{44}O_{24}$ (872), respectively. Full assignment of the ^{1}H and the ^{13}C NMR signals using $^{1}H\{^{13}C\}$–HSQC, $^{1}H\{^{13}C\}$–HMBC, TOCSY, and DQF–COSY techniques are shown in Table 2. After enzymatic hydrolysis with β-glucosidase and naringinase, both **4** and **6** gave kaempferol as the aglycone. The ^{1}H NMR spectrum of **4** was similar to that of **6** except for the presence of methylene (δ 2.32 and 2.47), and the methine (δ 4.15) proton signals were due to the presence of the malyl moiety. Analysis of the MS fragmentation patterns of **4** and **6** revealed the presence of two molecules of glucose and one molecule of rhamnose. Furthermore, the molecular ion in the MS of **6** was 116 mass units larger than that of **4**. Therefore, **6** was considered to be a malyl ester of **4**.

In order to distinguish the linkages of glucose, rhamnose, and malic acid, the $^{1}H\{^{13}C\}$–HMBC spectra were determined. As shown in Fig. 3a, the $^{1}H\{^{13}C\}$–HMBC spectra of **4** and **6** showed a cross-peak between the H-1 of Glc-1 and the C-3 of kaempferol, between the H-1 of Glc-2 and the C-2 of Glc-1, and between the H-1 of rhamnose and the C-6 of Glc-1, respectively.

The ^{1}H NMR of **6** showed the methylene proton signals (δ 4.05 and 4.14) at C-6 of Glc-2 being shifted to a lower magnetic field, and the $^{1}H\{^{13}C\}$–HMBC spectrum of **6** also showed a cross-peak between the H-6 of Glc-2 and the carbonyl C-4 of the malyl moiety, as shown in Fig. 3a. From the spectral data described earlier, the structures of **4** and **6** were determined to be kaempferol 3-(6‴-rhamnosyl-

