

Quantitative Evaluation of the Contribution of Four Major Anthocyanins to Black Flower Coloring of Dahlia Petals

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The black flower color of dahlias (*Dahlia variabilis*) has been suggested to be attributed to a high accumulation of cyanidin (Cy)-based anthocyanins. A possible explanation for this effect is that Cy-based anthocyanins in dahlias contribute more to the black flower color than pelargonidin (Pg)-based anthocyanins by lowering petal lightness (L^*) and chroma (C^*), but no obvious evidence has been reported. In this study, four major anthocyanins accumulated in dahlia petals, 3,5-diglucoside (3,5diG) and 3-(6''-malonylglucoside)-5-glucoside (3MG5G) of Pg and Cy, were purified and their colors were evaluated *in vitro* at various pHs (3.0, 4.0, 4.5, 5.0, 5.5, 6.0, or 7.0) and various concentrations (0.25, 0.5, 1.0, 2.0, or 3.0 mg·mL⁻¹ at pH 5.0 or pH 3.0). The color of solution of purified anthocyanins varied depending on pH. At pH 5.0, which is approximately the same as pH of dahlia petals, and at pH 3.0, at which anthocyanins are relatively stable, the L^* and C^* of Cy 3,5diG were similar to or higher than those of Pg 3,5diG, suggesting that Cy 3,5diG did not contribute more to the black flower coloring than Pg 3,5diG. On the other hand, the L^* and C^* of Cy 3MG5G were significantly lower than those of Pg 3MG5G, particularly above 2.0 mg·mL⁻¹, suggesting that Cy 3MG5G contributed more than Pg 3MG5G. A similar tendency was observed in the color measurement of mixed anthocyanins in various proportion of Pg and Cy. The L^* and C^* of Pg 3MG5G were much higher than those of the other three anthocyanins; therefore, its color was considered to be the farthest from black among the four anthocyanins. The accumulated amount of 3MG5G-type anthocyanins was much higher than that of 3,5diG-type anthocyanins in all nine cultivars, although the proportion of Pg- and Cy-based anthocyanins varied among the cultivars. Considering these results, it was suggested that because 3MG5G-type anthocyanins predominantly accumulate in petals, and Cy 3MG5G has a significantly higher contribution to lowering L^* and C^* than Pg 3MG5G, the high accumulation of Cy-based anthocyanins is critical for the black flower coloring of dahlias. The contribution of each anthocyanin is considered to depend on the structure; therefore, identifying the anthocyanin with the highest contribution to lowering L^* and C^* may enable the production of black flowers in various species through the high accumulation of the anthocyanin in petals.

Key Words: CIE $L^*a^*b^*C^*$, cyanidin, pelargonidin.

Introduction

Black flower color is a rare and attractive trait, but the underlying mechanism of its appearance is unclear. Black is the deepest (darkest) flower color that shows the lowest lightness (L^*) and lower chroma (C^*) among

cyanic colors; therefore, it has been considered to be attributed to an extremely high amount of anthocyanins. Black cultivars of dahlias (*Dahlia variabilis*) have a relatively high amount of anthocyanins; however, the black flower coloring of dahlias cannot be explained only by the high accumulation of total anthocyanins [cyanidin (Cy) and pelargonidin (Pg)-based] (Deguchi et al., 2013), although the flower color intensity of other cyanic (pink, purple, orange, and red) cultivars is explained to some extent by the amount of total anthocyanins (Ohno et al., 2013). In our previous study, three notable characteristics were observed in black dahlia cultivars: comparatively high accumulation of total anthocyanins, high accumulation (proportion) of Cy-based

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anthocyanins, and low accumulation of flavones, which are nearly colorless pigments and synthesized from the same substrate as anthocyanins (Deguchi et al., 2013). Therefore, it was suggested that high accumulation of Cy-based anthocyanins as well as total anthocyanins was important for the black flower coloring of dahlias. High accumulation of total and Cy-based anthocyanins in most black cultivars was induced by post-transcriptional gene silencing (PTGS) of *flavone synthase II* (*DvFNS*) (Deguchi et al., 2013, 2015). Abolishment of substrate competition between anthocyanin biosynthesis and flavone biosynthesis is probably related to the increase in anthocyanin accumulation.

When black dahlia cultivars were infected with tobacco streak virus (strain dahlia: TSV_{dahlia}), their flower color changed from black to purple except for ‘Fidalgo Blacky’ (Deguchi et al., 2015). This was because of the change of pigment accumulation in petals, increase of flavones and reduction of anthocyanins, particularly Cy-based anthocyanins, resulted from the suppression of *DvFNS* PTGS by TSV_{dahlia}. Only ‘Fidalgo Blacky’ retained black color (lower L^* and C^*) despite the change in pigment accumulation and consequential slight bluing probably due to co-pigmentation between anthocyanins and flavones. When black cultivars were infected with TSV_{dahlia}, the amount of total anthocyanins was nearly the same among the black cultivars. However, the proportion of Cy varied among the cultivars, wherein ‘Fidalgo Blacky’ had the highest proportion. This characteristic suggested that Cy-based anthocyanins contribute more to black flower coloring [described as the difference of “darkness unit” in Deguchi et al. (2013)]; petal L^* and C^* are lowered more by Cy-based anthocyanins than Pg-based anthocyanins in dahlias.

Many studies have reported relationships between flower color (L^* and C^*) and the proportion of each anthocyanin in various plant species (Sakata et al., 1995; Torskangerpoll et al., 2005; Uddin et al., 2004). In these reports, the structure and amount of anthocyanins varied depending on the cultivar, indicating that it was difficult to interpret the contribution of Cy and Pg to lowering L^* and C^* from these reports. The color of anthocyanins can be evaluated and compared *in vitro* (Cabrita et al., 2000; Giusti et al., 1999; Stintzing et al., 2002; Torskangerpoll and Andersen, 2005). Some of these previous evaluations have suggested that anthocyanins with different modifications showed different colors even when they were based on the same anthocyanidin. In dahlias, it was expected that some Cy-based anthocyanins would show lower L^* and C^* , thereby contributing more to the black flower coloring than Pg-based anthocyanins. In the present study, to determine the contributions of anthocyanins in dahlia petals to the black flower coloring, four major anthocyanins were purified from a black dahlia cultivar ‘Kokucho’, and the colors (CIE $L^*a^*b^*C^*$) of their an-

thocyanin solutions were evaluated *in vitro*. We also assessed the reason why high accumulation of Cy-based anthocyanins is important for the black flower coloring of dahlias.

