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Flavonoid composition related to petal color in different lines of *Clitoria ternatea*

Kohei Kazuma*[,1], Naonobu Noda, Masahiko Suzuki*

Division of Cell Engineering, Aomori Green BioCenter, 221-10 Nogi-Yamaguchi, Aomori, Aomori 030-0142, Japan

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Abstract

Flavonoids in the petals of several *C. ternatea* lines with different petal colors were investigated with LC/MS/MS. Delphinidin 3-*O*-(2″-*O*-α-rhamnosyl-6″-*O*-malonyl)-β-glucoside was newly isolated from the petals of a mauve line (wm) together with three known anthocyanins. They were identified structurally using UV, MS, and NMR spectroscopy. Although ternatins, a group of 15 (poly)acylated delphinidin glucosides, were identified in all the blue petal lines (WB, BM-1, 'Double Blue' and 'Albiflora'), WM accumulated delphinidin 3-*O*-(6″-*O*-malonyl)-β-glucoside instead. The white petal line (WW) did not contain anthocyanins. Quantitative data showed that the total anthocyanin contents in WB and 'Double Blue' were ca. 8- and 10-fold higher than that in BM-1, a bud mutant of 'Double Blue', respectively. The total anthocyanin content in 'Albiflora' was less than 2×10^{-3} times those in WB or 'Double Blue'. While all the lines contained the same set of 15 flavonol glycosides in similar relative ratios, the relative ratio of myricetin glycosides in ww and 'Albiflora' was ca. 30–70 times greater than those in the other lines. The change in flower color from blue to mauve was not due to a change in the structure of an anthocyanidin from delphinidin, but to the lack of (polyacylated) glucosyl group substitutions at both the 3′- and 5′-positions of ternatins. This implies that glucosylation at the 3′- and 5′-positions of anthocyanin is a critical step in producing blue petals in *C. ternatea*.

1. Introduction

Many ornamental flowering plants have lines or cultivars with different flower colors. The variety of flower colors is mainly due to the chemical structures of different anthocyanins accumulated in the flower. In *Gentiana* spp., a change of the delphinidin skeleton (blue flower lines) to a cyanidin skeleton (pink flower lines) in the structure of gentiodelphin, delphinidin 3-glucoside-3′,5′-di-(6-caffeoylglucoside), affects flower color (Hosokawa et al., 1995, 1997). The various flower colors in *Pharbitis nil* lines result from both a change in the peonidin skeleton (violet-blue flowers) to a pelargonidin skeleton (pale red-purple flowers) in the structure of heavenly blue anthocyanin (HBA) and the length of the acylated glucoside chain in HBA and pelargonidin-type anthocyanins (Lu et al., 1992a, b). Moreover, in *Lobelia erinus*, the change in the delphinidin skeleton (blue flowers) to the cyanidin skeleton (purple-red flowers), i.e., the loss of the 5′-hydroxyl group, with the simultaneous loss of a 5′-caffeoylglucosyl group in the structure of lobelinin A, delphinidin 3-*p*-coumaroylrutinoside-5-malonylglucoside-3′,5′-di-caffeoylglucoside, affected flower color (Kondo et al., 1989; Saito et al., 1995).

Clitoria ternatea has several lines with different flower colors: dark blue, light blue, mauve, and white. Of these, 'Double Blue' is well-known to accumulate ternatins, a group of (poly)acylated anthocyanins, in the petals. These are delphinidin 3-(6″-malonyl)glucoside derivatives that have acylated glucose chains of various lengths or simply glucosyl groups at both the 3′- and 5′-positions (Terahara et al., 1998). Moreover, the detailed structures of 15 flavonol glycosides from the petals of the same cultivar were identified recently (Kazuma et al., 2003). As a result, almost all of the flavonoid glycosides in a crude 'Double Blue' petal extract can now be identified using LC/MS. However, the compositions of

* Corresponding author. Tel.: +81-17-728-1015; fax: +81-17-728-1017.
E-mail addresses: kohei_kazuma@pref.aomori.jp (K. Kazuma), masahiko_suzuki@ags.pref.aomori.jp (M. Suzuki).
[1] Research Fellow of the Japan Society for the Promotion of Science (JSPS Research Fellow).

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anthocyanins and flavonol glycosides in the petals of other lines have not been investigated in relation to petal color.

This study identified the anthocyanins and flavonol glycosides in the petals of several lines of *C. ternatea* using LC/MS/MS. In addition, the anthocyanins in WM were isolated and their structures were determined. Moreover, each anthocyanin and flavonol glycoside in the petals of these lines was quantified. The causes of differences in petal color among lines and the early stages of ternatins biosynthesis in *C. ternatea* are discussed.