Table 2
^{1}H and ^{13}C NMR spectral data of **4**,**5** and **6**

	4			**5**			**6**		
	^{1}H (δ)	J (Hz)	^{13}C (δ)	^{1}H (δ)	J (Hz)	^{13}C (δ)	^{1}H (δ)	J (Hz)	^{13}C (δ)
Kaempferol				**Apigenin**			**Kaempferol**		
2	–		159.28	–		164.29	–		156.17
3	–		134.75	6.89	*s*	103.09	–		132.71
4	–		179.53	–		182.02	–		177.32
5	–		163.04	–		159.34	–		159.74
6	6.19	*d* 2.0	100.05	–		110.48	6.18	*d* 2.0	98.537
7	–		166.25	–		162.30	–		163.81
8	6.38	*d* 2.0	94.98	6.84	*s*	93.54	6.38	*d* 2.0	93.61
9	–		158.58	–		156.42	–		156.32
10	–		105.65	–		104.87	–		103.924
1′	–		122.92	–		120.77	–		120.76
2′	8.00	*d* 8.8	132.38	7.94	*d* 8.5	128.49	7.96	*d* 8.8	130.83
3′	6.90	*d* 8.8	116.23	6.96	*d* 8.5	116.04	6.87	*d* 8.8	115.06
4′	–		161.47	–		161.34	–		159.74
5′	6.90	*d* 8.8	116.23	6.96	*d* 8.5	116.04	6.87	*d* 8.8	115.06
6′	8.00	*d* 8.8	132.38	7.94	*d* 8.5	128.49	7.96	*d* 8.8	130.83
Glc-1	**(C-3 of kaempferol)**			**Glc-1 (C-7 of apigenin)**			**Glc-1 (C-3 of kaempferol)**		
1	5.33	*d* 7.6	101.12	5.01	*d* 7.3	100.94	5.40	*d* 7.0	98.16
2	3.73	*dd* 9, 8	81.94	3.37		73.63	3.42	*dd* 9, 7	82.64
3	3.578	*t* 9	77.87	3.34	*t* 9	75.29	3.43	*t* 9	76.24
4	3.26	*t* 9	71.39	3.252	*t* 9	69.43	3.120	*t* 9	69.34
5	3.39	*m*	77.87	3.80	*ddd* 9, 6.5, 1	73.92	3.21		75.51
6a	3.78	*dd* 12, 5	68.16	4.41	*dd* 12, 1	63.53	3.58	*dd* 12, 5	65.97
6b	3.31	*dd* 12, 3		4.14	*dd* 12, 6.5		3.21		
Glc-2	**(C-2 of Glc-1)**			**Glc-2 (C-6 of apigenin)**			**Glc-2 (C-2 of Glc-1)**		
1	4.75	*d* 7.2	104.48	4.63	*d* 9.8	72.64	4.57	*d* 7.8	103.84
2	3.375	*dd* 9, 7	75.38	3.94	*dd* 10, 9	70.66	3.078	*dd* 9, 8	74.16
3	3.295	*t* 9	78.29	3.21	*t* 9	78.86	3.17	*t* 9	76.08
4	3.366	*t* 9	71.29	3.304	*t* 9	69.43	3.124	*t* 9	69.53
5	3.302	*m*	76.96	3.17	*ddd* 9, 4, 2	80.89	3.36	*ddd* 9, 6, 2	73.81
6a	3.785	*dd* 12, 3	62.59	3.59	*brd*	60.22	4.15	*brd* 12	63.71
6b	3.69	*dd* 12, 5		3.59	*brd*		4.05	*dd* 11.7, 5.7	
Rham	**(C-6 of Glc-1)**						**Rham (C-6 of Glc-1)**		
1	4.47	*d* 0.9	102.21				4.31	*d* 0.8	100.3
2	3.57	*dd* 1, 3.5	72.07				3.34	*dd* 1.3	70.20
3	3.47	*dd* 10, 3.5	72.28				3.22	*dd* 10, 3	70.47
4	3.23	*t* 10	73.85				3.03	*t* 9.4	71.72
5	3.41	*dd* 10, 6	69.71				3.19	*ddb* 0, 6	68.08
6	1.10	*d* 6.2	17.85				0.90	*d* 6.2	17.54
Malic acid				**(C-6 of Glc-1)**			**(C-6 of Glc-2)**		
1				–		174.38			174.09
2				4.08	*dd* 4, 8	67.54	4.15	*dd* 7.8, 4.6	66.61
3a				2.71	*dd* 15, 4	39.60	2.32	*dd* 15.7, 7.8	38.65
3b				2.52	*dd* 15.8		2.47	*dd* 15.7, 4.6	
4				–		170.86			170.13

Solvents: **4**, CD_3OD; **5** and **6**: DMSO-d_6.

2‴-glucosyl-glucoside) and kaempferol 3-(6‴-rhamnosyl-2‴-(6-malyl-glucosyl)-glucoside), respectively.

On both HCl and enzymatic hydrolysis, **5** did not give the aglycone but gave compound **7**, which was determined to be isovitexin (apigenin 6-*C*-glucoside) by co-chromatography on HPLC. The detailed analyses of ^{1}H and ^{13}C NMR spectra of **5** revealed the presence of two glycosyl moieties due to the *C*-glucosyl and the *O*-glucosyl residues bound to an apigenin nucleus and the presence of a malyl moiety bonded to C-6 of Glc-2 through the ester bond. The ^{1}H{^{13}C}–HMBC spectrum showed cross-peaks between the H-1 of Glc-2 and the C-7 of the apigenin, between the H-1 of Glc-1 and the C-6 of the apigenin, and between the H-6 of Glc-2 and