Materials and Methods

Plant materials

Four black cultivars (‘Black Cat’, ‘Fidalgo Blacky’, ‘Kokucho’, and ‘Ms. Noir’), four purple cultivars (‘Atom’, ‘Cupid’, ‘Evelyn Rumbold’, and ‘Yukino’), and one pink cultivar (‘Jyunn-ai’) were grown in the experimental field at Kyoto University (Kyoto, Japan). All cultivars were purchased from Akita International Dahlia Park (Akita, Japan). The fully expanded petals were collected for high-performance liquid chromatography (HPLC) analysis. The petals of ‘Kokucho’ were freeze-dried, and used for the purification of anthocyanins.

HPLC analysis

Detection of anthocyanins in dahlia petals was performed using HPLC. Fresh petals (100 mg) of each cultivar were homogenized using a mortar and pestle under liquid nitrogen. After the addition of 1 mL of extraction solution (5% v/v acetic acid), the mixture was stored overnight at 4°C in the dark. The mixture was then centrifuged at 4°C at $20600 \times g$ for 15 min, and the supernatant was collected and diluted 10 times using the same solvent. A 20- μ L aliquot of the solution was injected into the HPLC system. The analysis was performed according to Deguchi et al. (2015) using a Hitachi HPLC system with a C18 column (4.6×250 mm, Nihon Waters K. K., Tokyo, Japan). The detection wavelength was 520 nm. The assay was performed using petals from three different inflorescences. For the standard curves of each anthocyanin, 1 mg of purified anthocyanin was dissolved in 1 mL of extraction solution and a one-half dilution series was made. A 20- μ L aliquot of each dilution was injected, and the relationship between peak areas and pigment concentrations was calculated.

Extractive purification of four anthocyanins

Twenty grams of freeze-dried petals of ‘Kokucho’ were immersed in 4 L of 5% acetic acid for 24 h at room temperature, and flavonoid pigments were extracted. The extract was transferred to a Diaion HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan) packed column, and washed using 5% acetic acid. The pigments were eluted from the column using 5% v/v acetic acid in methanol. After concentration, the eluates were fractionated by paper chromatography using BAW (*n*-butanol:acetic acid:water = 4:1:2, v/v/v). The crude fractionated pigments were further purified by preparative HPLC, which was performed on an LC 10A system (Shimadzu Corporation, Kyoto, Japan) with a Waters C18 prep column (19×150 mm) at 40°C with a flow

rate of 4 mL·min⁻¹ and monitoring at 530 nm according to a study by Tatsuzawa et al. (2012). Each fraction was transferred to a Diaion HP-20 column again, and each anthocyanin was eluted from the column using 5% acetic acid in methanol, concentrated, and dried. More than 80 mg of purified powder of each anthocyanin was obtained, and used for identification and color evaluation.

Identification of each anthocyanin

Two of the four purified anthocyanins were identified as Pg 3,5-diglucoside (Pg 3,5diG) and Cy 3,5-diglucoside (Cy 3,5diG) by HPLC, by comparing with identified anthocyanins purified from petals of rose

(*Rosa hybrida* L.) (Willstätter and Mallison, 1915) and cornflower (*Centaurea cyanus* L.) (Saito et al., 1964). The other two anthocyanins were identified as Pg 3-(6"-malonylglucoside)-5-glucoside (Pg 3MG5G) and Cy 3-(6"-malonylglucoside)-5-glucoside (Cy 3MG5G) by high-resolution fast atom bombardment mass spectroscopy (HR-FABMS) and nuclear magnetic resonance (NMR). NMR spectra were recorded on JNM AL-400 (JEOL Ltd., Tokyo, Japan) at 400 MHz for ¹H spectra and 100 MHz for ¹³C spectra in CD₃OD-TFA (9:1). Chemical shifts are reported on the δ -scale from tetramethylsilane as the internal standard, and coupling constants (*J*) are in Hz. Pg 3MG5G HR-FABMS calc. for

Table 1. NMR spectroscopic data of pelargonidin 3-(6"-malonylglucoside)-5-glucoside and cyanidin 3-(6"-malonylglucoside)-5-glucoside in CD₃OD-TFA (9:1).

	Pelargonidin 3-(6"-malonylglucoside)-5-glucoside			Cyanidin 3-(6"-malonylglucoside)-5-glucoside		
	¹³ C (ppm) ^z	¹ H (ppm) ^z		¹³ C (ppm)	¹ H (ppm)	
<i>Anthocyanidin</i>						
2	165.4			164.7		
3	146.0			146.1		
4	135.9	9.00	s	134.8	8.92	s
5	156.9			156.8		
6	105.6	7.05	d(1.2)	105.5	7.02	d(2.0)
7	170.3			169.8		
8	97.6	7.12	d(1.2)	97.5	7.06	d(2.0)
9	157.5			157.2		
10	113.5			113.2		
1'	120.8			121.1		
2'	136.3	8.61	d(9.3)	118.6	8.02	d(2.4)
3'	118.2	7.08	d(9.3)	147.7		
4'	167.4			156.7		
5'	118.2	7.08	d(9.3)	117.7	7.02	d(8.8)
6'	136.3	8.61	d(9.3)	129.0	8.25	dd(2.4, 8.8)
<i>Glucose(3)</i>						
1	102.7	5.45	d(8.1)	102.4	5.49	d(7.8)
2	74.5	3.71	t(8.7)	74.8	3.75	t(8.4)
3	78.1	3.60	t(9.0)	78.0	3.62	t(8.2)
4	71.5	3.47	t(8.5)	71.3	3.47	t(9.4)
5	75.8	3.91	ddd(2.2, 6.7, 9.6)	75.7	3.93	ddd(2.2, 7.3, 9.6)
6a	65.6	4.37	dd(7.2, 11.8)	65.7	4.36	dd(7.2, 12.0)
6b		4.49	dd(2.0, 11.8)		4.48	dd(1.9, 12.0)
<i>Glucose(5)</i>						
1	102.9	5.22	d(7.8)	102.8	5.22	d(7.8)
2	74.8	3.73	t(8.5)	74.5	3.73	t(8.3)
3	78.0	3.62	m	78.2	3.60	t(8.9)
4	71.3	3.49	t(8.5)	71.6	3.49	t(9.3)
5	78.9	3.61	m	78.9	3.62	m
6a	62.6	3.78	dd(5.5, 11.8)	62.6	3.78	dd(6.0, 12.0)
6b		3.99	dd(2.2, 11.8)		3.99	dd(2.0, 12.0)
<i>Malonic acid</i>						
CH ₃	49.4	3.43	s	49.4	3.49	m
COOH	168.9			170.4		
COOH	170.0			168.9		

^z 400 MHz for ¹H and 100 MHz for ¹³C.