2. Results and discussion

Wild *C. ternatea* (wb) have dark blue bilateral flowers (Fig. 1a). Various flower color mutants are known, including mauve and white bilateral flower mutants (wm and ww, Fig. 1b and c, respectively). In addition, there are two cultivars: 'Double Blue' with dark blue radial flowers (Fig. 1d) and 'Albiflora' with white bilateral flowers with a thin blue region around the petal edge (not shown). Moreover, BM-1, a bud mutant of 'Double Blue' with light blue radial flowers (Fig. 1e), arose spontaneously from one of many buds formed in a 'Double Blue' plant.

To investigate the relationship between petal color and flavonoid composition, crude petal extracts from each of these lines were analyzed using LC/MS/MS simultaneously detected with a PDA detector (see Section 3). In 'Double Blue', WB, and BM-1, all the known ternatins were detected and there were no remarkable differences in ternatin composition among these lines. In contrast, in 'Albiflora', only ternatins A1, A2, B1-3, D1, and D2 were detected. In WM, no ternatins were detected. Instead, a delphinidin malonylhexoside (*m/z* 551 $[M+H]^+$; MS/MS of $[M+H]^+$, *m/z* 507 $[M-CO_2+H]^+$, 465 $[M\text{-malonyl}+H]^+$, and 303 $[M\text{-malonylhexosyl}+H]^+$) predominated, along with three minor delphinidin glycosides. In WW, no anthocyanins were detected. Furthermore, the fifteen flavonol glycosides identified recently from the petals of 'Double Blue' (Kazuma et al., 2003) were detected in the petals of all lines.

To identify the structures of anthocyanins in the petals of wm, anthocyanins were extracted and isolated. A new anthocyanin, delphinidin 3-*O*-(2″-*O*-α-rhamnosyl-6″-*O*-malonyl)-β-glucoside (**1**), was identified structurally, in addition to three known anthocyanins: delphinidin 3-*O*-(6″-*O*-malonyl)-β-glucoside (**2**), delphinidin 3-neohesperidoside (**3**), and delphinidin 3-*O*-β-glucoside (**4**) (Fig. 2). ^{1}H NMR data for **2** were in good agreement with reported data (Kidøy et al., 1997). In addition, **2** was identified as the major anthocyanin in the petals of wm using co-chromatography and LC/MS/MS. The structure of **3** was identified from ^{1}H NMR data measured in 10% TFA-*d*–methanol-d_4 (Table 2 and Section 3) because no NMR data for **3** have been published. To our knowledge, this is the second time that **3** has been reported since it was first reported in the receptacles of several *Podocarpus* spp. (Andersen, 1989). The structure of **4** was identified by co-chromatography with authentic **4**. The structural determination of the new compound (**1**) is described below.

Compound **1** was obtained as a dark red powder. On acid hydrolysis of **1**, delphinidin, glucose, and rhamnose (glucose/rhamnose, 1/1) were identified by HPLC. The UV spectrum of **1** in 0.1% HCl–MeOH had λ_{max} of 542 and 278 nm. On adding $AlCl_3$, the λ_{max} at 542 nm shifted to 588 nm bathochromically, which demonstrated the presence of *ortho*-oriented free hydroxyl groups at

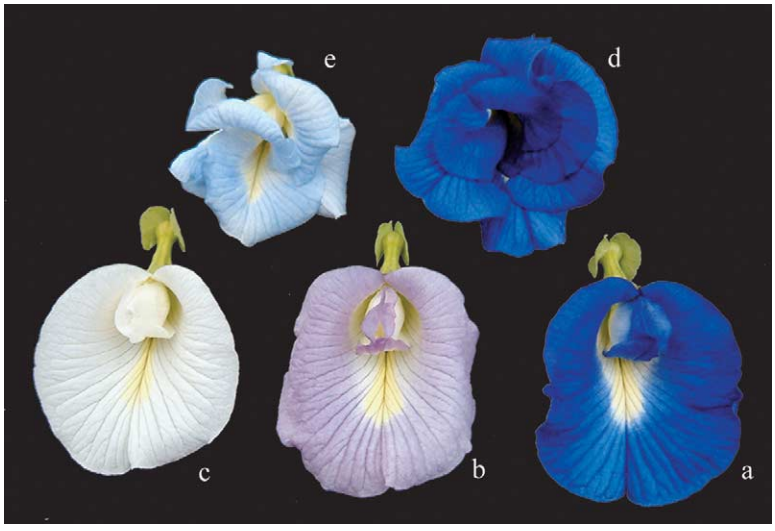
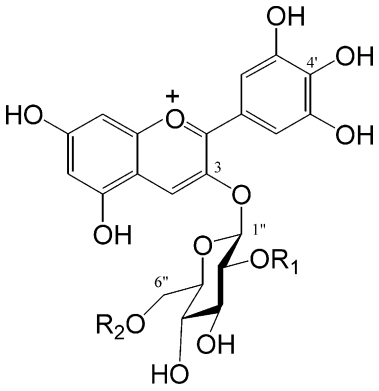


Fig. 1. Flowers of the *C. ternatea* lines. a, wild-type (WB); b, mauve petal line (WM); c, white petal line (WW); d, 'Double Blue;' and e, the bud mutant (BM-1).