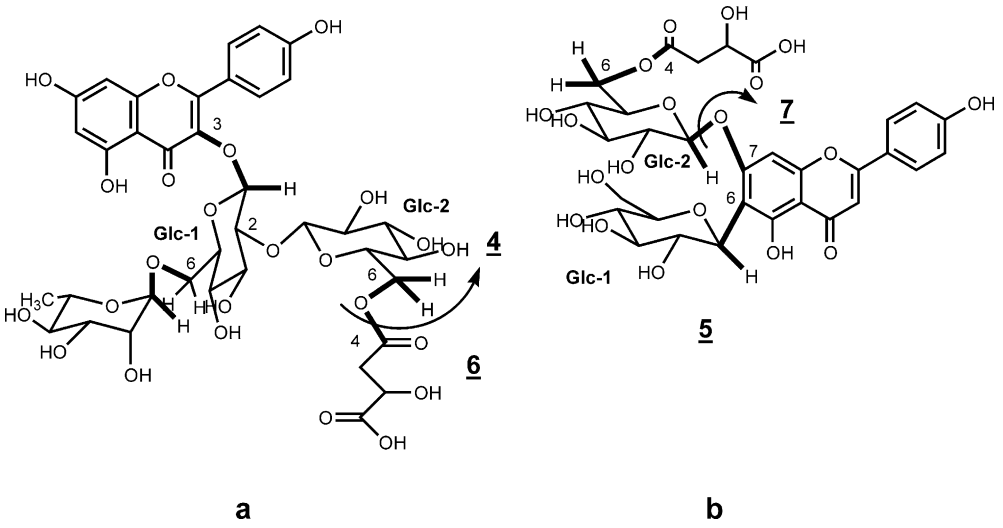

Fig. 3. Structure of co-pigments. (a) Structure of **4** and **6**. (b) Structure of **5** and **7**. $^{1}H\{^{13}C\}$–HMBC connections are indicated by thick lines.

the carbonyl C-4 of the malic acid. Thus, the structure of **5** was determined to be apigenin 6-*C*-glucosyl-7-*O*-(6-malyl-glucoside), as shown in Fig. 3b. The three co-pigments **4**–**6** accumulated in host carnation in same concentration as in transgenic carnations.

Although **4** is a known compound found in some plants, e.g. *Hosta vertricosa* and *Camellia oleifera*, malyl moieties bound to flavone and flavonol, such as in **5** and **6**, have not, to the best of our knowledge, been reported. 6-*C*-glucosyl-7-*O*-glucosyl-flavone, which was a deacyl form of **5**, is known as saponarin and was found in *Saponaria offinalis* of the Caryophyllaceae family (Harborne, 1999).

2.3. Co-pigmentation test

The results of the co-pigment effects of **4**, **5**, and **6** on the absorption spectra of **2** are shown in Fig. 4. The absorbance maximum (λ_{max}) of **2** in the McIlvaine buffer (pH 5.0) was 535 nm. The addition of **4** or **6** to **2** in this buffer solution caused little or no changes in the λ_{max} and the intensity of absorbance. However, the λ_{max} of a mixture of 2 mM of **5** and 1 mM of the **2** in the buffer solution of pH 5.0 exhibited a remarkable bathochromic shift to 563 nm; $\Delta\lambda_{max}$ was 28 nm longer (Fig. 4a), and the absorbance of the mixture solution increased by 1.61 times compared with that of the 1 mM solution of **2** (Fig. 4b). Moreover, the hue of the buffer solution containing two equivalents of **5** and **2** was 312.4° in $L^*a^*b^*$ colorimetric value ($L^* = 84.5$, $a^* = 8.74$, $b^* = -9.59$, $C = 12.98$, $H = 312.4$), and its value nearly reappeared with the hue of an intact FMS petal (308.65°). Furthermore, the $\lambda_{max} = 564$ nm of an intact plant petal measured by a Shimadzu spectrophotometer UV-2500 matched well with the pH 5.0 buffer solution mixture containing **2** and **5**.