$C_{30}H_{33}O_{18}$: 681.1667. Found: 681.1674; Cy 3MG5G HR-FABMS calc. for $C_{30}H_{33}O_{19}$: 697.16149. Found: 697.1627. NMR data of Pg 3MG5G and Cy 3MG5G are shown in Table 1. All four purified anthocyanins were confirmed to be of high purity (more than 90%) by HPLC measured at 520 nm.

In vitro color evaluation

A 0.5-mg sample of each purified anthocyanin was dissolved in 1 mL of phosphate-citrate buffer solution (pH = 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, or 7.0), and the color (CIE $L^*a^*b^*$) of the solution was measured using a spectroscopic colorimeter CM-5 (Konica Minolta, Inc., Tokyo, Japan) immediately and 30 min after dissolution. Different amounts (0.25, 0.5, 1.0, 2.0, or 3.0 mg) of each anthocyanin were dissolved in 1 mL of phosphate-citrate buffer solution (pH = 3.0 or 5.0), and the color of the solution was measured immediately and 30 min after dissolution. The same modified anthocyanins (3,5diG- or 3MG5G-type) were mixed in various proportions (Pg: Cy = 5:0, 4:1, 3:2, 2:3, 1:4, or 0:5) based on mass to give a total of 1.0 mg. The mixed pigment was dissolved in 500 μ L of phosphate-citrate buffer solution (pH = 5.0), and the color of the solution was measured immediately and 30 min after dissolution. These measurements were performed three times. C^* was calculated as $(a^{*2} + b^{*2})^{1/2}$.

Results

Major anthocyanins in dahlia petals

Five to eight peaks were detected from the extract of dahlia petals using HPLC (520 nm). Although the number of peaks varied among the cultivars, there were only four major peaks in each of the chromatograms of the cultivars. The four anthocyanins indicated by these four major peaks were extracted and purified from 20 g of freeze dried petals of ‘Kokucho’, and identified as Pg 3,5diG, Cy 3,5diG, Pg 3MG5G, and Cy 3MG5G. The amount of each of the four anthocyanins in 100 mg of fresh petals of each cultivar was analyzed (Table 2). Black cultivars contained high amounts of total anthocyanins (2.3–3.4 mg in 100 mg of fresh petals); how-

ever, their amounts in ‘Black Cat’ were not higher than those in a purple cultivar, ‘Yukino’. The proportion of Cy-based anthocyanins varied among the cultivars; ‘Fidalgo Blacky’, ‘Atom’, and ‘Cupid’ had higher amounts of Cy-based anthocyanins, and the other cultivars had higher amounts of Pg-based anthocyanins. The proportion of Cy-based anthocyanins in ‘Black Cat’ was higher than that in ‘Yukino’. Regardless of flower color, all cultivars contained higher amounts of the 3MG5G-type anthocyanins than the 3,5diG-type anthocyanins of both Pg and Cy.

In vitro color evaluation of anthocyanin solutions

The colors of anthocyanin solutions at various pHs were measured by spectrophotometric colorimetry. All purified anthocyanins started rapid discoloring after they were dissolved in buffer solution from pH 4.0 to 6.0, and the discoloring was completed within 30 min after dissolution. Discoloring was not observed visually at pH 3.0, and occurred very slowly (over ~1 day) at pH 7.0. The 3MG5G-type anthocyanins (Pg 3MG5G and Cy 3MG5G) were very soluble at all prepared pHs, whereas the 3,5diG-type anthocyanins (Pg 3,5diG and Cy 3,5diG) were relatively less soluble, and part of dissolved anthocyanin showed gradual recrystallization. Therefore, to compare the contributions to black flower coloring, the solution color of each anthocyanin was measured immediately after dissolution for the not discolored solutions and 30 min after dissolution for the discolored solutions without recrystallization. Immediately after dissolution, the L^* of the Pg 3MG5G solution was significantly higher than that of the other three anthocyanins from pH 3.0 to 4.5 (Fig. 1A). The C^* of Pg 3MG5G was highest in all pH buffer solutions due to high absolute values of both a^* and b^* , and that of Cy 3MG5G tended to follow a similar pattern (Fig. 1B; Table 3). At 30 min after dissolution, the L^* of all four anthocyanins had increased from the values immediately after dissolution, and the L^* of Pg 3MG5G was slightly higher than that of the other anthocyanins (Fig. 1C). The C^* of Pg 3,5diG, Cy 3,5diG, and Cy 3MG5G had increased from the values immediately

Table 2. The content of anthocyanins in 100 mg fresh petals of dahlia cultivars (means $\pm$ SE of three replicates).

Flower color	Cultivar	Pg 3,5diG (mg)	Pg 3MG5G (mg)	Cy 3,5diG (mg)	Cy 3MG5G (mg)	Total anthocyanin (mg)	Cy/Pg	3MG5G/3,5diG
Black	‘Black Cat’	0.293 $\pm$ 0.004	1.001 $\pm$ 0.007	0.215 $\pm$ 0.003	0.838 $\pm$ 0.010	2.347 $\pm$ 0.015	0.814 $\pm$ 0.005	3.625 $\pm$ 0.071
	‘Fidalgo Blacky’	0.289 $\pm$ 0.013	0.376 $\pm$ 0.020	0.463 $\pm$ 0.003	2.248 $\pm$ 0.116	3.376 $\pm$ 0.130	4.080 $\pm$ 0.164	3.488 $\pm$ 0.084
	‘Kokucho’	0.377 $\pm$ 0.015	1.638 $\pm$ 0.099	0.242 $\pm$ 0.012	1.025 $\pm$ 0.079	3.282 $\pm$ 0.155	0.635 $\pm$ 0.054	4.295 $\pm$ 0.010
	‘Ms. Noir’	0.274 $\pm$ 0.014	1.267 $\pm$ 0.027	0.192 $\pm$ 0.010	1.109 $\pm$ 0.110	2.842 $\pm$ 0.145	0.842 $\pm$ 0.063	5.114 $\pm$ 0.279
Purple	‘Atom’	0.087 $\pm$ 0.002	0.152 $\pm$ 0.002	0.174 $\pm$ 0.003	0.703 $\pm$ 0.016	1.117 $\pm$ 0.096	3.267 $\pm$ 0.016	3.663 $\pm$ 0.040
	‘Cupid’	0.071 $\pm$ 0.000	0.250 $\pm$ 0.004	0.096 $\pm$ 0.004	0.577 $\pm$ 0.005	0.995 $\pm$ 0.005	2.095 $\pm$ 0.023	4.947 $\pm$ 0.178
	‘Evelyn Rumbold’	0.254 $\pm$ 0.016	0.516 $\pm$ 0.040	0.067 $\pm$ 0.007	0.146 $\pm$ 0.015	0.983 $\pm$ 0.072	0.276 $\pm$ 0.015	2.065 $\pm$ 0.093
	‘Yukino’	0.227 $\pm$ 0.038	1.572 $\pm$ 0.059	0.053 $\pm$ 0.001	0.547 $\pm$ 0.022	2.399 $\pm$ 0.086	0.334 $\pm$ 0.009	7.923 $\pm$ 0.958
Pink	‘Jyunn-ai’	0.011 $\pm$ 0.001	0.072 $\pm$ 0.007	0.004 $\pm$ 0.000	0.028 $\pm$ 0.003	0.115 $\pm$ 0.011	0.388 $\pm$ 0.002	6.684 $\pm$ 0.233

