J. Japan. Soc. Hort. Sci. 81 (1): 91–100. 2012.
Available online at www.jstage.jst.go.jp/browse/jjshs1

Flower Colors and their Anthocyanins in *Matthiola incana* Cultivars (Brassicaceae)

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The flower colors and anthocyanin constitution of eight cultivars of Vintage series bedding Stock (*Matthiola incana*) were surveyed to determine the relation between their flower colors and anthocyanin components. Thirteen anthocyanins were isolated from the flowers of these cultivars as major anthocyanins, and their structures were identified by chemical and spectroscopic techniques. Among them, a novel anthocyanin, cyanidin 3-caffeoyl-sambubioside-5-malonyl-glucoside (pigment 1) was found in single and double flowers in cultivars of 'Vintage Lavender' and 'Vintage Burgundy'. Furthermore, two anthocyanins, cyanidin 3-*p*-coumaroyl-sambubioside-5-malonyl-glucoside (pigment 2) and cyanidin 3-feruloyl-sambubioside-5-malonyl-glucoside (pigment 3), were also found in these cultivars for the first time in *Matthiola incana* flowers. Regarding the flower color variation in these cultivars, the hue values (b*/a*) of these flower colors were roughly responsible for the numbers of hydroxycinnamic acid residues in anthocyanin molecules and also hydroxyl patterns of the B-ring in anthocyanidins. These flower colors were classified into eight groups, A–H, based on the hue values of their flowers, and were arranged as follows. In violet flowers (hue values b*/a* = –0.66 and –0.69, V 84A) of group A, cyanidin 3-dihydroxycinnamoyl-sambubioside-5-malonyl-glucosides were major anthocyanin pigments. In purple flowers (–0.43 and –0.45, P 75A) and red-purple flowers (–0.14 and –0.16, RP 74A) of groups B and D, pelargonidin 3-dihydroxycinnamoyl-sambubioside-5-malonyl-glucosides were major anthocyanin pigments. In red-purple flowers (–0.21 and –0.24, RP 72A) of group C, cyanidin 3-monohydroxycinnamoyl-sambubioside-5-malonyl-glucosides were major anthocyanin pigments. In red flowers (0.05 and 0.06, RP 66A) of group E, pelargonidin 3-monohydroxycinnamoyl-sambubioside-5-malonyl-glucosides were major anthocyanin pigments. In copper (0.23 and 0.16, R 54A) and peach (2.37 and 2.09, R38C) of groups F and G, pelargonidin 3-glucoside was a major anthocyanin pigment, and a small amount of pelargonidin 3-glucoside was present in yellow flowers of group H. From these results, the relation between flower colors and the bluing effects of acylated anthocyanins with hydroxycinnamic acids was discussed in flowers of *Matthiola incana* cultivars of Vintage series.

Key Words: acylated cyanidin 3-sambubioside-5-glucoside, acylated pelargonidin 3-sambubioside-5-glucoside, Brassicaceae, *Matthiola incana*, pelargonidin 3-glucoside.

Introduction

Matthiola incana is a popular ornamental plant as a bedding or cut flower plant with various flower colors. Until 1987, the following nine anthocyanin pigments were reported to be present in the flowers of *M. incana*: 3-glucoside, 3,5-diglucoside and 3-feruloyl-*p*-coumaroylsambubioside-5-glucoside of pelargonidin and 3-glucoside, 3-sambubioside-5-glucoside, 3-caffeoylglucoside, 3-*p*-coumaroylglucoside, 3-*p*-coumaroylsambubioside, and 3-caffeoylsambubioside of cyanidin (Harborne, 1964, 1967; Seyffert, 1960; Teusch et al., 1987). Thereafter, Saito et al. (1995, 1996) reported four malonyl or malonyl free cyanidin 3-dihydroxycinnamoyl-sambubioside-5-glucosides from 15 purple-violet flower cultivars, and nine malonyl or malonyl free pelargonidin

Received; June 20, 2011. Accepted; September 22, 2011.
This work was supported in part by a Grant-in-Aid for Scientific Research (C) [No. 22580024, to F.T.] from the Japan Society for the Promotion of Science (JSPS).
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3-mono- or di-hydroxycinnamoyl-sambubioside-5-glucosides from 23 red flower cultivars, and also pelargonidin 3-glucoside and 3-sambubioside-5-glucoside from three red and copper cultivars as major anthocyanins. In this study, we selected sixteen type (eight flower color cultivars with single and double) of Vintage series bedding stock, and investigated their flower colors and anthocyanin constitutions because the flower color variation of Vintage series stock cultivars was rich. In this paper, we report the finding of one novel acylated cyanidin 3-sambubioside-5-glucoside along with 12 known anthocyanins in these cultivars. Additionally, the bluing effect on the hydroxylation of anthocyanin at its 3'-position and the acylation of anthocyanins with hydroxycinnamic acids are discussed for flower color variation in these cultivars.

Materials and Methods

1. General procedures

TLC was carried out on cellulose-coated plastic sheets (Merck, Germany) using seven mobile phases: BAW (*n*-BuOH/HOAc/H_2O, 4 : 1 : 2, v/v/v), BuHCl (*n*-BuOH/2N HCl, 1 : 1, v/v, upper layer), AHW (HOAc/HCl/H_2O, 15 : 3 : 82, v/v/v), and 1% HCl for anthocyanins, Forestal (HOAc/HCl/H_2O, 30 : 3 : 10, v/v/v) for anthocyanidin, and BAW, 15% HOAc, and BEW (*n*-BuOH/EtOH/H_2O, 4 : 1 : 2.2, v/v/v) for acids and sugars with detection using UV light and aniline hydrogen phthalate (AHP) spray reagent (Harborne, 1984).

Analytical HPLC (high performance liquid chromatography) was performed with a LC 10A system (Shimadzu, Japan) using a C18 (4.6ϕ × 250 mm) column (Waters, USA) at 40°C with a flow rate of 1 mL·min^{-1} and monitoring with photodiodearray detector. The eluant was applied as a linear gradient elution for 40 min from 20 to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O) for anthocyanidin and anthocyanin (530 nm) (Tatsuzawa et al., 2010a, b, c; Saito et al., 2011), and as an isocratic elution of solvent A for 10 min for aliphatic acid (210 nm) (Tatsuzawa et al., 2009). UV-Vis spectra were recorded on an MPS-2450 (Shimadzu) in 0.1% HCl-MeOH (from 200 to 700 nm). Spectral absorption of the fresh flowers was directly measured on intact petals using a recording spectrophotometer operated as a double-beam instrument (Type MPS-2450) (Saito, 1967; Yokoi and Saito, 1973). Fast atom bombardment (FAB) mass spectra were obtained in positive ion mode using the magic bullet (5 : 1 mixture of dithiothreitol and dithioerythritol) as a matrix. Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^{1}H spectra, and at 125.78 MHz for ^{13}C spectra in DMSO-CF_3COOD (9 : 1). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants (*J*) are in Hz.