As a result of the co-pigmentation test, it was revealed that **5** has a strong co-pigment effect. The *C*-glucosyl

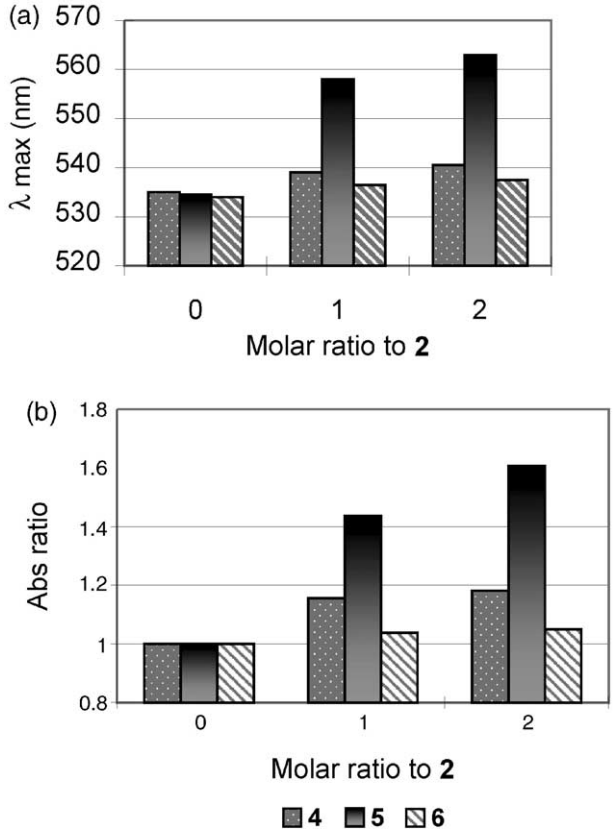

Fig. 4. Co-pigment analysis of **2** with **4**, **5**, and **6**, measured in a McIlvaine buffer (pH 5.0). Compound **2** was dissolved in 1 mM (725 μg/ml) conc. (a) Absorption maxima of the solutions. (b) Absorption intensity ratio of **2** and co-pigment mixed solution with **2** solution.

flavone isovitexin in the petals of the garden iris (*Iris ensata* Thunb.) was known to be a strong co-pigment co-existing with the iris anthocyanin, delphinidin 3-(*p*-coumaroyl)-rutinoside-5-glucoside (Yabuya et al., 1997). Luteolin 7-glycosides, such as luteolin-7-glucoside in torenia, are also known to be strong co-pigments

(Aida et al., 2000). These compounds also have a flavone nucleus, and the glycosyl moieties are substituted at the A-6 or A-7 positions, the same as **5**.

Both FMS and FMD contained **1**, **2** and **3** as pigments and **4**, **5** and **6** as co-pigments. The difference in chroma between these cultivars ($c^* = 32.36$ and 13.46, respectively) was caused by the difference in the amount of delphinidin. The delphinidin concentrations in their petals were 0.95 and 0.067 mg/g fresh petals, respectively. The colorimetric values of the intact petals of FMS were $L^* = 52.38$, $a^* = 20.21$, $b^* = -25.27$, $H = 308.65$, and $C^* = 32.36$, and those of FMD were $L^* = 75.45$, $a^* = 7.80$, $b^* = -10.97$, $H = 305.41$, and $C^* = 13.46$, which were measured with a Minolta colour meter CM-2022. The absorption maxima and absorbance of spectra of the petals of FMS were 564.5 nm and 1.705 and those of FMD were 564.0 nm and 1.160, respectively, measured with the Shimadzu UV-2500PC.

The pH values of the petal extract were estimated to be 5.59 for FMS and 5.64 for FMD, respectively. It is well known that anthocyanins change their colour depending on pH because pH changes the equilibrium structures. For example, petunia changes its colour from reddish purple to violet as the pH is elevated (Fukui et al., 1998). The Heavenly Blue (*Ipomoea nil*) petal colour changes from purple to blue when the vacuolar pH is elevated from 6.6 to 7.7 during flower opening (Yoshida, 1995). The pH of Moondust and Moonshadow were high enough to give the flower a violet hue.

