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Copigmentation with Acylated Anthocyanin and Kaempferol Glycosides in Violet and Purple Flower Cultivars of *Aubrieta* × *cultorum* (Brassicaceae)

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Flavonoid pigments and flower colors of the violet and purple flower cultivars 'Royal Violet' and 'Royal Red', respectively, of *Aubrieta* × *cultorum* in the family Brassicaceae were investigated. Three acylated cyanidin 3-sambubioside-5-glucosides (1–3) were isolated as the main anthocyanins from violet and purple flowers of both cultivars. In particular, 1 was the dominant anthocyanin in both cultivars. Furthermore, an acylated flavonol glycoside (4) and another acylated flavonol glycoside (5) were isolated as the main flavonol pigments from flowers of 'Royal Violet' and 'Royal Red', respectively. The structures of 1–5 were elucidated by chemical and spectroscopic methods. Pigments 1, 2, 4, and 5 were found to be new flavonoid pigments. The structures of 1 and 2 were determined to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] and its demalonyl derivative, respectively. The structures of 4 and 5 were determined to be kaempferol 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-7-*O*-[4-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside] and its desinapoyl-cellobiosyl derivative, respectively. Pigment 3 was identified to be the known compound cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside]. The flower color of 'Royal Violet' was Violet 87A and that of 'Royal Red' was Purple 78A according to the R.H.S. Color Chart. On the visible absorption spectral curve, fresh petals and pressed petal juices of 'Royal Violet' in pH 5.0 buffer solution showed two characteristic absorption maxima at 560 and 604 nm and those of 'Royal Red' showed a single broad absorption maximum near 546 nm. In the reproducible experiments of their flower colors in pH 5.0 buffer solution, the violet solution was produced by the mixture of 1 and 4 (1 : 2) and exhibited characteristic absorption maxima at 560 and 604 nm, similar to those of the flower of 'Royal Violet'. In contrast, a mixture of 1 and 5 or that of 1 and other common copigments produced a purple solution with only one absorption maximum near 547 nm. From these results, it was revealed that 4 was more appropriate for the bluing effect of *A.* × *cultorum* cultivars by intermolecular copigmentation with 1 than 5.

Key Words: acylated cyanidin 3-sambubioside-5-glucosides, acylated kaempferol 3,7-tetraglucoside, *Aubrieta* × *cultorum* Bergmans, copigmentation, flower color.

Introduction

The genus *Aubrieta* Adans. (Brassicaceae), comprising about 12 species of low evergreen perennials, is native to Europe and Central Asia. *A.* × *cultorum* Bergmans is best known in gardens for a large number of cultivars with lilac, pink, and purple to violet flowers.

In our continued work on flower color variations caused by acylated anthocyanins in the Brassicaceae, we have already reported the distribution of structurally complicated anthocyanidin glycosides in the flowers of *Matthiola incana* (Saito et al., 1995, 1996; Tatsuzawa et al., 2012), *Orychophragmus violaceus* (Honda et al., 2005), *Cheiranthus cheiri*, *Lobularia maritima*, *Lunaria annua* (Tatsuzawa et al., 2006, 2007, 2010c), *Malcolmia maritima* (Tatsuzawa et al., 2008a), *Heliophila*

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coronopifolia (Saito et al., 2011b), *Iberis umbellata* (Saito et al., 2008), and *Raphanus sativus* (Tatsuzawa et al., 2008b). In this study, we report the structural elucidations of two new acylated cyanidin 3-sambubioside-5-glucosides and two new acylated kaempferol glycosides along with a known anthocyanin isolated from violet and purple flowers of *A.* × *cultorum* cultivars 'Royal Violet' and 'Royal Red', respectively. The bluing effects of these flavonol glycosides on flower colors of both cultivars are also discussed.

Materials and Methods

1. General procedures

Thin layer chromatography (TLC) was carried out on plastic coated cellulose sheets (Merck, Germany) using seven mobile phases: BAW (*n*-BuOH-HOAc-H_2O, 4 : 1 : 2), BuHCl (*n*-BuOH-2N HCl, 1 : 1, upper layer), AHW (HOAc-HCl-H_2O, 15 : 3 : 82), and 1% HCl (HCl-H_2O, 3 : 97) for anthocyanins and flavonols, and BAW, EAA (EtOAc-HOAc-H_2O, 3 : 1 : 1), ETN (EtOH-NH_4OH-H_2O, 16 : 1 : 3), and EFW (EtOAc-HCOOH-H_2O, 5 : 2 : 1) for sugars and organic acid with UV light and aniline hydrogen phthalate spray reagent (Harborne, 1984).

Analytical high performance liquid chromatography (HPLC) was performed on a LC 10A system (Shimadzu, Japan), using a Waters C18 (4.6ϕ × 250 mm) column (Waters, USA) at 40°C with a flow rate of 1 mL·min^{-1} and monitoring at 530 nm for anthocyanins and at 350 nm for flavonols by a photo diode array 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) (Saito et al., 2011a; Tatsuzawa et al., 2010a, b).

UV-Vis spectra were recorded on UV-Vis Multi Purpose Spectrophotometer (MPS-2450, Shimadzu, Japan) in 0.1% HCl-MeOH (from 200 to 700 nm). The spectral absorption of flowers was directly measured on intact tissue using a recording spectrophotometer operating as a double-beam instrument (Type: MPS-2450) (Saito, 1967; Yokoi and Saito, 1973).

High resolution FAB mass (HR FABMS) spectra were obtained in positive ion mode using the magic bullet (5 : 1 mixture of dithiothreitol and dithioerythritol) as a matrix. NMR spectra were determined at 500 MHz for ^{1}H spectra and at 125.78 MHz for ^{13}C spectra in DMSO-d_6-CF_3COOD (9 : 1) for anthocyanins and DMSO-d_6 for flavonols. Chemical shifts are reported relative to a TMS internal standard (δ) and coupling constants are in Hz.