2. Plant materials

The *Matthiola incana* Vintage series ('Vintage Lavender', 'Vintage Burgundy', 'Vintage Lilac', 'Vintage Rose', 'Vintage Red', 'Vintage Copper', 'Vintage Peach', and 'Vintage Yellow') were purchased from PanAmerican Seed Co. (USA). The seeds were sown in February 2009 and 2010, and the plants grown in a greenhouse at Iwate University. The fresh petals were collected in June 2009 and 2010, dried overnight at 45°C, and kept at −10°C until use. The flower colors of these cultivars were recorded by comparing them directly with the Royal Horticultural Society (R.H.S.) color chart and their CIE L*a*b* chromaticity values were recorded on a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd., Japan) (Yokoi, 1975). Five flowers from each cultivar were measured and their average was obtained.

3. Isolation, purification, and identification of anthocyanins

1) Isolation and purification of anthocyanins

Dried petals of 'Vintage Burgundy' (*ca*. 50 g), 'Vintage Red' (*ca*. 10 g), and 'Vintage Copper' (*ca*. 10 g) were immersed in 5% HOAc-H_2O (1 L) at room temperature for 12 h and extracted, respectively. These extracts were passed through a Diaion HP-20 Ion Exchange Resin column (90ϕ × 150 mm, Mitsubishi Chemical, Japan), on which anthocyanins were absorbed. The column was then thoroughly washed with 5% HOAc-H_2O (5 L) and eluted with 5% HOAc-MeOH (300 mL) to recover the anthocyanins. After concentration, these eluates were separated and purified with paper chromatography (No. 590, ADVANTEC, Japan) using BAW. The separated pigments were further purified with preparative HPLC. Preparative HPLC was performed on a Waters C18 (19ϕ × 150 mm) column at 40°C with a flow rate of 1 mL·min^{-1} and monitoring at 530 nm. The solvent used was as follows: linear gradient elution for 30 min from 20 to 85% solvent B in solvent A. Concentrated pigment fractions were dissolved in a small volume of 5% HOAc-EtOH, followed by the addition of excess Et_2O to give precipitated pigments (**1**; *ca*. 10 mg, **2**; *ca*. 15 mg, **3**; *ca*. 20 mg, **4**; *ca*. 2 mg, **5**; *ca*. 2 mg, **6**; *ca*. 5 mg, **7**; *ca*. 2 mg, **8**; *ca*. 2 mg, **9**; *ca*. 5 mg, **10**; *ca*. 2 mg, **11**; *ca*. 2 mg, **12**; *ca*. 5 mg, **13**; *ca* 5 mg).

2) Hydrolyses of anthocyanins

Acid hydrolysis of purified pigments (*ca*. 0.5 mg each) were achieved by 2N HCl (1 mL) at 90°C for 2 h and anthocyanidins, sugars and organic acids were confirmed by direct comparison of TLC and HPLC with the hydrolysates of authentic samples (Saito et al., 1995). Cyanidin [TLC Rf value, 0.42 (Forestal), HPLC retention time (Rt) (min), 23.9] from pigments **1–6**, pelargonidin [TLC Rf value, 0.65 (Forestal), HPLC Rt(min), 28.1] from pigments **7–13**, glucose [TLC Rf value; 0.23 (BAW), 0.86 (15%HOAc), 0.21 (BEW), brown coloration with AHP] from pigments **1–13**, xylose [TLC

Rf value; 0.31 (BAW), 0.88 (15%HOAc), 0.28 (BEW), reddish-brown coloration with AHP] from pigments **1–12**, caffeic acid [TLC Rf value; 0.79 (BAW), 0.36, 0.53 (15%HOAc), 0.80 (BEW)] from pigments **1**, **4**, **7**, and **10**, *p*-coumaric acid [TLC Rf value; 0.91 (BAW), 0.49, 0.74 (15%HOAc), 0.93 (BEW)] from pigments **2**, **5**, **8**, and **11**, ferulic acid [TLC Rf value; 0.87 (BAW), 0.46, 0.65 (15%HOAc), 0.89 (BEW)] from pigments **3**, **6**, **9**, and **12**, sinapic acid [TLC Rf value; 0.83 (BAW), 0.40, 0.56 (15%HOAc), 0.82 (BEW)] from pigments **4–6** and **10–12**, and malonic acid [HPLC for aliphatic acid, Rt(min), 3.2 min] from pigments **1–12**.

Alkalin hydrolysis of purified pigments (*ca*. 0.5 mg each) was achieved by 2N NaOH (1 mL) using a degassed syringe to stir for 15 min. The solution was then acidified with 2N HCl (1.1 mL). The solution was used for TLC and HPLC with authentic 3-sambubioside-5-glucosides of cyanidin and pelargonidin (Saito et al., 1995, 1996).

3) Identification of pigments

The structure of pigments **2–13** was confirmed by TLC, HPLC, and UV-Vis measurements with authentic anthocyanins purified from the flowers of *Matthiola incana* 'Sosei', 'Higan Oh', 'Souka', and 'Chrismas Apricot' and *Lunaria annua* (Saito et al., 1995, 1996; Tatsuzawa et al., 2006); however, pigment **1** was not identical with any of the other known pigments, and was presumed to be an acylated cyanidin 3-sambubioside-5-glucoside; therefore, its structure was elucidated by measuring FAB mass and NMR spectra.

4) Quantitative analyses of pigments in the flowers

Dried petals (*ca*. 10 mg) from each cultivar were immersed in 5% HOAc (0.5 mL) at 4°C for 24 h in order to avoid the production of deacyl anthocyanins, and the pigment solutions were extracted for HPLC analysis of anthocyanin distribution. Five individuals from each cultivar were measured and the averages of cultivars were obtained.