B-ring of delphinidin (Markham, 1982). HR-MALDI-TOF-MS of **1** showed a molecular ion at *m/z* 697.1637 $[M+H]^+$, which corresponded to the formula $C_{30}H_{33}O_{19}$ (697.1616). On ESI-MS of **1** in positive mode, a peak was observed at *m/z* 697 $[M+H]^+$. The 86 mass unit difference from the $[M+H]^+$ of **3** (*m/z* 611) suggested the existence of a malonyl group in the structure of **1**. A malonyl group was also inferred from MS/MS data; MS/MS of *m/z* 697 produced fragment ions at *m/z* 653 $[M\text{-}CO_2+H]^+$ and 611 [M-malonyl+H]$^+$, which is the typical fragment pattern of a malonyl residue.



	R_1	R_2
1	rhamnosyl	malonyl
2	H	malonyl
3	rhamnosyl	H
4	H	H

Fig. 2. The structures of anthocyanins. **1**, delphinidin 3-*O*-(2″-*O*-α-rhamnosyl-6″-*O*-malonyl)-β-glucoside; **2**, delphinidin 3-*O*-(6″-*O*-malonyl)-β-glucoside; **3**, delphinidin 3-neohesperidoside; **4**, delphinidin 3-*O*-β-glucoside.

The ^{1}H NMR spectrum of **1** in 10% TFA-*d*–methanol-d_4 was similar to that of **3**, except for the signals derived from the hydroxymethyl protons (H-6a and -6b) and H-5 of the glucose residue (Table 1). In the ^{1}H NMR spectrum of **1**, δ_H 7.71 (*s*, H-2′ and -6′) was observed, which is the typical signal for a 3′,4′,5′-trihydroxyphenyl group on the B-ring of a flavonoid aglycone. Considering the other signals derived from an aglycone, δ_H 6.67 (*d*, 1.6 Hz, H-6), 6.87 (*d*, 1.6 Hz, H-8), and 8.86 (*s*, H-4), the presence of a delphinidin skeleton was obvious in the structure of **1**. Anomeric protons for two sugar residues were also observed at δ_H 5.52 (*d*, $J=7.4$; H-1 of glucose) and 5.31 (*d*, $J=1.7$, H-1 of rhamnose). Other signals for these sugar residues were assigned from the decoupling experiment. From the ^3J coupling constants of the two anomeric protons, **1** contains β-linked glucosyl and α-linked rhamnosyl groups. In the differential NOE spectra (See Section 3), NOE was observed between the H-4 of delphinidin and the H-1 of the glucosyl group, and between the H-2 of the glucosyl group and the H-1 of the rhamnosyl group. The NOE data showed that **1** was a delphinidin 3-*O*-(2″-*O*-α-rhamnosyl)-β-glucoside derivative.

Table 1
^{1}H NMR data for **1**–**4**[a]