2. Plant materials

Seeds of *Aubrieta* × *cultorum* 'Royal Violet' and 'Royal Red' were purchased from M&B Flora Co., Ltd. (Japan), and grown in greenhouses at Iwate University (Japan) and Minami Kyushu University (Japan). Flowers were collected during winter and spring seasons. The flowers of 'Royal Violet' and 'Royal Red' exhibited a violet color [Violet 87A by Royal Horticultural Society (R.H.S.) Color Chart, chromaticity value b*(−49.44)/a*(59.76) = −0.83 and L*(30.87)], and a purple color [Purple 78A by R.H.S. Color Chart, chromaticity value b*(−22.47)/a*(55.40) = −0.41 and L*(27.93)], recorded on a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd., Japan), respectively. The collected flowers were dried at 40°C overnight and stored at −20°C in the refrigerator until needed.

3. Isolation of flower pigments

Dried violet and purple flower mixture (ca. 30 g each) of *A.* × *cultorum* was immersed in 5% HOAc (acetic acid-water; 5 : 95, v/v) (5 L) at room temp. for 5 h. The extract was passed through a Diaion HP-20 (Mitsubishi Chemical, Japan) column (90 × 150 mm), on which acylated pigments were absorbed. The column was thoroughly washed with 5% HOAc (15 L), and eluted with 5% HOAc-MeOH (1 L) to recover the pigments. After concentration, eluates were separated and purified as anthocyanins and flavonols with paper chromatography using BAW.

For further purification of the anthocyanins, the crude anthocyanin pigment powder extracted from separated paper was subjected to HPLC on a Waters C18 (19 × 150 mm) at 40°C with a flow rate of 1 mL·min^{-1} and monitoring at 530 nm. The solvent used was as follows; a linear gradient elution for 10 min from 70 to 80% solvent B in solvent A. By this procedure, **1–3** were completely separated from the crude anthocyanin pigment powder as three different fractions. Each fraction was transferred to a Diaion HP-20 column, on which pigments were adsorbed. **1** and **3** were eluted with 5% HOAc-MeOH followed by the addition of excess Et_2O, and then dried. Purified **1** (*ca.* 30 mg), **2** (*ca.* 5 mg) and **3** (*ca.* 7 mg) were obtained.

For further purification of the flavonols, the crude flavonol pigment powder extracted from separated paper was subjected to HPLC on a Waters C18 (19 × 150 mm) at 40°C with a flow rate of 1 mL·min^{-1} and monitoring at 350 nm. The solvent used was as follows; a linear gradient elution for 10 min from 70 to 80% solvent B in solvent A. By this procedure, **4** and **5** were completely separated from the crude flavonol pigment powder as two different fractions. Each fraction was transferred to a Diaion HP-20 column, on which pigments were adsorbed. Pigments **4** and **5** were eluted with MeOH followed by the addition of excess Et_2O, and then dried. Purified **4** (*ca.* 30 mg) and **5** (*ca.* 25 mg) were obtained.

4. Analyses of pigments

Anthocyanins were identified by standard procedures involving deacylation, demalonylation, and both alkaline and acid hydrolyses (Harborne, 1984; Saito et al., 2008).

*1) Pigment **1**: cyanidin 3-O-[2-O-(β-xylopyranosyl)-6-O-(trans-sinapoyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside]*

Dark red powder: UV-Vis (in 0.1% HCl-MeOH):

Table 1. NMR[z] spectral data of acylated anthocyanins isolated from *Aubrieta* × *cultorum*.

	1			2		3	
	^{1}H		^{13}C	^{1}H		^{1}H	
Cyanidin							
2			162.4				
3			144.6				
4	8.79	s	131.5	8.80	s	8.72	s
5			154.9				
6	6.96	s	104.6	6.98	s	6.98	d (1.8)
7			167.3				
8	7.07	s	96.3	7.07	s	7.03	d (1.8)
9			155.2				
10			111.7				
1'			119.5				
2'	8.03	d (2.2)	117.7	8.03	d (2.3)	8.03	d (2.4)
3'			146.4				
4'			155.3				
5'	7.06	d (8.9)	116.8	7.04	d (8.9)	7.05	d (8.8)
6'	8.39	dd (2.2, 8.9)	128.3	8.38	dd (2.3, 8.9)	8.38	dd (2.4, 8.8)
Glucose A							
1	5.68	d (7.3)	98.8	5.69	d (7.3)	5.71	d (7.3)
2	4.02	t (8.1)	80.4	4.01	t (7.9)	4.01	t (8.0)
3	3.51	t (9.0)	69.7	3.49	t (7.8)	3.75	t (9.2)
4	3.41	t (7.3)	75.8	3.42	m	3.45	t (9.2)
5	4.00	m	73.9	3.93	m	4.01	m
6a	4.26	dd (6.3, 12.0)	63.1	4.30	m	4.28	dd (7.3, 12.2)
6b	4.47	brd (12.0)		4.42	brd (12.0)	4.42	brd (12.2)
Glucose B							
1	5.18	d (7.7)	101.6	5.10	d (8.0)	5.17	d (7.6)
2	3.55	t (7.6)	73.2	3.53	m	3.56	t (8.4)
3	3.39	m	75.8	3.39	t (8.7)	3.41	t (8.7)
4	3.20	t (9.5)	69.6	3.23	m	3.27	t (9.5)
5	3.80	m	76.6	3.68	m	3.80	m
6a	3.87	dd (7.2, 12.0)	64.1	3.40–3.80		4.07	dd (6.3, 11.8)
6b	4.40	brd (12.0)		3.40–3.80		4.40	brd (11.8)
Xylose							
1	4.73	d (7.6)	104.8	4.73	d (7.7)	4.73	d (8.0)
2	3.02	t (8.2)	74.3	3.02	t (8.6)	3.02	t (8.4)
3	3.15	t (8.9)	76.6	3.15	t (8.9)	3.15	t (8.9)
4	3.26	m	69.5	3.31	m	3.26	m
5a	3.55	m	66.1	3.55	m	3.55	m
5b	2.97	t (11.0)		2.95	t (11.2)	2.99	t (11.0)
Hydroxycinnamic acid (I)							
1			124.4				
2, 6	6.83	s	106.4	6.87	s	7.32	d (8.6)
3, 5			148.0			6.72	d (8.6)
4			138.5				
7(α)	6.38	d (15.9)	114.5	6.40	d (15.6)	6.26	d (15.9)
8(β)	7.45	d (15.9)	145.8	7.44	d (15.6)	7.37	d (15.9)
9			166.7				
-CH_3 × 2	3.75	s	56.1	3.78	s		
Malonic acid							
-CH_2-	3.41	s	41.2			3.35	s
COO			166.9				
COO			168.2				