Also, as a similar process, dried petals (*ca*. 10 mg) of each cultivar were immersed in 0.1% HCl-MeOH (10 mL) at 4°C for 24 h and extracted for quantitative analysis of total anthocyanins. The total anthocyanins in these flowers were measured by a UV-Vis spectrophotometer (MPS-2450, Shimadzu). The main visible absorption maximum (nm) of each cultivar and its absorbance were measured.

Furthermore, dried petals (*ca*. 10 mg) of each cultivar were immersed in acetone : methanol = 1 : 1 (v/v) (10 mL) at 4°C for 24 h and extracted for quantitative analysis of total flavonoids (excluding anthocyanins) or carotenoids. The total flavonoids (excluding anthocyanins) and carotenoids in the flowers were measured by UV-Vis spectrophotometer (MPS-2450, Shimadzu). Absorbance at 360 nm was observed for the flavonoid contents of cultivars. For carotenoid pigments, three characteristic absorption maxima were observed in some flowers of cultivars and the absorbance of their main absorption maxima was measured. Moreover, three absorption maxima of carotenoid pigments in 400–500 nm and their main absorbance at 450 nm were compared in each cultivar as carotenoids.

Results and Discussion

1. Analysis of flower colors of Vintage series bedding stock cultivars

1) Flower color measurement by R.H.S. Colour Chart and the colorimetric method

The flower colors of sixteen Vintage series bedding stock cultivars fell into eight groups **A–H**, as follows: violet (V 84A and V 84A) of group **A**, purple (P 75A and P 75A) of group **B**, red-purple (RP 72A and RP 72A) of group **C**, red-purple (RP 74A and RP 74A) of group **D**, red-purple (RP 66A and RP 66A) of group **E**, red (R 54A and R 54A) of group **F**, red (R 38C and R 38C) of group **G**, and yellow (Y 8D and Y 8D) of group **H** (Table 1).

In Table 1 and Figure 1, color data by the CIE L*a*b* system are presented, showing that the color ranges observed in these cultivars are distributed in to violet, purple, red-purple, red, and yellow ranges on the CIE chromaticity diagram. From these results, a significant difference was not observed between single and double petal cultivars.

2. Light absorption spectral curves of intact flowers of these cultivars

The absorption spectral curves of fresh petals of these cultivars were measured in the range of 400–700 nm and the results are given in Table 1 and Figure 2. As shown in Figure 2, the light absorption curves of these fresh petals fell into four types as follows: the first type was composed of group **A**, **B**, and **D** and exhibited a marked shoulder in the region of 574–584 nm as well as one λmax at 541–554 nm, the second type was composed of group **C** and **E** and exhibited one strong λmax at 537–550 nm with a very weak shoulder at near 570–580 nm, the third type was composed of group **F** and exhibited one λmax at 532 nm, and the last was composed of group **G** and **H** and exhibited a weak shoulder at 500–530 nm of anthocyanin pigments and two λmaxs at 453 and 483 nm, indicating the presence of carotenoid pigments.

3. Analysis of anthocyanin pigments

In a survey of eight single and double flower cultivars of *M. incana* by HPLC analysis, 13 anthocyanin peaks were observed as major anthocyanins (Table 2).

1) Pigments 1–6 isolated from the flowers of 'Vintage Lavender' and 'Vintage Burgundy'

Six major pigment (**1–6**) peaks were observed in 5% HOAc extracts of the flowers of 'Vintage Lavender' and 'Vintage Burgundy' by HPLC analysis. Among these pigments, **2**, **3**, and **6** were dominantly distributed in 'Vintage Burgundy', whereas **5** and **6** were dominantly distributed in 'Vintage Lavender' (Table 2). Chromato-

Table 1. Flower color and spectral data of fresh flowers of *Matthiola incana* cultivars.

Group & No.		Cultivars	L*	b*/a*[z]	RHSCC[y]	λmax (nm)[x]				
A	1	Vintage Lavender (Double)[w]	53.47 ± 2.58	−0.66 ± 0.02	V84A				554	(584)
	2	Vintage Lavender (Single)[w]	50.46 ± 2.18	−0.69 ± 0.02	V84A					
B	5	Vintage Lilac (Double)[w]	75.47 ± 2.70	−0.43 ± 0.05	P75A				545	(578)
	6	Vintage Lilac (Single)[w]	74.56 ± 1.45	−0.45 ± 0.05	P75A					
C	3	Vintage Burgundy (Double)[w]	25.88 ± 2.00	−0.24 ± 0.02	RP72A				550	
	4	Vintage Burgundy (Single)[w]	26.35 ± 1.19	−0.21 ± 0.02	RP72A					
D	7	Vintage Rose (Double)[w]	41.52 ± 0.80	−0.16 ± 0.01	RP74A				541	(574)
	8	Vintage Rose (Single)[w]	37.72 ± 0.22	−0.14 ± 0.01	RP74A					
E	9	Vintage Red (Double)[w]	34.05 ± 2.33	0.06 ± 0.02	RP66A				537	
	10	Vintage Red (Single)[w]	30.39 ± 1.33	0.05 ± 0.02	RP66A					
F	11	Vintage Copper (Double)[w]	57.83 ± 2.37	0.23 ± 0.06	R54A				532	
	12	Vintage Copper (Single)[w]	54.07 ± 1.15	0.16 ± 0.02	R54A					
G	13	Vintage Peach (Double)[w]	84.29 ± 0.62	2.37 ± 0.39	R38C	(424)	452	488	(533)	
	14	Vintage Peach (Single)[w]	83.18 ± 1.29	2.09 ± 0.30	R38C					
H	15	Vintage Yellow (Double)[w]	87.50 ± 1.80	−8.84 ± 1.07	Y8D	(424)	453	483	(532)	
	16	Vintage Yellow (Single)[w]	88.93 ± 0.53	−7.82 ± 0.60	Y8D					

[z] Hue (CIE). Mean ± SE (n = 5).
[y] RHS Colour Chart (The Royal Horticultural Society).
[x] ( ) = shoulder.
[w] Flower type.