No.	**1**	**2**	**3**	**4**
Aglycone				
4	8.86 (1H, *s*)	8.86 (1H, *s*)	8.91 (1H, *s*)	8.96 (1H, *s*)
6	6.67 (1H, *d*, 1.6)	6.66 (1H, *d*, 2.0)	6.66 (1H, *d*, 1.7)	6.65 (1H, *d*, 2.0)
8	6.87 (1H, *d*, 1.6)	6.84 (1H, *d*, 2.0)	6.86 (1H, *d*, 1.7)	6.85 (1H, *d*, 2.0)
2′ and 6′	7.71 (2H, *s*)	7.73 (2H, *s*)	7.74 (2H, *s*)	7.75 (2H, *s*)
3-Glucosyl				
1	5.52 (1H, *d*, 7.4)	5.26 (1H, *d*, 7.7)	5.57 (1H, *d*, 7.7)	5.28 (1H, *d*, 7.8)
2	3.97 (1H, *dd*, 7.4, 9.2)	3.71 (1H, *dd*, 7.7, 9.0)	3.96 (1H, *dd*, 7.7, 8.9)	3.71 (1H, *dd*, 7.8, 9.1)
3	3.69 (1H, *t*, 9.2)	3.56 (1H, *t*, 9.0)	3.71 (1H, *t*, 8.9)	3.56 (1H, *t*, 9.1)
4	3.47 (1H, *t*, 9.2)	3.43 (1H, *dd*, 9.0, 9.8)	3.47 (1H, *t*, 8.9)	3.47 (1H, *dd*, 9.1, 9.5)
5	3.72 (1H, *ddd*, 1.9, 6.3, 9.2)	3.81 (1H, *ddd*, 1.9, 7.2, 9.8)	3.50 (1H, *ddd*, 1.8, 5.1, 8.9)	3.56 (1H, *ddd*, 2.1, 5.6, 9.5)
6a	4.42 (1H, *dd*, 1.9, 11.9)	4.54 (1H, *dd*, 1.9, 12.0)	3.83 (1H, *dd*, 1.8, 12.1)	3.91 (1H, *dd*, 2.1, 12.0)
6b	4.24 (1H, *dd*, 6.3, 11.9)	4.28 (1H, *dd*, 7.2, 12.0)	3.66 (1H, *dd*, 5.1, 12.1)	3.73 (1H, *dd* , 5.6, 12.0)
2″-Rhamnosyl				
1	5.31 (1H, *d*, 1.7)		5.33 (1H, *d*, 1.7)	
2	3.99 (1H, *dd*, 1.7, 3.2)		4.00 (1H, *dd*, 1.7, 3.4)	
3	3.65 (1H, *dd*, 3.2, 9.5)		3.63 (1H, *dd*, 3.4, 9.4)	
4	3.31 (1H, *t*, 9.5)		3.30 (1H, *t*, 9.4)	
5	3.64 (1H, *dq*, 6.3, 9.5)		3.64 (1H, *dq*, 6.1, 9.4)	
6	0.92 (3H, *d*, 6.3)		0.91 (3H, *d*, 6.1)	
6″-Malonyl				
C$\underline{H_2}$	missing[b]	missing[b]		

[a] 400 MHz in 10% TFA-*d*–methanol-d_4.
[b] The signals were missing due to rapid exchange with deuterium atoms.

Although no signal derived from the methylene protons of a malonyl group was observed, it has been reported that malonyl protons disappeared in the same NMR solvent due to rapid exchange with deuterium (Kidøy et al., 1997). With respect to the chemical shifts of hydroxymethyl proton signals in **1** and **3**, the H-6″a and -6″b signals of the glucosyl group in **1** were shifted 0.59 and 0.58 ppm to a lower field than those of **3**, respectively. The magnitude of the lower field shift in **1** and **3** was comparable to that in delphinidin 3-(6″-malonyl)glucoside (**2**) and delphinidin 3-glucoside (**4**): malonylation at 6″-OH of the glucosyl group shifted the H-6″a and -6″b signals of the glucosyl group in **2** lower by 0.63 and 0.55 ppm, respectively, versus those in **4** due

Table 2
The amounts (pmol/mg fw petal) of anthocyanins and flavonol glycosides in the petals of *C. ternatea* lines[a]