We conclude that the reasons for the bluish hue of the carnation flowers are (1) the high accumulation of delphinidin-type anthocyanins, as a result of flavonoid 3′,5′-hydroxylase gene expression, (2) the presence of a strong co-pigment, and (3) the relatively high petal pH.

3. Experimental

3.1. Plant materials

Dianthus caryophyllus cv. Florigene Moondust™: FMD, and Florigene Moonshadow™:FMS, (International Flower Development, Ltd., Australia) were grown by Florequisa Ltd. (Quito, Ecuador). The flavonoids used for structural determination and co-pigment analysis were extracted from the FMS petals.

3.2. NMR and MS measurements

^{1}H NMR, ^{13}C NMR, ^{1}H{^{13}C}–HSQC, ^{1}H{^{13}C}–HMBC, TOCSY, and DQF–COSY spectra of **1** and **3** were obtained on a DMX-750 spectrometer (Bruker BIOSPIN, Germany). ^{1}H NMR, ^{13}C NMR, ^{1}H{^{13}C}–HSQC, ^{1}H{^{13}C}–HMBC, TOCSY, and DQF–COSY spectra of **2**, **4**, **5**, and **6** were obtained on a DMX-500 spectrometer (Brüker BIOSPIN, Germany). **1**, **2** and **3** were dissolved in a 10%(v/v) TFA-d/CD_3OD mixture. **4** was dissolved in CD_3OD; **5** and **6** were dissolved in DMSO-d_6. The residual proton peaks and ^{13}C peaks of CD_3OD or DMSO-d_6 were used as the internal standard (δ 3.30 and 2.50 for ^{1}H, δ 48.97 and 39.43 for ^{13}C, respectively).

High-resolution FAB-MS spectra of **1**, **2** and **3** were recorded on a JMS-HX/HX110A system (JEOL, Japan) in the positive mode. The mass spectra of **4**, **5**, and **6** were obtained using a nano ESI-Q-TOF MS equipped with a Z-spray ion source (Micromass, Manchester, UK) in both negative and positive modes.

3.3. Measurements of electronic spectra and colorimetric value of petals

Electronic absorption spectra of petals were measured on a UV-2500PC (Shimadzu Co., Ltd., Japan).

Colorimetric values in a CIE $L^*a^*b^*$ system of petals were measured on a CM-2022 spectrophotometer (Minolta Co., Ltd., Japan), and the data were calculated under a 10° observer and D65 illuminants (Commision Internationale d'Eclairage, 1986).

3.4. Measurement of petal pH

Fresh carnation petals (2 g) were frozen at −80 °C and homogenized. The pH of the pressed juice was measured with F-22 pH meter with a 6069-10C electrode (Horiba, Ltd., Japan).

3.5. Purification of flavonoids *1*, *2*, *3*, *4*, *5*, and *6*

Petals of FMS (65 g) were lyophilized, pulverized, and extracted with 500 ml of 50% aq. MeCN containing 0.1% TFA under a supersonic wave for 20 min. After filtration, the extract was evaporated up to half volume under vacuum. The solution was applied to 200 ml of HP-20 (Mitsubishi Chemical Co., Ltd., Japan), and afterwards, washed with water; the flavonoid fraction was eluted with 400 ml of 50% MeCN containing 0.1% TFA. The eluate was concentrated and lyophilized to give 2.1 g of dry matter.