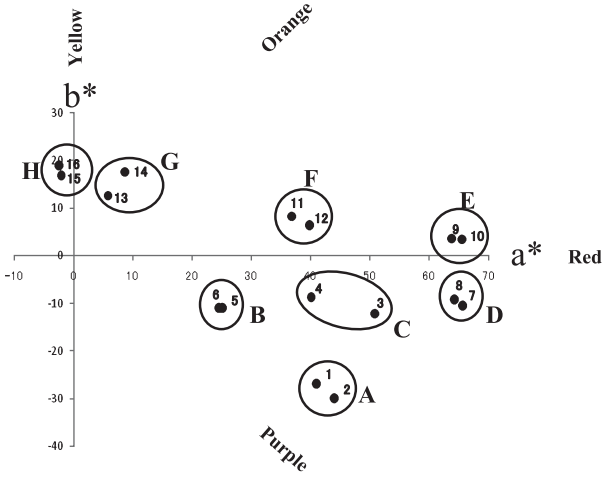

Fig. 1. Distribution of flower colors in *Matthiola incana* cultivars. CIE L*a*b* chromaticity diagram. Group and number are the same as in Table 1, Table 2, and Figure 2.
A: Vintage Lavender [cyanidin type]
B: Vintage Lilac [pelargonidin type]
C: Vintage Burgundy [cyanidin type]
D: Vintage Rose [pelargonidin type]
E: Vintage Red [pelargonidin type]
F: Vintage Copper [pelargonidin type]
G: Vintage Peach [pelargonidin type]
H: Vintage Yellow [pelargonidin type]

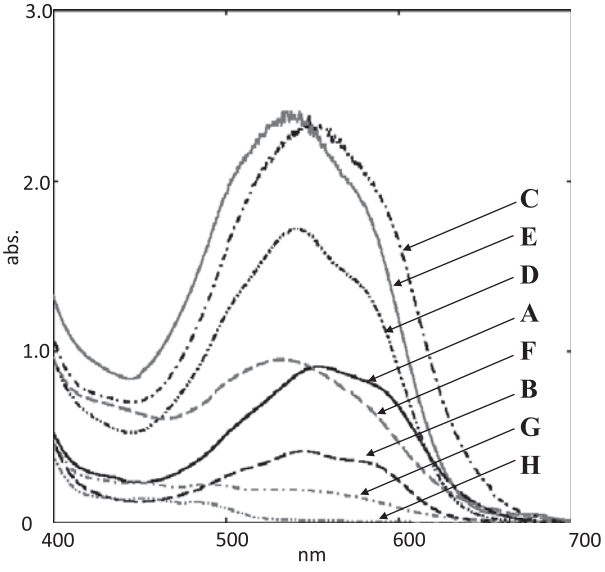

Fig. 2. Absorption spectral curves of fresh petals of *Matthola incana* cultivars. A–H are the same as in Table 1, Table 2, and Figure 1.
Vintage Lavender (A) [(584), 554 nm]
Vintage Lilac (B) [(578), 545 nm]
Vintage Burgundy (C) [550 nm]
Vintage Rose (D) [(574), 541 nm]
Vintage Red (E) [537 nm]
Vintage Copper (F) [532 nm]
Vintage Peach (G) [533, 488, 452, (424) nm
Vintage Yellow (H) [(532), 483, 453, (424) nm]

graphic and spectroscopic properties of pigments **1–6** are summarized in Table 3. The structures of these pigments were confirmed by the following process.

On acid hydrolysis (Saito et al., 1995) all pigments **1–6** yielded cyanidin as the aglycone, glucose and xylose as sugars. Moreover, pigments **1** and **4** gave caffeic acid, pigments **2** and **5** gave *p*-coumaric acid, pigments **3** and **6** gave ferulic acid, and also the pigments **4–6** gave sinapic acid in addition to malonic acid as their acid components. These compounds of cyanidin, glucose, xylose, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, and malonic acid were confirmed by direct

Table 2. Distribution of anthocyanins in *Matthiola incana*.

Group & No.[z]		Cultivars	HPLC data of anthocyanins (as %)[y]												
			1	2	3	4	5	6	7	8	9	10	11	12	13
A	1	Vintage Lavender (Double)[w]	0.5	5.8	6.6	5.4	26.8	42.3							
	2	Vintage Lavender (Single)[w]	0.4	4.0	4.6	7.3	29.9	39.0							
B	5	Vintage Lilac (Double)[w]							0.1	3.1	7.6	6.7	20.8	52.4	
	6	Vintage Lilac (Single)[w]							0.1	3.7	6.5	6.6	31.8	43.8	
C	3	Vintage Burgundy (Double)[w]	3.2	11.4	30.9	1.0	8.4	16.3							
	4	Vintage Burgundy (Single)[w]	2.3	8.0	27.1	1.6	4.4	19.7							
D	7	Vintage Rose (Double)[w]							0.7	5.4	18.2	7.0	14.6	38.6	
	8	Vintage Rose (Single)[w]							1.3	5.1	16.7	5.6	13.3	30.5	
E	9	Vintage Red (Double)[w]							2.5	9.9	33.6	1.4	6.4	21.0	
	10	Vintage Red (Single)[w]							2.8	8.7	25.9	1.4	4.8	21.7	
F	11	Vintage Copper (Double)[w]													78.9
	12	Vintage Copper (Single)[w]													75.6
G	13	Vintage Peach (Double)[w]													81.8
	14	Vintage Peach (Single)[w]													84.0
H	15	Vintage Yellow (Double)[w]													81.1[x]
	16	Vintage Yellow (Single)[w]													82.8[x]

[z] Group & No. is same as Table 1, Figure 1, and Figure 2.
[y] Dried flowers (100 mg) were extracted with 5% HOAc (5 mL) at 4°C for 12 hour. Anthocyanin numbers and their structures are the same as in Table 3.
[x] Trace anthocyanins with pale yellow petals.
[w] Flower type.

Table 3. Chromatographic and spectral properties of anthocyanins from flowers of *Matthiola incana*.