Compounds	Lines					
	'Double Blue' ($n=3$)	WB ($n=3$)	BM-1[b] ($n=3$)	'Albiflora' ($n=3$)	WM ($n=6$)	WW ($n=3$)
Anthocyanins						
Delphinidin 3-(2″-rhamnosyl-6″-malonyl)glucoside (**1**)	n.d.[c]	n.d.	n.d.	n.d.	142.7±23.4	n.d.
Delphinidin 3-(6″-malonyl)glucoside (**2**)	n.d.	n.d.	n.d.	n.d.	1691.6±704.6	n.d.
Delphinidin 3-neohesperidoside (**3**)	n.d.	n.d.	n.d.	n.d.	5.2±2.4	n.d.
Delphinidin 3-glucoside (**4**)	n.d.	n.d.	n.d.	n.d.	51.5±24.5	n.d.
Ternatin A1	425.4±87.7	289.7±39.5	191.9±8.9	n.d.	n.d.	n.d.
Ternatin A2	539.6±124.2	416.8±6.3	42.5±1.7	0.3±0.1	n.d.	n.d.
Ternatin A3	100.2±25.6	311.3±39.3	0.5±0.4	n.d.	n.d.	n.d.
Ternatin B1	1544.5±281.0	769.5±27.2	277.4±7.7	1.1±0.1	n.d.	n.d.
Ternatin B2	999.4±254.4	928.8±51.7	52.1±1.0	0.9±0.1	n.d.	n.d.
Ternatin B3	444.8±105.6	295.5±16.4	42.1±17.3	n.d.	n.d.	n.d.
Ternatin B4	111.9±36.3	302.1±46.3	2.1±0.2	n.d.	n.d.	n.d.
Ternatin C1	160.7±65.0	157.3±9.2	11.5±0.7	n.d.	n.d.	n.d.
Ternatin C2	81.7±29.6	71.6±4.1	12.2±1.5	n.d.	n.d.	n.d.
Ternatin C3	14.4±25.2	104.4±4.3	n.d.	n.d.	n.d.	n.d.
Ternatin C4	41.4±16.7	186.0±11.1	n.d.	n.d.	n.d.	n.d.
Ternatin C5	16.5±13.5	71.1±24.3	n.d.	n.d.	n.d.	n.d.
Ternatin D1	1630.6±338.8	750.7±31.3	57.8±3.4	0.4±0.2	n.d.	n.d.
Ternatin D2	622.7±172.2	540.0±65.2	4.2±0.3	0.2±0.1	n.d.	n.d.
Ternatin D3	127.7±41.4	206.6±27.5	7.1±1.7	n.d.	n.d.	n.d.
Flavanol glycosides						
Kaempferol 3-(2^G-rhamnosylrutinoside)	1738.2±280.3	3571.7±61.0	1805.8±58.8	973.4±38.3	2418.8±181.3	1342.1±123.3
Kaempferol 3-neohesperidoside	6454.9±1125.0	8225.1±386.6	5146.4±150.4	2367.1±90.0	8547.4±811.5	4218.4±464.7
Kaempferol 3-(2″-rhamnosyl-6″-malonyl)glucoside	567.3±93.6	841.5±17.9	571.3±20.8	180.5±9.4	712.4±66.6	417.8±46.2
Kaempferol 3-rutinoside	23.5±9.6	72.4±5.0	68.9±5.7	53.4±4.1	42.8±1.5	81.5±19.0
Kaempferol 3-glucoside	n.q.[d]	n.q.[d]	n.q.[d]	46.9±4.3	86.1±2.1	71.0±5.8
Quercetin 3-(2^G-rhamnosylrutinoside)	388.3±61.0	501.0±13.0	245.5±2.5	229.6±8.9	289.2±15.4	291.6±30.5
Quercetin 3-neohesperidoside	1012.7±199.9	631.0±37.3	357.7±4.9	324.8±7.8	764.3±46.6	613.7±63.4
Quercetin 3-(2″-rhamnosyl-6″-malonyl)glucoside	n.q.[e]	n.q.[e]	n.q.[e]	n.q.[e]	n.q.[e]	n.q.[e]
Quercetin 3-rutinoside	231.9±63.4	449.0±78.8	802.5±108.2	436.8±39.5	94.2±8.2	321.2±105.4
Quercetin 3-glucoside	249.9±119.2	335.9±66.0	692.6±93.1	335.6±42.9	55.2±7.1	325.3±147.2
Myricetin 3-(2^G-rhamnosylrutinoside)	56.8±10.0	37.4±7.1	10.3±0.5	188.8±3.4	28.1±2.3	246.0±26.2
Myricetin 3-neohesperidoside	n.d.	n.d.	11.3±0.7	257.3±6.2	41.5±4.0	328.0±42.8
Myricetin 3-(2″-rhamnosyl-6″-malonyl)glucoside	n.d.	n.d.	n.d.	21.9±0.6	n.d.	38.3±4.1
Myricetin 3-rutinoside	n.d.	n.d.	n.d.	389.8±9.3	n.d.	384.5±45.3
Myricetin 3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	391.3±56.5
The total amount (nmol/mg fw petal)						
Flavonoids	17.59±3.30	20.07±0.55	10.41±0.24	5.81±0.26	14.97±0.54	9.07±0.83
Anthocyanins	6.86±1.61	5.40±0.23	0.70±0.04	<0.01	1.89±0.75	n.d.
Flavonol glycosides	10.72±1.69	14.66±0.33	9.71±0.21	5.81±0.26	13.08±1.13	9.07±0.83
Kaempferol glycosides	8.78±1.49	12.71±0.46	7.59±0.21	3.62±0.14	11.81±1.06	6.13±0.65
Quercetin glycosides	1.88±0.26	1.92±0.12	2.10±0.20	1.33±0.10	1.20±0.07	1.55±0.28
Myricetin glycosides	0.06±0.01	0.04±0.01	0.02±0.00	0.86±0.02	0.07±0.01	1.39±0.17

[a] Each anthocyanin and flavonol glycoside was quantified as ternatin A1 and kaempferol 3-neohesperidoside, respectively. The data are the means of three or six replicates ±S.E.
[b] A bud mutant of 'Double Blue'.
[c] Not detected.
[d] Not quantified because it overlapped with ternatin B1 peak.
[e] Not quantified because it overlapped with kaempferol 3-neohesperidoside peak.

(Table 1) (Markham and Geiger, 1994). These observations and MS data indicate that a malonyl group was attached to the 6″-OH of the glucosyl group in **1**. Considering all the data, the structure of compound **1** was determined to be delphinidin 3-*O*-(2″-*O*-α-rhamnosyl-6″-*O*-malonyl)-β-glucoside (Fig. 2).