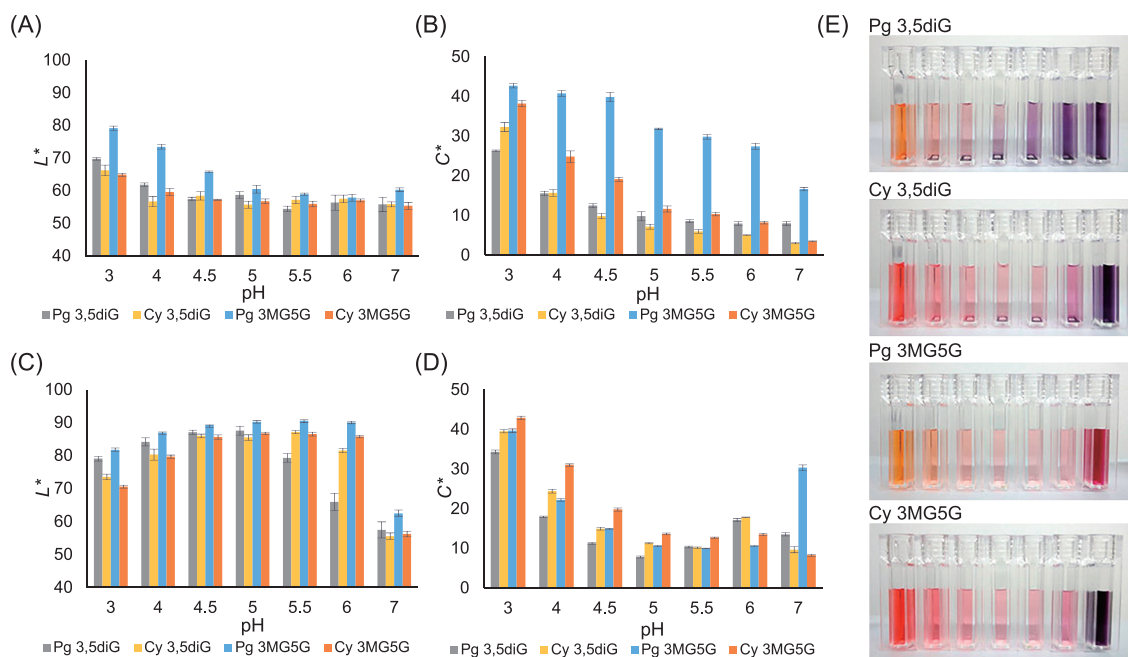


Fig. 1. The color of the purified anthocyanin solutions at various pHs measured immediately or 30 min after dissolution. (A) lightness (L^*) immediately after dissolution, (B) chroma (C^*) immediately after dissolution, (C) L^* at 30 min after dissolution, (D) C^* at 30 min after dissolution. Pg 3,5diG, pelargonidin 3,5-diglucoside; Cy 3,5diG, cyanidin 3,5-diglucoside; Pg 3MG5G, pelargonidin 3-(6"-malonylglucoside)-5-glucoside; Cy 3MG5G, cyanidin 3-(6"-malonylglucoside)-5-glucoside. A 0.5 mg sample of each anthocyanin was dissolved in 1 mL of phosphate-citrate buffer solution. C^* was calculated as $(a^{*2} + b^{*2})^{1/2}$. All data represent the mean $\pm$ SE of three replications. (E) Color photographs at 30 min after dissolution. The buffer pH is 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0 from left to right in each photograph.

Table 3. CIE a^* and b^* of purified anthocyanin solutions ($0.5 \text{ mg} \cdot \text{mL}^{-1}$) at various pHs.