Anthocyanins[z]	R_f values (× 100)				Spectral data in 0.1% HCl-MeOH				HPLC
	BAW	BuHCl	1%HCl	AHW	λmax (nm)	E_{acyl}/E_{max}	E_{440}/E_{max}	$AlCl_3$	R_t (min)
1	15	23	18	48	529, 324, 295, 282	87	12	+	27.0
2	18	35	23	58	529, 314, 295, 281	63	12	+	31.0
3	17	28	21	58	529, 326, 296, 281	68	12	+	31.8
4	41	21	25	61	528, 326, 296, 281	131	11	+	28.7
5	47	27	26	67	529, 320, 297, 281	114	12	+	32.1
6	46	24	27	68	528, 327, 297, 281	114	11	+	32.9
7	34	18	27	60	511, (425), 330, 287	79	22	0	29.8
8	43	24	36	69	511, (427), 320, 289	78	25	0	34.0
9	41	19	35	67	511, (425), 325, 289	75	23	0	34.8
10	46	32	30	64	510, (425), 329, 289	104	22	0	30.8
11	56	44	29	64	511, (420), 320, 289	92	24	0	35.1
12	52	35	32	70	510, (420), 329, 289	98	27	0	35.9
13	53	26	11	35	511, 432, 275	—	41	0	17.7
Cy3Sa5G	10	7	29	52	527, 277	—	16	+	12.2
Pg3Sa5G	14	10	40	59	507, 282	—	28	0	14.9

[z] 1: cyanidin 3-[2-(xylosyl)-6-(caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside]
2: cyanidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]
3: cyanidin 3-[2-(xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside]
4: cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside]
5: cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]
6: cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside]
7: pelargonidin 3-[2-(xylosyl)-6-(caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside]
8: pelargonidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]
9: pelargonidin 3-[2-(xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside]
10: pelargonidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside]
11: pelargonidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside]
12: pelargonidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside]
13: pelargonidin 3-glucoside
Cy3Sa5G: cyanidin 3-sambubioside-5-glucoside
Pg3Sa5G: pelargonidin 3-sambubioside-5-glucoside

comparison of TLC and HPLC analyses with authentic samples which were commercially available and also obtained from the hydrolysates of authentic samples, which were isolated from stock flowers (Saito et al., 1995).

By alkaline hydrolysis, pigments **2–6** yielded only one anthocyanin as their deacylanthocyanin. This anthocyanin was identified to be cyanidin 3-sambubioside-5-glucoside by TLC and HPLC analysis with an authentic sample obtained from *M. incana* (Saito et al., 1995) (Table 3).

Furthermore, based on their Rf values on TLC, their spectral properties by UV-Vis, and their Rts of HPLC (Table 3) by direct comparison with authentic anthocyanins (Saito et al., 1995; Tatsuzawa et al., 2006) (Table 3) the following detailed structures of pigments **2–6** were determined: cyanidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **2**; cyanidin 3-[2-(xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **3**; cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **4**; cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **5**; cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **6**. These results revealed findings for pigments **2** and **3** for the first time in the genus *Matthiola*. Moreover, the structure of pigment **1** has not been reported until now. The structure of pigment **1** was determined by analysis of its high-resolution FAB mass spectra (HR-FABMS) and NMR spectra as follows (Tatsuzawa et al., 2006).

The molecular ion $[M]^+$ of pigment **1** was observed at *m/z* 991.2 ($C_{44}H_{47}O_{26}$) using FABMS, indicating that pigment **1** is composed of cyanidin with two molecules of glucose and one molecule each of xylose, malonic acid, and caffeic acid. These elemental components of pigment **1** were confirmed by measuring its HR-FABMS (calc $C_{44}H_{47}O_{26}$: 991.2356. Found: 991.2371).

The structure of pigment **1** was further elucidated on the basis of analysis of its ^{1}H and ^{13}C NMR spectra, including 2D COSY, 2D NOESY, HMQC, and HMBC spectra according to the process described before (Tatsuzawa et al., 2006). ^{1}H NMR δ cyanidin: 8.78 (*s*, H-4), 7.02 (*brs*, H-6), 7.07 (*brs*, H-8), 8.07 (*d*, *J*=2.5 Hz, H-2'), 7.08 (*d*, *J*=9.2 Hz, H-5'), 8.39 (*dd*, *J*=2.2, 9.2 Hz, H-6'). Caffeic acid I: 6.29 (*brd*, *J*=1.9 Hz, H-2), 6.74 (*d*, *J*=8.3 Hz, H-5), 6.87 (*dd*, *J*=1.9, 8.3 Hz, H-6), 6.22 (*d*, *J*=15.9 Hz, H-7 (α)), 7.35 (*d*, *J*=15.9 Hz, H-8 (β)). Malonic acid: 3.39 (*s*, -CH_2-). Glucose A (Glc A): 5.72 (*d*, *J*=7.7 Hz, H-1), 4.04 (*t*, *J*=8.2 Hz, H-2), 3.77 (*t*, *J*=9.0 Hz, H-3), 3.50 (*t*, *J*=9.5 Hz, H-4), 4.02 (*t*, *J*=8.2 Hz, H-5), 4.32 (*dd*, *J*=6.7, 11.9, H-6a), 4.45 (*brd*, *J*=11.9, H-6b). Glucose B (Glc B): 5.19 (*d*, *J*=7.7 Hz, H-1), 3.57 (*t*, *J*=9.5 Hz, H-2), 3.45 (*t*, *J*=9.2 Hz, H-3), 3.30 (*t*, *J*=9.3 Hz, H-4), 3.80 (*t*, *J*=7.9 Hz, H-5), 4.12 (*dd*, *J*=6.4, 11.9, H-6a), 4.46 (*brd*, *J*=11.9, H-6b). Xylose: 4.75 (*d*, *J*=8.0 Hz, H-1), 3.05 (*t*, *J*=8.5 Hz, H-2), 3.18 (*t*, *J*=8.9 Hz, H-3), 3.28 (*t*, *J*=9.2 Hz, H-4), 3.00 (*dd*, *J*=10.7, 11.0 H-5a), 3.59 (*dd*, *J*=7.6, 13.0 H-5b). ^{13}C NMR δ cyanidin: 162.4 (C-2), 144.6 (C-3), 131.9 (C-4), 155.4 (C-5), 105.2 (C-6), 167.7 (C-7), 96.5 (C-8), 155.1 (C-9), 111.5 (C-10), 119.7 (C-1'), 118.0 (C-2'), 146.6 (C-3'), 155.5 (C-4'), 117.0 (C-5'), 128.4 (C-6'). Caffeic acid: 125.8 (C-1), 115.9 (C-2), 145.8 (C-3), 148.5 (C-4), 116.0 (C-5), 121.2 (C-6), 114.0 (C-7 (α)), 145.6 (C-8 (β)), 166.9 (C-9 (COO)). Malonic acid: 166.9 (C-1), 41.4 (C-2), 167.1 (C-3). Glucose A: 98.8 (C-1), 80.7 (C-2), 76.8 (C-3), 69.9 (C-4), 74.2 (C-5), 63.2 (C-6). Glucose B: 102.0 (C-1), 73.4 (C-2), 76.1 (C-3), 69.7 (C-4), 74.5 (C-5), 64.3 (C-6). Xylose: 104.9 (C-1), 74.5 (C-2), 76.8 (C-3), 69.7 (C-4), 66.3 (C-5).