[z] ^{1}H NMR 500 MHz and ^{13}C NMR 125.78 MHz in DMSO-d_6-CF_3COOD (1 : 9) TMS as an internal standard, coupling constants (*J* in Hz) in parentheses.

Table 2. NMR[z] spectral data of acylated flavonols isolated from *Aubrieta* × *cultorum*.

	4			5		
	^{1}H		^{13}C	^{1}H		^{13}C
kaempferol						
2			155.8			155.4
3			133.0			131.0
4			177.6			177.5
5			160.9			161.1
6	6.22	d (1.9)	98.8	6.00	d (2.0)	98.6
7			162.2			164.0
8	6.71	d (1.9)	94.6	6.37	d (2.0)	93.5
9			155.6			156.2
10			105.4			103.8
1'			120.7			120.9
2', 6'	8.09	d (8.9)	131.0	8.07	d (8.9)	131.0
3', 5'	6.88	d (8.9)	115.3	6.90	d (8.9)	115.3
4'			160.1			160.0
Glucose C						
1	5.72	d (7.1)	98.0	5.67	d (7.3)	98.2
2	3.76	m	81.0	3.77	t (8.5)	80.7
3	3.72	m	73.0	3.71	m	73.0
4	3.45	m	72.0	3.49	m	69.8
5	3.72	m	73.2	3.71	m	73.1
6a	4.00	dd (4.0, 11.3)	62.9	4.02	dd (4.3, 11.3)	63.0
6b	4.10	dd (7.3, 11.0)		4.07	brd (11.3)	
Glucose D						
1	4.57	d (7.6)	104.6	4.57	d (7.6)	104.4
2	3.09	t (9.6)	74.4	3.09	t (9.6)	74.5
3	3.17	t (8.9)	70.1	3.18	t (8.3)	69.6
4	3.20	t (9.8)	69.5	3.23	m	69.5
5	3.45	m	76.5	3.21	m	77.1
6a	3.70	m	61.1	3.47	m	60.7
6b	3.78	m		3.60	m	
Glucose E						
1	4.94	d (7.7)	99.2			
2	3.35	m	76.9			
3	3.35	m	76.9			
4	3.52	t (7.1)	79.8			
5	3.92	m	72.7			
6a	4.34	dd (5.9, 11.9)	62.8			
6b	4.57	brd (12.2)				
Glucose F						
1	4.30	d (7.6)	103.3			
2	3.06	t (8.7)	73.3			
3	3.17	t (8.9)	70.1			
4	3.25	t (8.1)	77.1			
5	3.35	m	76.9			
6a	3.53	m	60.6			
6b	3.60	brd (10.7)				
Sinapic acid (II)						
1			124.3			124.3
2, 6	6.98	s	106.2	6.91	s	106.1
3, 5			148.0			147.9
4			138.4			138.3
7(α)	6.51	d (15.9)	114.6	6.26	d (15.9)	114.1
8(β)	7.45	d (15.9)	145.4	7.37	d (15.9)	145.4
-CH_3 × 2	3.97	s	56.1	3.81	s	56.1
CO			166.0			166.2
Sinapic acd (III)						
1			124.3			
2, 6	6.91	s	106.2			
3, 5			147.9			
4			138.3			
7(α)	6.24	d (15.9)	114.2			
8(β)	7.34	d (15.9)	145.2			
-CH_3 × 2	3.81	s	56.1			
CO			166.4			

[z] ^{1}H NMR 500 MHz and ^{13}C NMR 125.78 MHz in DMSO-d_6 TMS as an internal standard, coupling constants (J in Hz) in parentheses.

λmax 527, 328, 294, 280 nm, E_{acyl}/E_{max} (%) = 47, E_{440}/E_{max} (%) = 13, $AlCl_3$ shift + (λvis·max 559 nm); TLC: Rf (× 100) BAW 33, BuHCl 7, 1%HCl 29, AHW 56; HPLC: *R*t (min) 32.8. HR FABMS: calc. $C_{46}H_{51}O_{27}$ 1035.2618. Found 1035.2616; for ^1H and ^{13}C NMR spectroscopic assignments, see Table 1.

*2) Pigment **2**: cyanidin 3-O-[2-O-(β-xylopyranosyl)-6-O-(trans-sinapoyl)-β-glucopyranoside]-5-O-β-glucopyranoside (= demalonyl **1**)*

Dark red powder: UV-Vis (in 0.1% HCl-MeOH): λmax 528, 330, 298, 281 nm, E_{acyl}/E_{max} (%) = 61, E_{440}/E_{max} (%) = 11, $AlCl_3$ shift + (λvis·max 567 nm); TLC: Rf (× 100) BAW 32, BuHCl 6, 1%HCl 20, AHW 52; HPLC: *R*t (min) 30.9. HR FABMS: calc. $C_{43}H_{49}O_{24}$ 949.2614. Found 949.2602; for ^1H NMR spectroscopic assignments, see Table 1.