			pH						
			3.0	4.0	4.5	5.0	5.5	6.0	7.0
0 min	a^*	Pg 3,5diG	23.17 $\pm$ 0.10	15.56 $\pm$ 0.56	12.24 $\pm$ 0.54	9.26 $\pm$ 1.05	8.22 $\pm$ 0.38	7.43 $\pm$ 0.32	6.84 $\pm$ 0.43
		Cy 3,5diG	30.52 $\pm$ 1.07	15.59 $\pm$ 0.83	9.81 $\pm$ 0.64	7.15 $\pm$ 0.64	5.90 $\pm$ 0.46	4.94 $\pm$ 0.07	2.56 $\pm$ 0.21
		Pg 3MG5G	31.81 $\pm$ 0.41	35.76 $\pm$ 0.51	37.28 $\pm$ 1.05	30.44 $\pm$ 0.13	28.64 $\pm$ 0.52	26.40 $\pm$ 0.66	16.34 $\pm$ 0.38
		Cy 3MG5G	35.09 $\pm$ 0.63	23.82 $\pm$ 0.18	18.61 $\pm$ 0.51	11.54 $\pm$ 0.71	10.28 $\pm$ 0.40	8.20 $\pm$ 0.34	3.46 $\pm$ 0.11
	b^*	Pg 3,5diG	12.46 $\pm$ 0.29	0.09 $\pm$ 0.17	-2.50 $\pm$ 0.20	-3.22 $\pm$ 0.45	-2.34 $\pm$ 0.38	-2.83 $\pm$ 0.44	-4.19 $\pm$ 0.25
		Cy 3,5diG	10.54 $\pm$ 0.47	1.39 $\pm$ 0.41	-0.32 $\pm$ 0.29	-0.62 $\pm$ 0.11	-0.95 $\pm$ 0.16	-1.13 $\pm$ 0.05	-1.61 $\pm$ 0.09
		Pg 3MG5G	28.46 $\pm$ 0.41	19.50 $\pm$ 0.51	14.06 $\pm$ 0.53	9.35 $\pm$ 0.10	8.26 $\pm$ 0.25	7.31 $\pm$ 0.35	3.11 $\pm$ 0.20
		Cy 3MG5G	15.04 $\pm$ 0.43	6.98 $\pm$ 0.52	4.40 $\pm$ 0.08	1.59 $\pm$ 0.38	1.16 $\pm$ 0.23	0.65 $\pm$ 0.21	-0.48 $\pm$ 0.07
30 min	a^*	Pg 3,5diG	28.08 $\pm$ 0.30	17.06 $\pm$ 0.21	11.09 $\pm$ 0.25	7.79 $\pm$ 0.26	9.94 $\pm$ 0.14	13.39 $\pm$ 0.32	9.60 $\pm$ 0.36
		Cy 3,5diG	37.11 $\pm$ 0.39	24.16 $\pm$ 0.46	14.82 $\pm$ 0.35	11.17 $\pm$ 0.15	10.14 $\pm$ 0.17	17.21 $\pm$ 0.02	7.62 $\pm$ 0.68
		Pg 3MG5G	29.76 $\pm$ 0.25	20.19 $\pm$ 0.25	14.28 $\pm$ 0.09	10.37 $\pm$ 0.13	9.80 $\pm$ 0.09	10.49 $\pm$ 0.06	30.32 $\pm$ 0.69
		Cy 3MG5G	39.43 $\pm$ 0.37	30.76 $\pm$ 0.30	19.77 $\pm$ 0.40	13.63 $\pm$ 0.22	12.67 $\pm$ 0.14	13.47 $\pm$ 0.30	8.15 $\pm$ 0.23
	b^*	Pg 3,5diG	19.71 $\pm$ 0.41	5.52 $\pm$ 0.13	1.91 $\pm$ 0.18	-0.16 $\pm$ 0.19	-2.73 $\pm$ 0.49	-10.67 $\pm$ 0.26	-9.49 $\pm$ 0.24
		Cy 3,5diG	13.55 $\pm$ 0.13	3.38 $\pm$ 0.36	1.77 $\pm$ 0.13	1.55 $\pm$ 0.17	0.91 $\pm$ 0.07	-4.67 $\pm$ 0.15	-5.88 $\pm$ 0.38
		Pg 3MG5G	26.21 $\pm$ 0.41	9.24 $\pm$ 0.21	4.40 $\pm$ 0.12	2.12 $\pm$ 0.07	1.77 $\pm$ 0.08	1.51 $\pm$ 0.12	0.64 $\pm$ 0.37
		Cy 3MG5G	16.91 $\pm$ 0.24	3.72 $\pm$ 0.16	0.84 $\pm$ 0.11	0.05 $\pm$ 0.16	-0.27 $\pm$ 0.01	-1.11 $\pm$ 0.07	-1.18 $\pm$ 0.09

0 min indicates immediately after dissolution and 30 min indicates 30 min after dissolution.

All data represent the mean $\pm$ SE of three replications.

after dissolution, whereas that of Pg 3MG5G had decreased due to decrease of absolute values of both a^* and b^* , except at pH 7.0 (Fig. 1D; Table 3). From pH 3.0 to 5.5, the C^* of Cy 3MG5G was significantly higher than that of the other anthocyanins, and from

pH 3.0 to 5.0, that of Pg 3,5diG was significantly lower. The visual color of Pg 3MG5G solutions appeared the brightest among the four anthocyanins at all pHs (Fig. 1E).

The cellular pH of petals has been shown to be ap-

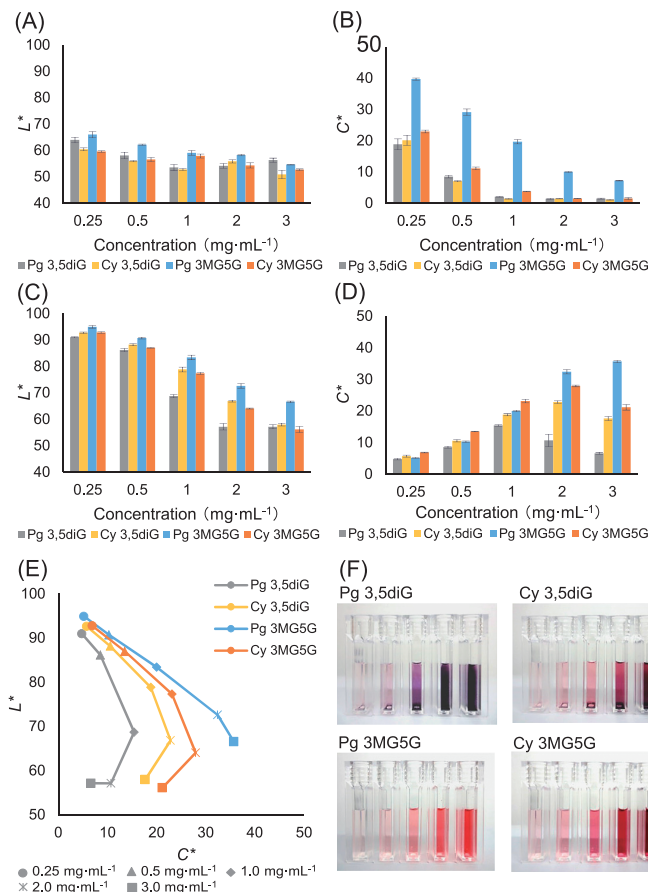


Fig. 2. The color of the purified anthocyanin solutions (pH 5.0) at various anthocyanin concentrations measured immediately, or 30 min after dissolution. (A) lightness (L^*) immediately after dissolution, (B) chroma (C^*) immediately after dissolution, (C) L^* at 30 min after dissolution, (D) C^* at 30 min after dissolution. Pg 3,5diG, pelargonidin 3,5-diglucoside; Cy 3,5diG, cyanidin 3,5-diglucoside; Pg 3MG5G, pelargonidin 3-(6"-malonylglucoside)-5-glucoside; Cy 3MG5G, cyanidin 3-(6"-malonylglucoside)-5-glucoside. C^* was calculated as $(a^{*2} + b^{*2})^{1/2}$. All data represent the mean $\pm$ SE of three replications. (E) Color plot pattern of C^* (X-axis) and L^* (Y-axis) at 30 min after dissolution. (F) Color photographs at 30 min after dissolution. Anthocyanin concentrations are 0.25, 0.5, 1.0, 2.0, and 3.0 mg·mL⁻¹ from left to right in each photograph.