Almost all of the flavonoid constituents in the petals of *C. ternatea* lines were identified, and their contents in each line were quantified (Table 2). The major anthocyanins in the petals of the blue petal lines were roughly consistent, although the major species differed in each line. In 'Double Blue', the major anthocyanin was ternatin D1 (1631 pmol/mg fw petal), followed by ternatin B1 and B2 (1545 and 999 pmol/mg fw petal, respectively), whereas ternatin B2 (929 pmol/mg fw petal) was the major species in WB, followed by ternatin B1 and D1 (770 and 751 pmol/mg fw petal, respectively). In BM-1, the major species was ternatin B1 (277 pmol/mg fw petal), followed by ternatin A1 and D1 (192 and 58 pmol/mg fw petal). In 'Albiflora', ternatin B1 (1.1 pmol/mg fw petal) was the major species, followed by ternatin B2 and D1 (0.9 and 0.4 pmol/mg fw petal, respectively). In this quantitative experiment, ternatins A1 and B3 were not detected in 'Albiflora' as HPLC peaks, but only by MS, although they were detected in another LC/MS/MS experiment. Moreover, the total anthocyanin content in BM-1 (0.7 nmol/mg fw petal) was ca. 9.8- and 7.7-fold lower than those of 'Double Blue' and WB, respectively. Of the blue petal lines, 'Albiflora' contained the fewest total anthocyanins (2.9 pmol/mg fw petal). In wm, the major anthocyanin was delphinidin 3-(6″-malonyl)glucoside (**2**) (1692 pmol/mg fw petal), which accounted for ca. 90% of the total anthocyanin content. The new compound delphinidin 3-(2″-rhamnosyl-6″-malonyl)glucoside (**1**) (143 pmol/mg fw petal) accounted for only ca. 8% of the total anthocyanin content. Although delphinidin 3-glucoside (**4**) constituted ca. 2.7% of the total anthocyanin content, **4** accounted for ca. 33% of the total anthocyanins in the isolation experiment due to demalonylation of **2** during the purification process.

The total amount of flavonol glycosides differed in each line. 'Albiflora' contained the smallest amount of flavonol glycosides (5.8 nmol/mg fw petal); 'Double Blue', BM-1, and WW contained ca. 1.7-fold higher levels than 'Albiflora;' and WB and WM contained ca. 2.4-fold more than 'Albiflora'. The relative ratios of flavonol glycosides in each line were similar to those reported in a previous study (Kazuma et al., 2003), except for myricetin glycosides. In 'Double Blue', WB, BM-1, and WM, myricetin glycosides accounted for ca. 0.21–0.55% of the total flavonol glycosides, while they accounted for ca. 15% of the total flavonol glycosides in 'Albiflora' and WW.

These results showed that the marked differences in the flavonoid contents of blue and mauve petals were due to accumulation of ternatins and non-polyacylated delphinidin glycosides in the petals, respectively. White petals result from a lack of anthocyanin production. In addition, the quantitative data suggested that the petal shade is proportional to the total anthocyanin content, not the total flavonol content. The composition of flavonol glycosides might be independent of the color and tone of the petals, as suggested by Saito et al. (1985) for the contribution of flavonol glycosides to the petal color of 'Double Blue'.

The mauve petal line (WM) accumulated delphinidin 3-(6″-malonyl)glucoside (**2**), which did not have substitution with an acylated glucosyl group at either the 3′- or 5′-position, although all the blue lines accumulated a mixture of 3′,5′-disubstituted delphinidin glucosides with (acylated) glucosyl groups, *i.e.*, ternatins. A case similar to that in *C. ternatea* lines was reported in *Delphinium×hybridum* lines (Hashimoto et al., 2002). The pink flower cultivar 'Blue Spring' accumulates mainly delphinidin 3-rutinoside in the sepals, while blue flower lines, such as 'Blue Mirror', accumulate mainly cyanodelphin, a 7-polyacylglucoside of delphinidin 3-rutinoside.

Intramolecular co-pigmentation with anthocyanidin and aromatic acid chromophores causes a visible absorption maximum shift bathochromically, resulting in a bluer petal color due to polyacylated anthocyanidin glycosides (Lu et al., 1992a; Honda and Saito, 2002). Hence, the presence of glucose residues at the 3′- and 5′-positions of delphinidin is important, not only as linkers to *p*-hydroxycinnamic acids, but as the structural basis for intramolecular co-pigmentation between anthocyanidin and *p*-hydroxycinnamic acid chromophores.

In addition, the lack of 3′- and 5′-substitutions in the anthocyanins of wm predicted a lack of glucosyltransferase activity in transferring a glucosyl group to the 3′-position of **2** from a glucosyl donor, such as UDP-glucose, which intercepts the biosynthetic pathway to ternatins. Therefore, the accumulation of **2** in wm suggested the prior malonylation of delphinidin 3-glucoside (**4**) to glucosylation at the 3′- and 5′-positions of **4**, although delphinidin 3,3′,5′-triglucoside (preternatin C5) has been proposed as a precursor of delphinidin 3-(6″-malonyl)glucoside-3′,5′-diglucoside (ternatin C5) (Terahara et al., 1998). Glucosylation at the 3′- and 5′-positions of **2** might be critical steps for producing blue color in the petals of *C. ternatea*.