*3) Pigment **3**: cyanidin 3-O-[2-O-(β-xylopyranosyl)-6-O-(trans-p-coumaroyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside]*

Dark red powder: UV-Vis (in 0.1% HCl-MeOH): λmax 529, 312, 296, 281 nm, E_{acyl}/E_{max} (%) = 67, E_{440}/E_{max} (%) = 12, $AlCl_3$ shift + (λvis·max 566 nm); TLC: Rf (× 100) BAW 35, BuHCl 9, 1%HCl 31, AHW 58; HPLC: *R*t (min) 31.8. FABMS: $[M]^+$ 975 *m/z*; HR FABMS: calc. $C_{44}H_{47}O_{25}$ 975.2406. Found 975.2407; for ^1H NMR spectroscopic assignments, see Table 1.

*4) Pigment **4**: kaempferol 3-O-[2-O-(β-glucopyranosyl)-6-O-(trans-sinapoyl)-β-glucopyranoside]-7-O-[4-O-(β-glucopyranosyl)-6-O-(trans-sinapoyl)-β-glucopyranoside]*

Pale yellow powder: TLC: Rf (× 100), BAW 32, BuHCl 12, 1% HCl 4, AHW 32, UV: λmax (nm) MeOH 325, 266; +NaOMe 385 (inc.), 329, 266sh; +$AlCl_3$ 350sh, 324, 305sh, 266sh; +$AlCl_3$/HCl 350sh, 324, 305sh, 267sh; +NaOAc 390sh, 326, 266; +NaOAc/H_3BO_3 325, 266. HPLC: *R*t (min) 35.3; HR FABMS: $[M+H]^+$ calc. $C_{61}H_{71}O_{34}$ 1347.3827. Found 1347.3810; for ^1H and ^{13}C NMR spectroscopic assignments, see Table 2.

*5) Pigment **5**: kaempferol 3-O-[2-O-(β-glucopyranosyl)-6-O-(trans-sinapoyl)-β-glucopyranoside]*

Pale yellow powder: TLC: Rf (× 100), BAW 53, BuHCl 18, 1% HCl 12, AHW 56, UV: λmax (nm) MeOH 328, 266; +NaOMe 380 (inc.), 327, 273; +$AlCl_3$ 380sh, 333, 306, 272; +$AlCl_3$/HCl 380sh, 330, 305, 271; +NaOAc 324, 270; +NaOAc/H_3BO_3 328, 266. HPLC: *R*t (min) 34.9; HR FABMS: $[M+H]^+$ calc. $C_{38}H_{41}O_{20}$ 817.2191. Found 817.2193; for ^1H and ^{13}C NMR spectroscopic assignments, see Table 2.

*6) Deacyl pigments **1–3** (= cyanidin 3-sambubioside-5-glucoside)*

Dark red powder: UV-Vis (in 0.1% HCl-MeOH): λmax 527, 278 nm, E_{440}/E_{max} (%) = 13, $AlCl_3$ shift + (λvis·max 565 nm); TLC: Rf (× 100) BAW 21, BuHCl 3, 1%HCl 30, AHW 62; HPLC: *R*t (min) 13.1. FABMS: $[M]^+$ 743 *m/z*, HR FABMS $[M]^+$ calc. $C_{32}H_{39}O_{20}$ 734.2035. found 743.2045.

Results and Discussion

Three anthocyanin peaks (**1–3**) were observed in both 5% HOAc extracts of violet flowers of *A.* × *cultorum* 'Royal Violet' and purple flowers of 'Royal Red' as main anthocyanin peaks by HPLC analysis with monitoring at 530 nm (Fig. 1). One flavonol (**4**) was observed in the same extract from the flowers of 'Royal Violet' as a main pigment, and another flavonol (**5**) was observed in the same extract from the flowers of 'Royal Red' by HPLC analysis with monitoring at 350 nm (Fig. 1). The proportions of anthocyanin peaks were 70.3% (violet cultivar: 'Royal Violet') and 73.7% (purple cultivar: 'Royal Red') (**1**), 5.3% and 1.7% (**2**), and 17.8% and 15.7% (**3**), based on the percentage of total absorbance at 530 nm of anthocyanin peaks of each cultivar. Moreover, the proportions of other flavonoid peaks were 53.5% (violet cultivar: 'Royal Violet') (**4**) and 48.2% (purple cultivar: 'Royal Red') (**5**), based on the percentage of total absorbance at 350 nm peaks. From the extract with 5% HOAc, these pigments were isolated and purified using the process described in previous reports (Saito et al., 2011b; Tatsuzawa et al., 2007). The chromatographic and spectroscopic properties of these acylated anthocyanins and flavonol glycosides are summarized in Section 4 of Materials and Methods.

Acid hydrolysis of three anthocyanins (**1–3**) resulted in cyanidin, glucose, xylose, and hydroxycinnamic acids (sinapic acid for **1** and **2** and *p*-coumaric acid for **3**). In addition, malonic acid was detected in the hydrolysates of **1** and **3**. Acid hydrolysis of flavonols (**4** and **5**) resulted in kaempferol, glucose, and sinapic acid. Alkaline hydrolysis of **4** resulted in deacyl kaempferol glycoside and sinapic acid, and similar treatment of **5** resulted in another deacyl kaempferol glycoside and sinapic acid.