The chemical shifts of 9 aromatic protons of cyanidin and caffeic acid moieties with their coupling constants were assigned by analysis of its 2D COSY spectrum as shown in the section of *Pigment **1***. The ^{1}H NMR spectrum exhibited two olefinic proton signals of caffeic acid with large coupling constants (*J*=15.9 Hz). Caffeic acid was determined to be in the *trans* configuration. Chemical shifts of the sugar moieties were observed in the region of δ3.00–5.72, where the three anomeric proton resonated at δ5.72 (*d*, *J*=7.7 Hz, Glc A-H1), δ5.19 (*d*, *J*=7.7 Hz, Glc B-H1), and δ4.75 (*d*, *J*=8.0 Hz, Xyl-H1), respectively. Based on the observed coupling constants, these three sugars were assumed to be in β-pyranose forms. By analysis of the 2D COSY spectrum, a proton signal (δ4.04) shifting to a lower magnetic field was assigned to H-2 of Glc A, and four characteristic proton signals, shifted to a lower magnetic field, were assigned as methylene protons of Glc A (δ4.32 and 4.45, H-6a and -6b) and Glc B (δ4.12 and 4.46, H-6a and -6b), respectively. These results indicated that the two OH-6 groups of Glc A and Glc B were acylated with a caffeic acid and malonic acid, respectively. NOESY and HMBC spectra were used to distinguish the sites of attachment of glucose, xylose, caffeic acid, and cyanidin aglycone (Fig. 3). The signals of the proton H-4 (δ8.78) of cyanidin and H-1 (δ5.72) of Glc A, the proton H-6 (δ7.02) of cyanidin, and H-1 (δ5.19) of Glc B, the proton H-2 of Glc A and H-1 (δ4.75) of xylose, and the protons H-6a, b (δ4.32 and 4.45) of Glc A and H-6 (δ6.87) of caffeic acid correlated in the NOESY spectrum. Moreover, the signals of the anomeric proton of Glc B correlated to the signals of the C-5 (δ155.4) of cyanidin, H-2 proton of Glc A and H-1 (δ4.75) proton of xylose correlated to the signals of the C-1 (δ104.9) of xylose and C-2 (δ80.7) of Glc A, respectively, in the HMBC spectrum. These characteristic features revealed that the OH-3 and OH-5 positions of cyanidin are both bound to glucose molecules, and the OH-2 and OH-6 positions of Glc A are bonded to xylose and caffeic acid molecules, respectively. Consequently, the structure of pigment **1** was elucidated to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-caffeoyl)-β-glucopyranoside]-5-*O*-[6-*O*-

(malonyl)-β-glucopyranoside] (Fig. 3), which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999). The structures of pigments **1–6** and pigments **7–13** are illustrated in Figure 4.

2) Pigments 7-13 isolated from the flowers of 'Vintage Lilac', 'Vintage Rose', 'Vintage Red', 'Vintage Copper', 'Vintage Peach', and 'Vintage Yellow'

By HPLC analysis, seven peaks of anthocyanin pigments (**7–13**) were observed in 5% HOAc extracts of the flowers of 'Vintage Lilac', 'Vintage Rose', 'Vintage Red', 'Vintage Copper', 'Vintage Peach', and 'Vintage Yellow'. These pigment peaks were isolated and purified using Diaion HP-20 column chromatography, preparative paper chromatography, and preparative HPLC, according to the procedure described previously (Saito et al., 1996). Chromatographic and spectroscopic properties of pigments **7–13** are summarized in Table 3.

These pigments were identified in direct comparison with authentic anthocyanins obtained from *M. incana* (Saito et al., 1996) by TLC, HPLC, and spectroscopic analytical methods as follows (Table 3). The structures of these pigments were pelargonidin 3-[2-(xylosyl)-6-(caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **7**, pelargonidin 3-[2-(xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **8**, pelargonidin 3-[2-(xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **9**, pelargonidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(caffeoyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **10**, pelargonidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **11**, pelargonidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside] as pigment **12**, and pelargonidin 3-glucoside as pigment **13** (Fig. 4). In these pigments, pigments **7–9** (mono-hydroxycinnamoyl anthocyanins) were distributed dominantly as compared with pigments **10–12** (di-hydroxycinnamoyl anthocyanins) in 'Vintage Red' whereas the later pigment groups were shown dominantly in 'Vintage Lilac' and 'Vintage Rose' (Table 2). Pigment **13** was dominantly distributed in 'Vintage Copper', 'Vintage Peach', and 'Vintage Yellow' (Table 2).

4. Distribution of anthocyanins and their flower colors in 16 cultivars of Vintage series bedding stocks

As mentioned above, the sixteen types (eight flower color cultivars with single and double) of Vintage series bedding stock were classified into eight groups **A–H** depending upon their flower colors and anthocyanin components (Figs. 1, 2, and 5, Tables 1 and 2). In group **A**, the flowers of cultivars were violet (V 84A and V 84A) on the R.H.S. Colour Chart and their hue values (b*/a*) = −0.69 and −0.66 by SE-2000 Spectro color meter. As their major pigments, pigments **6** (average: 40.7%) and **5** (28.4%) were identified to be cyanidin 3-[2-(2-(acyl-I)-xylosyl)-6-(acyl-II)-glucoside]-5-[6-malonyl-glucoside], in which the acyl-I groups were sinapic acid for pigments **6** and **5**, and the acyl-II groups were ferulic acid for pigment **6** and *p*-coumaric acid for pigment **5**. So, both pigments in group A were dihydroxycinnamoyl cyanidin glycosides. In group **B**, the flowers were purple (P 75A and P 75A) with hue values = −0.43 and −0.45. As their major pigments, pigments **12** (48.1%) and **11** (26.3%) were determined to be pelargonidin3-[2-(2-(acyl-I)-xylosyl)-6-(acyl-II)-glucoside]-5-[6-malonyl-glucoside], in which the acyl-I groups were sinapic acid for pigments **12** and **11** and the acyl-II groups were ferulic acid for pigment **12** and *p*-coumaric acid for pigment **11**; therefore, both pigments in group B were also dihydroxycinnamoyl pelargonidin glycosides. In group **C**, the flowers were red-purple (RP 72A and RP 72A) with hue values = −0.24 and −0.21. As their major pigments in group **C**, mixtures of mono- and di-hydroxycinnamoyl cyanidin 3-sambubioside-5-malonyl-glucosides [pigment **3** (29.0%) and pigment **6** (18.0%)]