3. Experimental

3.1. General

^{1}H NMR spectra were recorded on a JEOL α-400 NMR spectrometer at 400 MHz at 30 °C. The chemical shifts are reported in δ unit (ppm) values relative to the

solvent (at δ_H 3.30 for methanol-d_4). 1-D differential NOE spectra were recorded with the Jeol NOEDF program. The ^{1}H NMR spectrum of each compound was simulated using SwaN-MR 3.6.1 (Balacco, 1994) to check the signal assignments. UV/vis spectra were recorded with a Gene Spec III spectrometer (Naka Instruments). HR-MS measurements were made on a PerSeptive Biosystems Voyager DE-STR. ESI-MS was measured on a Bruker Esquire3000 spectrometer. Preparative HPLC was performed using a Develosil-10/20 column (20 i.d.×250 mm, Nomura) on a Waters 616 LC system consisting of a 717 autosampler and a 486 UV/vis detector. CIELab coordinates for the petal colors were determined with a CR-300 chroma meter (Minolta). Delphinidin 3-glucoside was purchased from Polyphenol Lab. (Sandnes, Norway).

3.2. Plant material

Seeds of the mauve (WM) and white (WW) petal lines were kindly supplied by Prof. Y. Tachibana (Hanazono University, Kyoto, Japan). Seeds of the wild-type (WB) were kindly supplied by Ms. M. Aragane (Tokyo Metropolitan Medical Plant Garden, Tokyo, Japan). Seeds of 'Double Blue' were purchased from Sakata Seed Co. (Yokohama, Japan). Seeds of 'Albiflora' were purchased from Barbadine (La Réunion, France). All the plants were cultivated in a greenhouse. Voucher specimens (AGBC CLITE-B01 for WB, AGBC CLITE-M01 for WM, AGBC CLITE-W01 for WW, AGBC CLITE-DB01 for 'Double Blue', and AGBC CLITE-A01 for 'Albiflora') have been deposited in the laboratory of the Aomori Green BioCenter. No voucher specimen for the bud mutant of 'Double Blue' (BM-1) has been deposited because we had only one mutant bud. The CIELab coordinates for petal color measured with a chroma meter were as follows: for 'Double Blue' ($n=10$), $a^*=55.14\pm1.08$, $b^*=-57.03\pm0.60$, $L^*=23.91\pm0.82$; for WB ($n=10$), $a^*=58.66\pm0.73$, $b^*=-57.92\pm0.32$, $L^*=22.11\pm0.58$; for BM-1 ($n=11$), $a^*=10.82\pm0.96$, $b^*=-28.83\pm1.53$, $L^*=61.53\pm1.32$; for 'Albiflora' ($n=20$), $a^*=-3.17\pm0.12$, $b^*=6.29\pm0.47$, $L^*=85.80\pm0.73$; for WM ($n=10$), $a^*=27.62\pm1.06$, $b^*=-19.91\pm0.40$, $L^*=55.47\pm1.17$; and for WW ($n=10$), $a^*=-2.13\pm0.06$, $b^*=6.60\pm0.16$, $L^*=94.75\pm0.22$. For the LC/MS/MS experiments, the petals of mature opened flowers in each line were harvested and used immediately. For the isolation experiment, the petals of wm were collected and stored at −80 °C until use. For the quantitative experiment, the petals of each line were collected, frozen immediately with liquid N_2, and stored at −80 °C until use.

3.3. LC/MS/MS of petal extracts

Each petal extract of the six different lines was analyzed using the method described previously (Kazuma et al., 2003). For anthocyanins in wm, the results (t_R; MS; MS/MS of $[M+H]^+$) were as follows: delphinidin 3-neohesperidoside (**3**) (14.0 min; m/z 611 $[M+H]^+$; m/z 303 [M-rhamnosylglucosyl + H]$^+$), delphinidin 3-glucoside (**4**) (16.6 min; m/z 465 $[M+H]^+$; m/z 303 [M-glucosyl + H]$^+$), delphinidin 3-(2″-rhamnosyl-6″-malonyl)-glucoside (**1**) (19.3 min; m/z 697 $[M+H]^+$; m/z 653 [M-CO_2 + H]$^+$, 611 [M-malonyl + H]$^+$, 303 [M-malonyl-rhamnosylglucosyl + H]$^+$), and delphinidin 3-(6″-malonyl)glucoside (**2**) (23.0 min; m/z 551 $[M+H]^+$; m/z 303 [M-malonylglucosyl + H]$^+$).