Alkaline hydrolysis of **1–3** resulted in the same deacylated cyanidin glycoside and hydroxycinnamic acids. The deacylated cyanidin glycoside was identified to be cyanidin 3-sambubioside-5-glucoside by analysis using HPLC, TLC, UV-Vis, and HR FABMS data in comparison with the authentic anthocyanin specimen obtained from *Lunaria annua* (Tatsuzawa et al., 2006).

Also, the structure of **3** was easily identified to be cyanidin 3-*O*-[2-*O*-(xylosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] by analyses using chromatographic and spectroscopic methods in comparison with authentic pigments isolated from *Lunaria annua* (Tatsuzawa et al., 2006).

The structure elucidations of new anthocyanins (**1** and **2**) and new flavonols (**4** and **5**) were performed by analyses of their ^1H NMR (500 MHz) and ^{13}C NMR (125.78 MHz) spectra in DMSO-d_6-CF_3COOD (9 : 1) for anthocyanins and DMSO-d_6 for flavonol, including 2D COSY, NOESY, ^1H-^{13}C HMQC, and ^1H-^{13}C HMBC spectra, as described hereinafter.

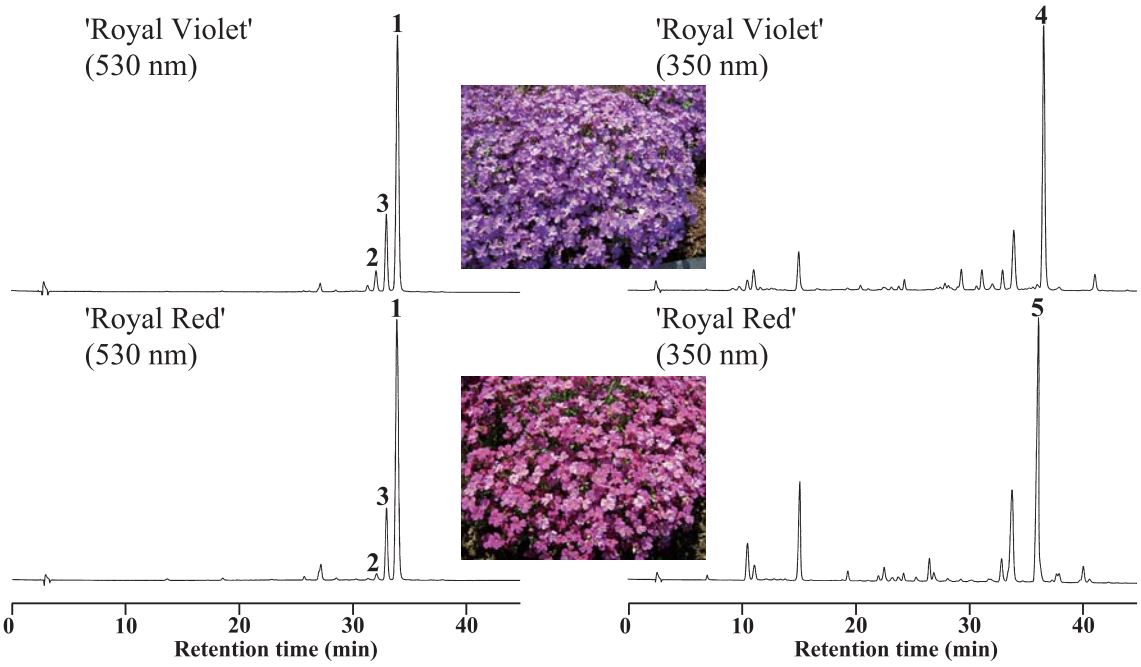

Fig. 1. HPLC profile of acylated flavonoids isolated from the violet and purple flowers of *Aubrieta* × *clutorum*. 1: cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], 2: cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-5-*O*-(β-glucopyranoside) = demalonyl **1,** 3: cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], 4: kaempferol 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-7-*O*-[4-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside], 5: kaempferol 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]

1. Structural determination of new flavonoid pigments

*1) Pigment **1***

The molecular ion $[M]^+$ of **1** as a major pigment in these cultivars was observed at 1035 *m/z* ($C_{46}H_{51}O_{27}$ calc. 1035.262), indicating that the pigment is composed of cyanidin with two molecules of glucose and one molecule each of xylose, sinapic acid, and malonic acid. The elemental components of this pigment were confirmed by measuring the HR FABMS (see Section 4-1 of Materials and Methods).

Its structure was further elucidated by analysis of its NMR spectra. The chemical shifts of 8 aromatic protons of cyanidin and sinapic acid moieties with their coupling constants were assigned as shown in Table 1. Six protons at δ3.75 were assigned to two methoxyl groups of sinapic acid. Two olefinic proton signals of sinapic acid with their large coupling constants (J = 15.9 and 15.9 Hz) showed *trans* configuration (Table 1). The chemical shifts of the sugar moieties were observed in the region of δ5.68–2.97, where the three anomeric protons were exhibited at δ5.68 (*d*, J = 7.3 Hz, Glc A), δ5.18 (*d*, J = 7.7 Hz, Glc B), and δ4.73 (*d*, J = 7.6 Hz, Xylose). Based on the observed coupling constants (Table 1), these three sugars were assumed to be the β-pyranose forms. The linkages and/or positions of the attachments of the sugar and acyl groups were determined by 2D COSY, NOESY, and HMBC experiments.

Four characteristic proton signals shifted to a lower magnetic field were assigned to the methylene protons of Glc A (δ4.26 and 4.47, H-6a and b) and Glc B (δ3.87 and 4.40, H-6a and b). Thus, two hydroxyl groups of the sugar moieties, OH-6s of Glc A and B, were assumed to be acylated with two molecules of acids.