The crude flavonoids were purified by prep HPLC. The HPLC was accomplished with the use of ODS (Develosil-ODS-UG, 50×5 cm, Nomura Chemical, Ltd., Japan) with a flow rate of 32 ml/min and monitoring absorbance at 280 nm. The solvent systems used included a linear gradient elution for 60 min using 20–80% of solvent B (50% MeCN, 0.5% TFA in H_2O) in solvent A (H_2O) and a further elution for 20 min with 80% of solvent B. This chromatogram gave seven fractions (fr.I–fr.VII). Fr. I contained **1** as a principal component, Fr. II contained **3**, Fr. III contained **2**, and Fr. IV was a flavone and flavonol fraction that contained **4**, **5**, and **6**.

Fr. I was purified by HPLC using ODS (Develosil-ODS-UG, 50×5 cm, Nomura Chemical, Ltd., Japan) with a flow rate of 32 ml/min and monitoring at 280 nm to give pure **1** (18 mg). The solvent systems used included a linear gradient elution for 60 min using 10–100% of solvent B (50% MeCN, 0.5% TFA in H_2O) in solvent A (H_2O).

Fr. II was purified by HPLC using ODS (YMC-D-ODS-5A, 25×2 cm, YMC Co., Ltd., Japan) with a flow rate of 6 ml/min and monitoring at 280 nm to give pure **3** (15 mg). The solvent systems used included a linear gradient elution for 60 min using 20–50% of solvent B (50% MeCN, 0.5% TFA in H_2O) in solvent A (H_2O).

Fr. III was purified by HPLC using Asahipak ODP-50 (30×2.15 cm) with a flow rate of 6 ml/min and monitoring absorbance at 280 nm to give **2** (20 mg). The solvent systems used included a linear gradient elution for 90 min 20–50% of solvent B (50% MeCN, 0.5%TFA in H_2O) in solvent A (50 mM KH_2PO_4, 0.5% TFA in H_2O) and a further isocratic elution for 20 min with 50% B.

Fr. IV was repeatedly purified by HPLC using a YMC pack polymer C18 (30×2 cm, YMC Co., Ltd., Japan) with a flow rate of 6 ml/min and monitoring absorbance at 340 nm. The solvent systems were used as follows: after a 25 min isocratic elution with 20% B, a linear gradient elution followed for 25 min using from 20 to 70% of solvent B (50% MeCN, 0.1% TFA in H_2O) in solvent A (0.1% TFA in H_2O). From these chromatograms, a fraction containing **4** and a fraction containing **5** and **6** were obtained.

The fraction containing **4** was further purified by HPLC using a YMC pack polymer C18 (30×2 cm, YMC Co., Ltd., Japan) with a flow rate of 6 ml/min and monitoring at 340 nm to yield **4** (10 mg). The solvent systems used were as follows: after a 25 min isocratic elution of 10% B, a linear gradient elution for 25 min using 10 to 60% of solvent B (50% MeCN in H_2O) in solvent A (H_2O).

The fraction containing **5** and **6** was finally subjected to HPLC using C30-UG-5 (Develosil-C30, 30×2 cm, Nomura Chemical, Ltd., Japan) with a flow rate of 6 ml/min and monitoring at 340 nm to give pure **5** (10 mg) and **6** (6 mg). The solvent systems used were as follows: a linear gradient elution for 40 min using 25–40% of solvent B (90% MeCN, 0.1% TFA in H_2O) in solvent A (0.1% TFA in H_2O).

3.6. HPLC analysis of anthocyanins in Moonshadow extract

HPLC was conducted using an RSpak DE-413L (25 cm×4.6 mm Shoko. Co., Ltd., Japan) column and a flow rate of solvent of 0.6 ml/min; the solvent system used was as follows: after 15 min linear gradient elution from 10 to 50% of MeCN containing 0.5% TFA in H_2O, 10 min of isocratic elution of 50% of MeCN containing 0.5% TFA in H_2O was carried out and detection was done with a photodiode array detector SPD-M10A (Shimadzu Co., Ltd., Japan) detecting absorbance at 600–250 nm. Under these conditions, the retention time, λ_{max} and ratio of **1**, **2** and **3** based on A_{520} were: 14.8 min, 525 nm, 33.1%; 16.2 min, 529 nm, 46.8%; and 15.1min, 525 nm, and 20.1%, respectively.