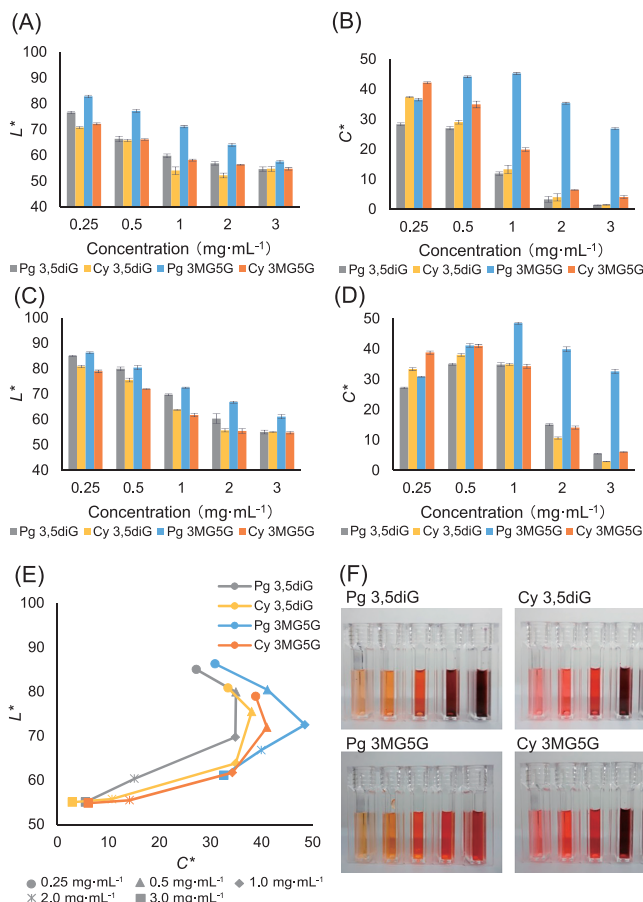


Fig. 3. The color of the purified anthocyanin solutions (pH 3.0) at various anthocyanin concentrations measured immediately or 30 min after dissolution. (A) lightness (L^*) immediately after dissolution, (B) chroma (C^*) immediately after dissolution, (C) L^* at 30 min after dissolution, (D) C^* at 30 min after dissolution. Pg 3,5diG, pelargonidin 3,5-diglucoside; Cy 3,5diG, cyanidin 3,5-diglucoside; Pg 3MG5G, pelargonidin 3-(6"-malonylglucoside)-5-glucoside; Cy 3MG5G, cyanidin 3-(6"-malonylglucoside)-5-glucoside. C^* was calculated as $(a^{*2} + b^{*2})^{1/2}$. All data represent the mean $\pm$ SE of three replications. (E) Color plot pattern of C^* (X-axis) and L^* (Y-axis) at 30 min after dissolution. (F) Color photographs at 30 min after dissolution. Anthocyanin concentrations are 0.25, 0.5, 1.0, 2.0, and 3.0 mg·mL⁻¹ from left to right in each photograph.

proximately 5.0 in 'Kokucho' and other black cultivars (Deguchi et al., 2013). Immediately after dissolution at this pH, the L^* and C^* of all anthocyanins decreased as the anthocyanin concentration increased (Fig. 2A, B). The L^* of Pg 3MG5G tended to be slightly higher than that of the other anthocyanins, although the values were not very different among the four anthocyanins (Fig. 2A). On the other hand, the C^* of Pg 3MG5G was markedly highest at all concentrations due to high absolute values of both a^* and b^* , whereas that of the other anthocyanins was similar (Fig. 2B; Table 4). At 30 min after dissolution, when the solutions had already discolored, the L^* decreased as the concentration increased (Fig. 2C, E); this was similar to the behavior immediately after dissolution. On the other hand, the C^* increased once, and then decreased as the concentration increased, as the absolute values of both a^* and b^* varied, except for that of Pg 3MG5G (Fig. 2D, E; Table 4). The decrease in C^* occurred at 2.0 mg·mL⁻¹ in Pg 3,5diG and at 3.0 mg·mL⁻¹ in Cy 3,5diG and Cy 3MG5G. The L^* of Pg 3MG5G was the highest among those of four anthocyanins at all concentrations, and that of Pg 3,5diG was the lowest except for at 3.0 mg·mL⁻¹. The C^* of Cy 3MG5G was the highest among those of four anthocyanins at lower concentration (0.5 mg·mL⁻¹ and 1.0 mg·mL⁻¹), while that of Pg 3MG5G was the highest at higher concentration (2.0 mg·mL⁻¹ and 3.0 mg·mL⁻¹). The C^* of Pg 3,5diG was the lowest except for at 0.5 mg·mL⁻¹. The color plot pattern of C^* (X-axis) and L^* (Y-axis) at 30 min

after dissolution exhibited a decreasing slope from top left to bottom right in Pg 3MG5G as the concentration increased and down-turned curves with an inflection on the right side in Pg 3,5diG, Cy 3,5diG, and Cy 3MG5G (Fig. 2E). The visual color of Pg 3,5diG solutions appeared the darkest and that of Pg 3MG5G appeared the brightest (Fig. 2F).