NOESY and HMBC spectra were used to determine the sites of attachment of the acid, sugar and cyanidin moieties (Fig. 2). The signal of H-1 of Glc A (δ5.68) was correlated to the signal of C-3 (δ144.6) carbon in its HMBC spectrum, and to the H-4 (δ8.79) proton signal of cyanidin moiety in the NOESY spectrum. The signal of H-1 of Glc B correlated to the signal of the C-5 (δ154.9) carbon in the HMBC spectra, and to the signal of the H-6 (δ6.96) proton in the NOESY spectrum of cyanidin. These characteristic features revealed that the OH-3 and OH-5 positions are both bound to glucose molecules. The signal of H-1 (δ4.73) of xylose was correlated to the signal of the C-2 (δ80.4) carbon of Glc A in the HMBC spectrum, and to the signal of proton H-2 (δ4.02) in the NOESY spectrum of Glc A. These results support that OH-3 and OH-5 of cyanidin are glycosylated with Glc A and Glc B, respectively, and also that OH-2 of Glc A is bonded with xylose, forming sambubiose (Fig. 2). The signals of H-6 of Glc B were correlated to the signal of the CO (δ166.9) carbon of malonic acid in the HMBC spectrum, and to the signal of methylene proton -CH_2- (δ3.41) of malonic acid in the NOESY spectrum, therefore, both OH-6 groups of Glc A and Glc B were acylated with sinapic acid and malonic acid, respectively.

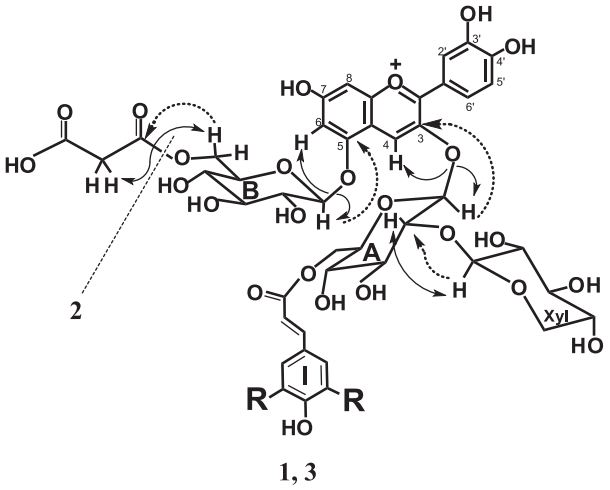

Fig. 2. Acylated anthocyanins isolated from the violet and purple flowers of *Aubrieta* × *clutorum*. Observed NOEs are indicated by arrows. Observed HMBC correlations are indicated by dotted arrows. 1: R = OCH_3, 2: R = OCH_3, 3: R = H

Consequently, the structure of **1** was determined to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside], which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002; Veitch and Grayer, 2008, 2011).

*2) Pigment **2***

The molecular ion $[M]^+$ of **2** as a minor pigment in these cultivars was observed at 949 *m/z* ($C_{43}H_{49}O_{24}$ calc. 949.261), indicating that the pigment is composed of cyanidin with two molecules of glucose and one molecule each of xylose and sinapic acid. The elemental components of this pigment were confirmed by measuring its HR FABMS (see Section 4-2 of Materials and Methods).

By analysis of its ^{1}H NMR spectra, it was revealed that proton chemical shifts of **2** were almost in agreement with those of **1** except for the proton signals of Glc B and malonic acid moieties, as shown in Table 1. In particular, upfield shifts of methylene protons (H-6a and b, δ3.40–3.80) of Glc B in **2** were observed in comparison to those (δ3.87 and δ4.40) of **1**, therefore, OH-6 of Glc B in **2** was free from malonic acid. Other proton signals of **2** were assigned by the same process as described for **1** (Table 1). Thus, **2** was determined to be cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside, which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002; Veitch and Grayer, 2008, 2011). In order to confirm the identity of both **2** and demalonyl **1**, demalonyl **1** was prepared from **1** by treatment with 1N HCl for 5 days. Direct comparison of demalonyl **1** with **2** by TLC and HPLC demonstrated that both pigments were exactly the same (Tatsuzawa et al., 2008a).

*3) Pigment **4***

The FAB mass spectrum of **4** gave a molecular ion $[M + H]^+$ at 1347 *m/z* ($C_{61}H_{71}O_{34}$, calc. 1347.383) indicating that **4** was composed of kaempferol with four molecules of glucose and two molecules of sinapic acid. The chromatographic and spectroscopic properties of **4** are summarized in Section 4-4 of Materials and Methods. The structure of **4** was presumed to be kaempferol 3,7-tetraglucoside by analytical results of its chromatographic and spectroscopic properties. From analysis of the ^{1}H NMR spectrum of **4**, ten aromatic protons of kaempferol and sinapic acids, and four anomeric protons of glucose were assigned (Table 2). The four glucose moieties were found to be the β-pyranose form from their coupling constants. Moreover, four olefinic protons of sinapic acids in **4** showed large coupling constants (J = 15.9, 15.9, 15.9 and 15.9 Hz) suggesting *trans*-configuration.

By analysis of its NOESY spectrum, three strong long-range NOEs were observed between H-8 and H-6 of kaempferol and H-1 of Glc E, H-4 of Glc E and H-1 of Glc F, and H-2 of Glc C and H-1 of Glc D, suggesting that kaempferol was glycosylated with Glc E at 7-OH, Glc F was bonded with Glc E at OH-4, and Glc C was bonded with Glc D at OH-2. The glycosylation at OH-3 of kaempferol with Glc C was concluded by the results of spectroscopic properties.