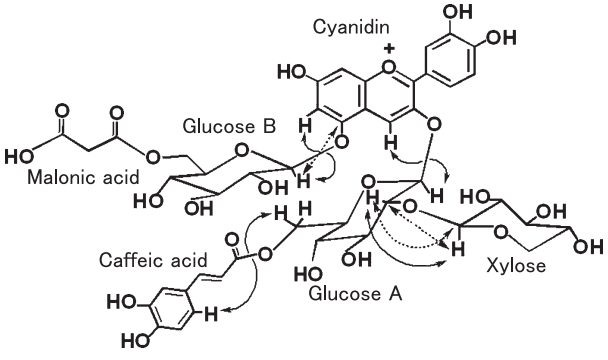

Fig. 3. New anthocyanin (pigment 1) from the flowers of *Matthiola incana* cultivars.
Observed NOE correlations are indicated by arrows.
Observed HMBC correlations are indicated by dotted arrows.

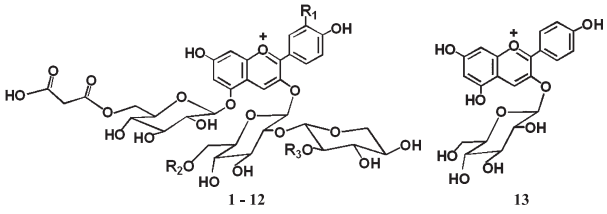

Fig. 4. Structures of anthocyanins isolated from the flowers of *Matthiola incana* cultivars.

	R_1	R_2	R_3
1:	OH	caffeoyl	H
2:	OH	*p*-coumaroyl	H
3:	OH	feruloyl	H
4:	OH	caffeoyl	sinapoyl
5:	OH	*p*-coumaroyl	sinapoyl
6:	OH	feruloyl	sinapoyl
7:	H	caffeoyl	H
8:	H	*p*-coumaroyl	H
9:	H	feruloyl	H
10:	H	caffeoyl	sinapoyl
11:	H	*p*-coumaroyl	sinapoyl
12:	H	feruloyl	sinapoyl
13:	Pelargonidin 3-glucoside		

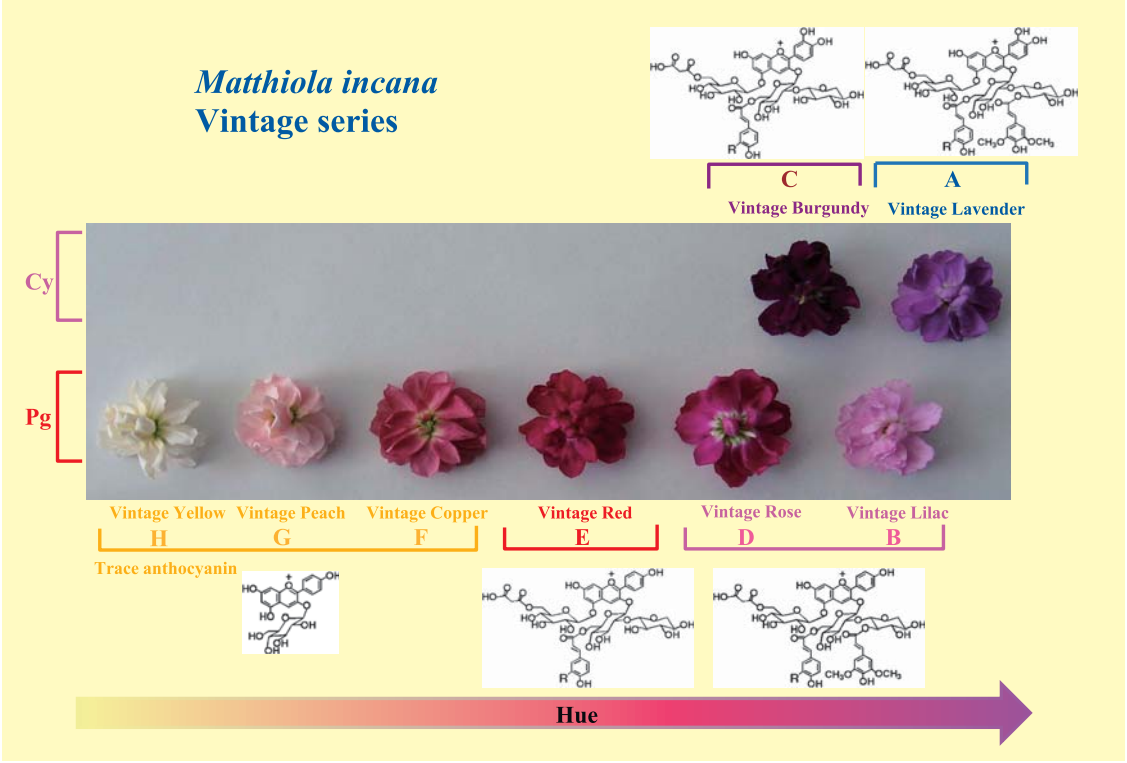

Fig. 5. Fresh flower colors of *Matthiola incana* cultivars, and their major anthocyanin components.

were observed, and the structure of pigment **3** was identified to be cyanidin 3-[2-(xylosyl)-6-(feruloyl)-glucoside]-5-[6-malonyl-glucoside]. In group **D**, the flowers were red-purple (RP 74A and RP 74A) with hue values = −0.16 and −0.14. As major pigments, mixtures of mono- and di-hydroxycinnamoyl pelargonidin 3-sambubioside-5-malonylglucosides [pigment **12** (34.6%), pigment **11** (14.0%), and pigment **9** (17.5%)] were observed in these cultivars, and the structure of pigment **9** was identified to be pelargonidin 3-[2-(xylosyl)-6-(feruloyl)-glucoside]-5-[6-malonyl-glucoside].