3.4. Extraction and isolation of flavonoids from the petals of the mauve petal line (WM)

Petals from line WM (212 g fresh wt.) were homogenized and extracted with 50% MeOH containing 1% TFA. Hydrophobic compounds were removed successively from the concentrated extract with $CHCl_3$ and EtOAc. The crude extract was separated by Sephadex LH-20 column chromatography (20-80% MeCN gradient in aq. 0.05 M TFA) to give 5 fractions (A to E are the 5–20, 20–30, 30–36, 36–53 and 53–80% MeCN fractions, respectively). Fractions B and C contained anthocyanins and several flavonol glycosides. Fractions A, D, and E contained flavonol glycosides and phenolic substances. Preparative HPLC (5–40% MeCN gradient in 0.01 M TFA) of B gave three compounds: **1** (8.9 mg), **3** (4.2 mg), and **4** (8.5 mg). Preparative HPLC (5–40% MeCN gradient in 0.01 M TFA) of C gave two compounds: **2** (58.1 mg) and **4** (26.0 mg).

3.5. Acid hydrolysis

Acid hydrolysis was performed using the method reported previously (Kazuma et al., 2003).

3.6. Delphinidin 3-O-(2″-O-α-rhamnosyl-6″-O-malonyl)-β-glucoside (1)

Dark red amorphous powder. UV (0.1% HCl–MeOH) λ_{max} nm (log ϵ): 542 (4.3), 279 (4.2); $AlCl_3$/HCl: 588, 360, 310, 279, and 236. ^{1}H NMR data: see Table 2. Differential NOE experiment data: enhanced H-2 of glucose (9.0% increase) and H-2 of 2″-rhamnose (5.4% increase) on irradiation of H-1 of rhamnose; enhanced H-4 of delphinidin aglycone (18.3% increase) on irradiation of H-1 of glucose. HR-MS: m/z 697.1637 $[M+H]^+$, calc. for $C_{30}H_{33}O_{19}$, 697.1616. ESI-MS: m/z 697 $[M+H]^+$. MS/MS of m/z 697: 653 [M-CO_2 + H]$^+$, 611 [M-malonyl + H]$^+$, 303 [M-malonyl-rhamnosylglucosyl + H]$^+$.

3.7. Delphinidin 3-neohesperidoside (2)

Dark red amorphous powder. ^{1}H NMR data: see Table 2. Differential NOE experiment data: enhanced

H-2 of glucose (9.5% increase) and H-2 of rhamnose (7.0% increase) on irradiation of H-1 of rhamnose; enhanced H-4 of delphinidin aglycone (16.2% increase) on irradiation of H-1 of glucose. ESI-MS: *m/z* 611 $[M+H]^+$. MS/MS of *m/z* 611: 465 [M-rhamnosyl + H]$^+$, 303 [M-rhamnosylglucosyl + H]$^+$.

3.8. Quantitative analyses of anthocyanins and flavonols in the petals of different lines

From the stocked petals of each line, several petals (9.21–170.76 mg) were homogenized and extracted with 50% MeOH containing 0.1% TFA (×1) (870–970 μl). The extracts were centrifuged; the supernatants were filtered with 0.45–μm filters and analyzed. Without any other pre-treatment, 10.0 μl of each extract was injected into a Nanospace semi-micro HPLC system with a photodiode array detector (Shiseido). For the general separation method, a Develosil C30-UG-3 (1.5 i.d.×150 mm, Nomura Chemical) was used with a 14–86% linear gradient of solvent B (40% MeCN containing 0.05 M TFA) in solvent A (5% MeCN containing 0.05 M TFA) for 30 min at a column temp. of 30 °C and a flow rate of 125 μl/min. To quantify anthocyanins and flavonol glycosides, data for two chromatograms (at 530 and 350 nm, respectively) extracted from the PDA data were used. The anthocyanins and flavonol glycosides were quantified as ternatin A1 and kaempferol 3-neohesperidoside, respectively. Since ternatins A2 and C3, and B1 and B2 did not separate well with the general separation method, their ratios were determined from the other separation conditions. To separate ternatin A2 from C3, a Develosil C30-UG-5 (1.5 i.d.×250 mm, Nomura Chemical) was used with a 0–60% linear gradient of solvent B in solvent A for 45 min at a column temp. of 30 °C, and a flow rate of 125 μl/min. To separate ternatin B1 from B2, a Develosil Ph-UG-5 (1.5 i.d.×150 mm, Nomura Chemical) was used with a 5–25% linear gradient of solvent D (80% MeCN containing 0.1% formic acid) in solvent C (5% MeCN containing 0.1% formic acid) for 45 min, at a column temp. of 30 °C, and a flow rate of 125 μl/min.

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