Moreover, by analysis of its COSY spectrum, four characteristic proton signals shifted to a lower magnetic field were also assigned to the methylene protons of Glc C (δ4.00 and 4.10, H-6a and b) and Glc E (δ4.34 and 4.57, H-6a and b). Thus, two hydroxyl groups of the sugar moieties, OH-6s of Glc C and E were assumed to be acylated with two molecules of sinapic acids (II and III). The signals of H-6a and b (δ4.00 and 4.10) of Glc C were correlated to the signal of the CO (δ166.0) carbon of sinapic acid (II) by analysis of its HMBC spectrum (Table 2) (Fig. 3). Moreover, the signal of the H-1 of Glc D (δ4.57) was correlated to the signal of the C-2 of Glc C (δ81.0), and also the signal of Glc F (δ4.30) was correlated to the C-4 of Glc E (δ79.8), respectively, in the HMBC spectrum, suggesting the presence of bonds between OH-2 of Glc C and OH-1 of Glc D, and OH-4 of Glc E and OH-1 of Glc F, respectively.

Therefore, the structure of **4** was determined to be kaempferol 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-7-*O*-[4-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside] (Fig. 3), which is a new kaempferol glycoside in plants (Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011; Williams, 2006). Regarding the glycosylation patterns of flavonol glycosides in the Brassicaceae, kaempferol 3-sophoroside-7-cellobioside have been found in plants of *Brassica oleracea* (Nielsen et al., 1998).

*4) Pigment **5***

The FAB mass spectrum of **5** gave a molecular ion $[M + H]^+$ at 817 *m/z* ($C_{38}H_{41}O_{20}$, calc. 817.291)

indicating that **5** was composed of kaempferol with two molecules of glucose and one molecule of sinapic acid. The chromatographic and spectroscopic properties of **5** are summarized in section 4-5 of Materials and Methods. The structure of **5** was presumed to be a kaempferol 3-diglucoside by analysis of its chromatographic and spectroscopic properties.

The proton chemical shifts of **5** in its ^{1}H NMR spectrum were identical to those of **4**, except for the signals of sinapic acid (III), Glc E, and Glc F moieties (Table 2). In detail, six proton signals of sinapic acid were assigned as shown in Table 2. Two olefinic protons of this acid with large coupling constants (J = 15.9 and 15.9 Hz) indicated that the geometry of the olefin was of *trans*-configuration. By analysis of its COSY spectrum, downfield shifts of methylene protons (δ4.02, H-6a and δ4.07, H-6b) of Glc C were observed, supporting that Glc C was acylated with sinapic acid at OH-6. By analysis of its NOESY spectrum, three strong long-range NOEs were observed between H-2 of Glc C and H-1 of Glc D, suggesting that Glc C was bonded with Glc D at OH-2. Consequently, the structure of **5** was determined to be kaempferol 3-*O*-[2-*O*-(6-*O*-(*trans*-sinapoyl)-β-glucopyranosyl)-β-glucopyranoside] (Fig. 3), which is a new kaempferol glycoside in plants (Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011; Williams, 2006). This structure was further confirmed by measuring its ^{13}C NMR spectrum (Table 2).

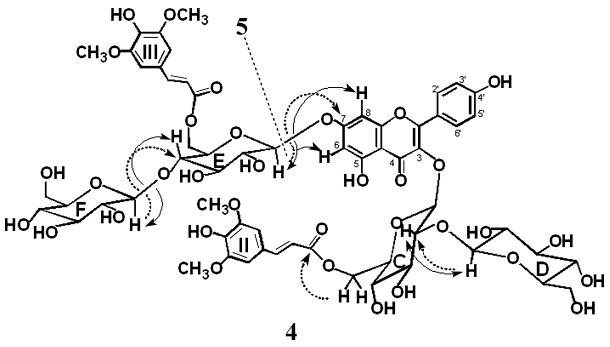

Fig. 3. Acylated flavonols isolated from the violet and purple flowers of *Aubrieta*×*clutorum*. Observed NOEs are indicated by arrows. Observed HMBC correlations are indicated by dotted arrows. 4: from 'Royal Violet', 5: from 'Royal Red'

2. Flower colors of both A. × *cultorum cultivars and copigmentation of* ***1*** *with acylated kaempferol glucosides*

Flower colors of the *A.* × *cultorum* 'Royal Violet' and 'Royal Red' were distinguishable, namely violet for 'Royal Violet' [Violet 87A according to the R.H.S. Colour Chart, with a chromaticity value of −0.83 = −49.4(b*)/59.8(a*) and 30.9(L*)] and purple for 'Royal Red' [Purple 78A according to the R.H.S. Colour Chart, with a chromaticity value of −0.41 = −22.5(b*)/55.4(a*) and 27.9(L*)] (Fig. 4a). The visible absorption spectral curve of intact petals of 'Royal Violet' showed λmax at 530sh, 560, and 604 nm and that of intact petals of 'Royal Red' showed λmax at 546 nm, however, flowers of both cultivars contained very similar anthocyanin components, such as cyanidin 3-*O*-[2-*O*-(β-xylopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (**1**) and its demalonyl derivative (**2**) as their main anthocyanins (Fig. 2). In contrast, flavonol components in flowers of both cultivars were clearly distinguishable from each other (Fig. 1). Flowers of 'Royal Violet' contained kaempferol 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside]-7-*O*-[4-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside] (**4**) as the dominant flavonol, whereas those of 'Royal Red' contained kaempferol 3-*O*-[2-*O*-(β-glucopyranosyl)-6-*O*-(*trans*-sinapoyl)-β-glucopyranoside] (**5**) as the dominant flavonol (Fig. 1).