The flower colors of group **D** were very similar to those of group **B** and **C**, but their color characteristics were clearly distinguished by the CIE L*a*b* chromaticity diagram (Fig. 1). In group **E**, the flowers were red-purple (RP 66A and RP 66A) with hue values = 0.06 and 0.05. Their major pigments were another mixture of mono- and di-hydroxycinnamoyl pelargonidin 3-sambubioside-5-malonylglucosides [pigment **9** (29.8%) and pigment **12** (21.4%)]. In groups **F** and **G**, the flowers of both groups were red (R 54A and R 54A for group **F**, and R 38C and R 38C for group **G**) with their hue values = 0.23 and 0.16 for group **F**, and 2.37 and 2.09 for group **G**. Pigment **13** was found in the flowers of both groups as their dominant anthocyanin, and was identified to be pelargonidin 3-glucoside. In group **H**, the flowers were yellow (Y 8D and Y 8D) with hue values = −8.84 and −7.82. As the anthocyanin pigment, a small amount of pigment **13** was detected in the flowers of this group. Furthermore, carotenoid pigments were detected in the flowers of this group as well as those of groups **F** and **G** (Table 4).

From these results, it was revealed that the flower colors and their hue values in these groups were shifted more to blue or red and decreased or increased depending on their percentages of di-hydroxycinnamoyl-anthocyanin contents as follows. Group **A** (V 84A, average: −0.68) in 75.4% and group **C** (RP 72A, −0.23) was 25.7% in the cyanidin glycoside types, and also in the pelargonidin glycoside types, group **B** (P 75A, −0.44) was 81.1%, group **D** (RP 74A, −0.15) was 54.8%, group **E** (RP 66A, 0.06) was 28.4%, and group **F** (R 54A, 0.20) was 0%. It is noteworthy that the bathochromic shift of group **B** is larger than that of group **C** (Fig. 2). Namely, the bluing effect of the acylation with di-hydroxycinnamic acid is more effective than that of the hydroxylation at 3' position of pelargonidin in these cultivars.

The absorption spectral curves of fresh petals of these cultivars are shown in the region of 400–700 nm in Figure 2. From the results of their spectroscopic analysis, it was revealed that the absorption maxima in these spectral curves were shifted into longer wavelength regions depending upon the increasing percentages of dihydroxycinnamoyl anthocyanin contents in these groups, including pelargonidin pigments, such that 81.1% was at λ_{max} 545 nm in group **B**, 54.8% was at λ_{max} 541 nm in group **D**, 28.4% was at λ_{max} 537 nm in group **E**, and 0% was at λ_{max} 532 nm in group **F**. Furthermore, in groups **A** and **C** of cyanidin pigments,

Table 4. Absorption maxima and their absorbances of *Matthiola incana* cultivars in 0.1% HCl-MeOH.

Group & No.[z]		Cultivars	Anthocyanins[y]		Carotenoids[x]		Other Flavonoids[w]
			λmax (nm)	ABS	λmax (nm)	ABS	ABS
A	1	Vintage Lavender (Double)[v]	530	0.360	—	—	0.785
	2	Vintage Lavender (Single)[v]	530	0.341	—	—	0.486
B	5	Vintage Lilac (Double)[v]	512	0.116	—	—	0.782
	6	Vintage Lilac (Single)[v]	512	0.113	—	—	0.595
C	3	Vintage Burgundy (Double)[v]	528	1.750	—	—	1.246
	4	Vintage Burgundy (Single)[v]	528	1.226	—	—	1.129
D	7	Vintage Rose (Double)[v]	511	1.331	—	—	1.248
	8	Vintage Rose (Single)[v]	511	1.088	—	—	1.280
E	9	Vintage Red (Double)[v]	511	1.887	—	—	1.236
	10	Vintage Red (Single)[v]	511	1.278	—	—	1.342
F	11	Vintage Copper (Double)[v]	509	0.523	(419), 442, 472	0.026	1.893
	12	Vintage Copper (Single)[v]	509	0.380	(419), 442, 472	0.037	1.652
G	13	Vintage Peach (Double)[v]	509	0.134	(419), 442, 472	0.028	1.365
	14	Vintage Peach (Single)[v]	509	0.104	(419), 442, 472	0.020	1.208
H	15	Vintage Yellow (Double)[v]	509 (shoulder)	0.017	(419), 442, 472	0.038	1.469
	16	Vintage Yellow (Single)[v]	509 (shoulder)	0.012	(419), 442, 472	0.032	1.495

[z] Group & No. is the same as in Table 1, Figure 1, and Figure 2.
[y] Dried flowers (10 mg) were extracted with 0.1% HCl-MeOH (10 mL) at 4°C for 24 hours. UV-Vis spectra of extracts (1.5 mL) were recorded on a MPS-2450 from 400 to 700 nm.
[x] Dried flowers (10 mg) were extracted with acetone : MeOH = 1 : 1 (v/v) (10 mL) at 4°C for 24 hours. UV-Vis spectra of extracts (1.5 mL) were recorded on a MPS-2450 from 400 to 500 nm.
[w] Dried flowers (10 mg) were extracted with acetone : MeOH = 1 : 1 (v/v) (10 mL) at 4°C for 24 hours. UV-Vis spectra of extracts (1.5 mL) were recorded on a MPS-2450 at 360 nm.
[v] Flower type.

75.4% of dihydroxycinnamoyl anthocyanin content was at λ_{max} 554 nm in group **A** and 25.7% was at λ_{max} 550 nm in group **C**. Moreover, the absorption spectral curves of the cultivars, which are in a high percentages of contents for dihydroxycinnamoyl anthocyanins exhibited marked strong shoulders in the region of 574–584 nm so that group **A** was near 584 nm, group **B** was near 578 nm, and group **D** was near 574 nm (Table 1, Fig. 2).

From these results, the acylation of anthocyanins with two molecules of hydroxycinnamic acids is considered to be responsible for the bathocromic shifts of the absorption maxima in spectral curves. This was thought to be the most important effect on flower colors in these *M. incana* cultivars. Moreover, in these plants, the bluing effect of dihydroxycinnamoyl pelargonidin glycosides is stronger than that of hydroxylation at 3' position of their aglycone of pelargonidin glycosides.

Regarding the yellow flower cultivars of stock, breeding deep yellow flowers is still one of the important subjects in this field. As some cultivars show rather low contents of carotenoid pigments in groups **F**, **G**, and **H**, the amplification of carotenoid pigment contents will be expected for stock flowers in the future.

Acknowledgements

We thank Mr. James Hall (Iwate University) and Dr. Kazumitsu Miyoshi (Akita Pref. University) for careful revision of the manuscript.

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