*3.7. HCl hydrolysis of **1**, **2** and **3** and HPLC analysis of anthocyanidins*

Compounds **1**, **2** and **3** (0.1 mg each) were dissolved in 6 N HCl (0.2 ml) and kept at 100 °C for 20 min. The hydrolyzed anthocyanidins were extracted with 0.2 ml 1-pentanol.

HPLC was performed using ODS-A312 (15 cm×6 mm, YMC Co., Ltd., Japan) column, a flow rate of solvent 1 ml/min, detection at an absorbance of 600–400 nm on a photodiode array detector SPD-M10A (Shimadzu Co., Ltd.). The solvent system used was as follows: AcOH: MeOH: H_2O = 15:20:65. Under these HPLC conditions, the R_t and λ_{max} of delphinidin were 4.0 min and 534 nm, which was identified in comparison with authentic delphinidin chloride (Funakoshi Co., Ltd., Japan).

*3.8. Enzymatic hydrolysis of **4**, **5**, and **6** or HCl hydrolysis of **5** and an HPLC condition of flavone and flavonol*

Compounds **4**, **5**, and **6** (0.1 mg each) was hydrolyzed in 0.2 ml of 0.1 M KPB (pH 4.5) with 6 units of β-glucosidase (Shin-nihon Chemical Co., Ltd., Japan) and 1 unit of naringinase (Sigma Chemical Co., MO, USA) under incubation for 16 h at 30 °C. MeCN (0.2 ml, 90%) was added to the reaction mixture which was then subjected to HPLC analysis. HPLC was conducted using a Develosil C30-UG-5 (15 cm×4.6 mm YMC Co., Ltd., Japan) column and a flow rate of solvent of 0.6 ml/min; the solvent system used was as follows; after 10 min a linear gradient elution using 18–63% of MeCN containing 0.1% TFA in H_2O, 5 min of isocratic elution of 63% MeCN containing 0.1% TFA in H_2O was carried out with absorbance at 400–250 nm detected using a photodiode array detector SPD-M10A (Shimadzu Co., Ltd., Japan). Under these conditions, the retention time and λ_{max} of kaempferol and apigenin were 11.6 min and 365 nm, and 11.2 min and 336 nm, respectively. After hydrolysis, both **4** and **6** showed the same peak, which was identified to be kaempferol, and **5** did not give the aglycone. Therefore, **5** was hydrolyzed by 2 N HCl at 100 °C for 20 min. The authentic sample of isovitexin (apigenin 6-*C*-glucoside) was purchased from Funakoshi Co., Ltd., Japan. Under these HPLC conditions, the retention time and λ_{max} of isovitexin were 6.8 min and 336 nm, respectively.

3.9. Co-pigment analysis

The co-pigment effects of **4**, **5**, and **6** with **2** were examined as follows. Of compound **2**, 1 mM was dissolved in McIlvaine buffer[1] (pH 5.0), and 1 mM or 2 mM of **4** was added to the solution. After 10 min, their visible absorption and the transmittance spectra at 780–380 nm were measured. Similarly 1 mM or 2 mM of **5** was added to 1 mM solution of **2**, and 1 or 2 mM of **6** was added to the 1 mM solution of **2**, and their spectra were measured.

The absorption and transmittance spectra for the observation of co-pigment effects were measured on a Shimadzu UV-2500PC (Shimadzu Co., Ltd., Japan). The transmittance spectrum values were converted into the colorimetric values of an $L^*a^*b^*$ system using Shimadzu colour soft for UVPC.

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[1] McIlvaine buffer was made by mixing of 0.1 M of citric acid and 0.2 M of Na_2HPO_4 solution adjusted to pH 5.0.