These results suggested that the divergence in flower colors of these cultivars was responsible for the difference in flavonol components of flowers (Fig. 1). A copigmentation study between **1** and flavonol was performed in pH 5.0 buffer solution of the same pH as

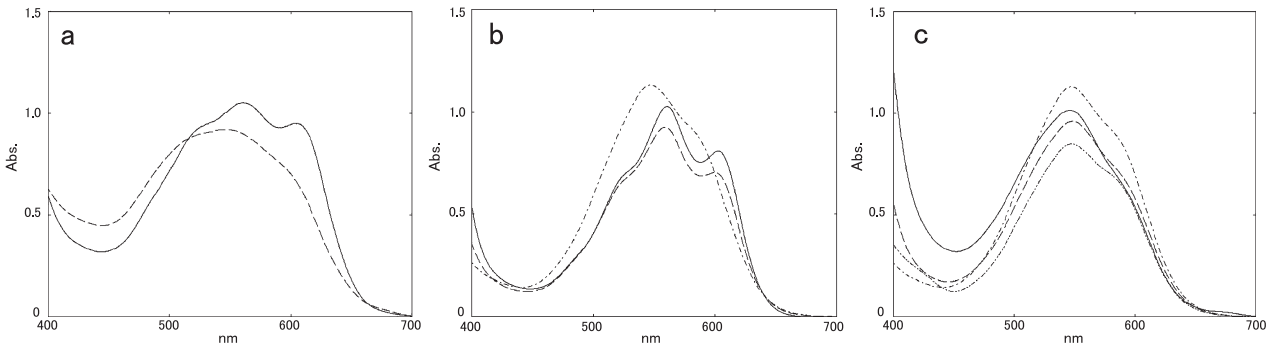

Fig. 4. Visible spectra of *Aubrieta*×*clutorum*. a —— Fresh flower tissue of 'Royal Violet' [604, 560, 530sh nm], – – – Fresh flower tissue of 'Royal Red' [590sh, 546, 520sh nm], b —— Flower pressed juice of 'Royal Violet' in pH 5.0 buffer solution [604, 560, 530sh nm], – – – Pigment **1** (0.25 mg/ml) + pigment **4** (0.5 mg/ml) in pH 5.0 buffer solution [604, 560, 530sh nm], –·–·– Pigment **1** (0.25 mg/ml) in pH 5.0 buffer solution [585sh, 547, 520sh nm], c —— Flower pressed juice of 'Royal Red' in pH 5.0 buffer solution [585sh, 546, 520sh nm], – – – Pigment **1** (0.25 mg/ml) + pigment **5** (0.5 mg/ml) in pH 5.0 buffer solution [585sh, 547, 520sh nm], –·–·– Pigment **1** (0.25 mg/ml) in pH 5.0 buffer solution [585sh, 547, 520sh nm], –··–·· Pigment **1** (0.25mg/ml) + rutin (0.5 mg/ml) in pH 5.0 buffer solution [585, 547, 520sh nm]

pressed juice as follows.

The solution of **1** in pH 5.0 buffer solution was purple with a broad λmax at 547 nm (Fig. 4c), however, the color was unstable and decayed rapidly. By the addition of **5** (anthocyanin : flavonol = 1 : 2; this ratio was given by quantitative analysis of pigment in crude extract using purified pigments of 'Royal Violet' used as a control), the purple color of the solution of **1** became stable, similar to that of the pressed flower juice of 'Royal Red', but did not change to violet, similar to that of the pressed flower juice of 'Royal Violet' (Fig. 4b). A similar reaction was observed in the purple solution of **1** by the addition of rutin and kaempferol 3-glucoside as common copigments (Asen et al., 1972; Chen and Hrazdina, 1981) (Fig. 4c). In contrast, the purple color of the solution of **1** changed to violet by the addition of **4** and became very stable, similar to that of the pressed flower juice of 'Royal Violet' in pH 5.0 buffer solution. The violet solution of the mixture of **1** and **4** exhibited two λmax at 604, 560, and 530sh nm, which were identical to those of the pressed flower juice in pH 5.0 buffer solution (Fig. 4b). These results suggested that both kaempferol glycosides (**4** and **5**) function as copigments in the flowers of both cultivars, and that **4** (dihydroxycinnamoyl kaempferol glycoside) forms a strong and stable association with **1**, showing λmax at 604 and 560 nm as observed for *Heliophila coronopifolia* (Saito et al., 2011b).

3. Concluding remarks

In this study, anthocyanins of the *A.* × *cultorum* 'Royal Violet' and 'Royal Red' were shown to comprise acylated cyanidin 3-sambubioside-5-glucosides as the main anthocyanins, and these anthocyanins (**1–3**) were classified into the group of 3-sambubioside-5-glucoside pattern as the glycosylation of anthocyanidins. To the best of our knowledge, plants containing this group of anthocyanins are *Arabidopsis* (Bloor and Abrahams, 2002; Nakabayashi et al., 2009), *Cheiranthus* (Tatsuzawa et al., 2006), *Heliophila* (Saito et al., 2011b), *Lobularia* (Tatsuzawa et al., 2006, 2007, 2010c), *Lunaria* (Tatsuzawa et al., 2006), *Matthiola* (Saito et al., 1995, 1996; Tatsuzawa et al., 2012), *Orychophragmus* (Honda et al., 2005), and *Sinapis* (Takeda et al., 1988) in the family Brassicaceae. The acylation pattern of the three anthocyanins (**1–3**) was rather simple, with **1** and **3** being acylated with one molecule each of hydroxycinnamic acid and malonic acid, and **2** being acylated with one molecule of hydroxycinnamic acid. Acylated kaempferol glycosides (**4** and **5**) were isolated as the dominant flavonol from 'Royal Violet' and 'Royal Red', respectively. The structures of **4** and **5** were determined to be disinapoyl kaempferol 3-sophoroside-7-cellobioside and monosinapoyl kaempferol 3-sophoroside, respectively. The structural difference in flavonol glycosides may be responsible for the divergence in flower colors of *A.* × *cultorum* cultivars, such as the violet flowers of 'Royal Violet' and purple flowers of 'Royal Red'.

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