The colors of anthocyanins are relatively stable in acidic conditions such as at pH 3.0. Immediately after dissolution at pH 3.0, the L^* tended to decrease as the anthocyanin concentration increased, and C^* also decreased due to decrease of absolute values of both a^* and b^* , except for that of Pg 3MG5G (Fig. 3A, B; Table 4). The C^* of Pg 3MG5G increased until 0.5 mg·mL⁻¹, held at 1.0 mg·mL⁻¹, and decreased from 2.0 mg·mL⁻¹. The L^* of Pg 3MG5G was significantly higher than that of the other three anthocyanins at the same concentration until 2.0 mg·mL⁻¹ (Fig. 3A). The C^* of Pg 3MG5G was also significantly higher than that of the other anthocyanins at 0.5 mg·mL⁻¹ or higher, and that of Cy 3MG5G tended to follow a similar pattern (Fig. 3B). At 30 min after dissolution, the L^* of all anthocyanins decreased as the concentration increased (Fig. 3C, E); this was similar to the behavior immediately after dissolution. The C^* of Pg 3,5diG, Cy 3,5diG, and Cy 3MG5G increased until 0.5 mg·mL⁻¹, and decreased at higher concentrations, whereas that of Pg 3MG5G increased until 1.0 mg·mL⁻¹, and then decreased as the absolute values of both a^* and b^* varied (Fig. 3D, E; Table 4). The L^* of each Cy-based anthocyanin tended to be

Table 4. CIE a^* and b^* of purified anthocyanin solutions at pH 5.0 and pH 3.0.

		pH 5.0					pH 3.0					
		Concentration (mg·mL ⁻¹)					Concentration (mg·mL ⁻¹)					
		0.25	0.5	1	2	3	0.25	0.5	1	2	3	
0 min	<i>a</i> *	Pg 3,5diG	18.19±1.70	8.10±0.43	1.68±0.07	0.28±0.08	0.13±0.05	24.66±0.26	23.67±0.42	11.42±0.48	3.13±0.95	0.99±0.12
		Cy 3,5diG	19.90±1.56	6.98±0.14	0.97±0.06	0.03±0.00	0.10±0.04	35.88±0.22	27.46±0.56	12.96±1.34	3.94±1.19	0.71±0.20
		Pg 3MG5G	38.74±0.34	27.89±0.97	19.05±0.61	9.89±0.11	7.13±0.07	27.89±0.23	32.99±0.06	37.04±0.21	32.35±0.24	25.49±0.30
		Cy 3MG5G	22.74±0.42	10.98±0.43	3.73±0.10	1.26±0.06	1.07±0.27	39.37±0.24	32.28±0.95	19.15±0.51	6.44±0.09	4.04±0.52
	<i>b</i> *	Pg 3,5diG	-4.97±0.27	-2.52±0.15	-1.10±0.10	-1.34±0.12	-1.47±0.15	13.94±0.26	13.01±0.31	3.43±0.35	-0.42±0.45	-0.93±0.08
		Cy 3,5diG	-3.06±0.37	-0.59±0.12	-0.98±0.09	-1.39±0.04	-1.06±0.06	10.47±0.08	9.49±0.30	3.04±0.30	-0.02±0.28	-1.22±0.08
		Pg 3MG5G	8.73±0.21	8.42±0.38	4.87±0.31	1.39±0.10	0.85±0.20	23.62±0.41	29.40±0.37	26.05±0.24	14.22±0.31	8.52±0.17
		Cy 3MG5G	3.07±0.21	1.82±0.18	-0.43±0.09	-0.76±0.07	-0.95±0.17	15.16±0.21	13.39±0.65	5.41±0.51	0.62±0.12	-0.15±0.19
30 min	<i>a</i> *	Pg 3,5diG	4.63±0.28	8.50±0.28	13.51±0.20	9.08±1.61	5.65±0.26	23.01±0.21	28.52±0.20	29.29±0.39	14.37±0.28	5.49±0.07
		Cy 3,5diG	5.62±0.34	10.47±0.33	18.79±0.33	22.76±0.38	17.54±0.61	32.63±0.35	35.90±0.39	32.21±0.28	10.43±0.36	2.86±0.14
		Pg 3MG5G	4.97±0.13	10.11±0.23	19.65±0.18	31.73±0.64	34.30±0.28	24.35±0.12	30.71±0.32	37.43±0.22	35.42±0.63	30.36±0.59
		Cy 3MG5G	6.85±0.14	13.51±0.09	23.09±0.55	27.90±0.26	20.88±0.82	37.51±0.48	37.86±0.45	31.70±0.56	13.74±0.47	6.05±0.12
	<i>b</i> *	Pg 3,5diG	0.98±0.19	-0.38±0.25	-7.42±0.20	-5.62±1.61	-3.36±0.26	14.48±0.20	20.21±0.26	18.88±0.47	4.52±0.24	0.24±0.01
		Cy 3,5diG	0.99±0.07	1.32±0.14	0.84±0.09	-1.57±0.04	-1.84±0.18	7.06±0.25	12.61±0.22	13.50±0.20	2.28±0.20	-0.65±0.10
		Pg 3MG5G	1.29±0.08	1.96±0.03	3.59±0.22	6.49±0.17	9.76±0.15	18.96±0.10	27.40±0.51	30.79±0.25	18.39±0.47	11.86±0.36
		Cy 3MG5G	0.31±0.02	0.18±0.07	-0.83±0.02	0.98±0.22	3.26±0.34	9.95±0.23	15.80±0.23	13.04±0.48	3.22±0.27	0.36±0.03

0 min indicates immediately after dissolution and 30 min indicates 30 min after dissolution.

All data represent the mean±SE of three replications.

slightly lower than that of each Pg-based anthocyanin with the same modification. The C^* of Pg 3MG5G was markedly higher than that of the other three anthocyanins at $1.0 \text{ mg} \cdot \text{mL}^{-1}$ or higher. The color plot of C^* and L^* at 30 min after dissolution exhibited down-turned curves with an inflection on the right side in all anthocyanins (Fig. 3E). The curve of Pg 3MG5G was the gentlest among the four anthocyanins, and the distances from the point of origin to each plotted point of Pg 3MG5G were the longest among those of the other anthocyanins at the same concentration. The visual color of Pg 3MG5G obviously appeared the brightest among the four anthocyanins (Fig. 3F). These results suggested that the color of Pg 3MG5G was the most dissimilar to black.

A mixture of Pg- and Cy-based anthocyanins that were subjected to the same modifications (3,5diG or 3MG5G) was prepared with different proportions of Pg: Cy, and the solution color was measured at pH 5.0. Regarding the 3,5diG-type anthocyanin mixture, both L^* and C^* of solution were not different among all Pg: Cy proportions immediately after dissolution (Fig. 4A, B). At 30 min after dissolution, the values slightly increased as the proportion of Cy increased (Fig. 4C, D). Regarding the 3MG5G-type anthocyanin mixture, the C^* markedly decreased as the proportion of Cy increased, and L^* also slightly decreased (Fig. 5). These values of the anthocyanin mixture solutions were generally consistent with expectations based on the results of color measurements of the single anthocyanin solutions. Therefore, it is suggested that, even if multiple anthocyanins are accumulated, the intrinsic contri-

bution of each anthocyanin to black flower coloring shown in Figures 2 and 3 may be reflected in petal color.

Discussion

The reason why high accumulation of Cy-based anthocyanins is important for the black flower coloring of dahlias

The four anthocyanins Pg 3,5diG, Cy 3,5diG, Pg 3MG5G, and Cy 3MG5G that we purified in this study corresponded to those in previous reports on dahlias (Takeda et al., 1986; Yamaguchi et al., 1999). Two of the other anthocyanins detected in some cultivars may be the 3,5-dimalonylglucosides of Pg and Cy, as previously reported (Takeda et al., 1986; Yamaguchi et al., 1999). Although 3,5-dimalonylglucoside-type anthocyanin accounted for a considerable portion of the total anthocyanins in the study by Yamaguchi et al. (1999), these anthocyanins and other unidentified ones were detected only in trace amounts in the present study. There were no specific anthocyanins that accumulated only in black cultivars, suggesting that the black flower color is a quantitative trait regulated by the amount and proportion of the four major anthocyanins. Although the proportion of Cy-based anthocyanins varied among the cultivars, the amount of 3MG5G-type anthocyanins was higher than the 3,5diG-type anthocyanins in all cultivars (Table 2). In dahlias, a bHLH transcription factor, *DvIVS*, which positively regulates the biosynthesis of anthocyanidins (Ohno et al., 2011, 2013), also regulates the expression of 3-malonyltransferase (3MT) identi-

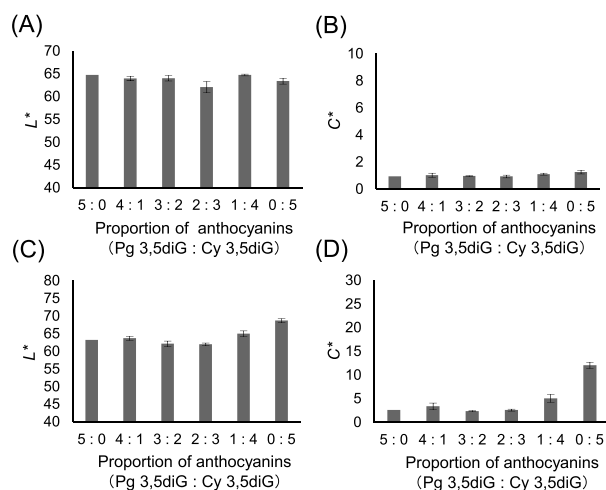


Fig. 4. The lightness (L^*) and chroma (C^*) of the solution of mixed 3,5-diglucoside-type anthocyanins at various proportions of Pg and Cy measured immediately (A, B) or 30 min after dissolution (C, D). Pg 3,5diG, pelargonidin 3,5-diglucoside; Cy 3,5diG, cyanidin 3,5-diglucoside. The buffer pH is 5.0, and the concentration of total anthocyanins is $1.0 \text{ mg} \cdot \text{mL}^{-1}$. C^* was calculated as $(a^{*2} + b^{*2})^{1/2}$. All data represent the mean $\pm$ SE of three replications (only the data for 5:0 represent the score of one sample).

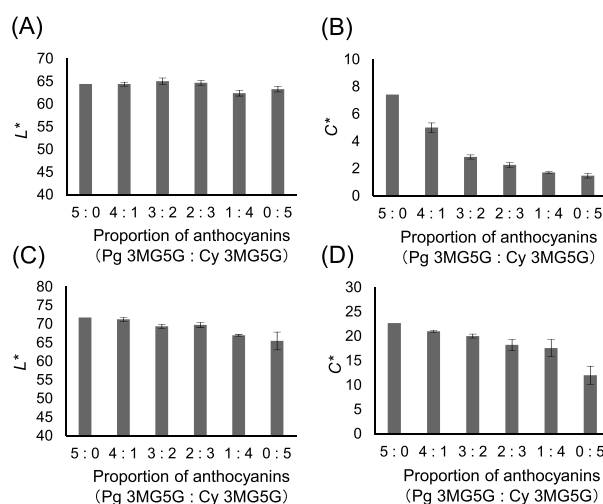


Fig. 5. The lightness (L^*) and chroma (C^*) of the solution of mixed 3-(6"-malonylglucoside)-5-glucoside-type anthocyanins at various proportions of Pg and Cy measured immediately (A, B) or 30 min after dissolution (C, D). Pg 3MG5G, pelargonidin 3-(6"-malonylglucoside)-5-glucoside; Cy 3MG5G, cyanidin 3-(6"-malonylglucoside)-5-glucoside. The buffer pH is 5.0, and the concentration of total anthocyanins is $1.0 \text{ mg} \cdot \text{mL}^{-1}$. C^* was calculated as $(a^{*2} + b^{*2})^{1/2}$. All data represent the mean $\pm$ SE of three replications (only the data for 5:0 represent the score of one sample).

fied by Suzuki et al. (2002) (unpublished data). This may be a reason that 3MG5G-type anthocyanins are predominant in dahlia petals.

The color and stability of purified anthocyanins varied depending on the pH (Fig. 1; Table 3), as previously reported (Brouillard, 1988; Fossen et al., 1998; Heredia et al., 1998; Hurtado et al., 2009; Torskangerpoll and Andersen, 2005). Under mildly acidic and neutral conditions, anthocyanins discolor because of the transformation to colorless pseudo bases by hydration. Therefore, the color of purified anthocyanins was evaluated immediately (before discoloring) and 30 min after dissolution (after discoloring).

At pH 5.0, which is approximately the pH of the black cultivar petals, Pg 3MG5G showed the highest C^* , and tended to show slightly higher L^* than the other three anthocyanins (Fig. 2). The highest C^* of Pg 3MG5G was due to the highest absolute values of a^* and b^* (Table 4). At 30 min after dissolution, Cy 3MG5G had the second brightest color after Pg 3MG5G when the concentration was at $1.0 \text{ mg} \cdot \text{mL}^{-1}$ or higher. Pg 3,5diG had almost the same L^* and C^* as Cy 3,5diG at low anthocyanin concentrations, but lower L^* and C^* at high concentrations 30 min after dissolution. Given the distance from the point of origin ($C^* = 0$, $L^* = 0$), which indicates the ideal black, to each plot in Figure 2E, the order of the four anthocyanins at the same mass concentration with respect to their contribution to black flower coloring at pH 5.0 appeared to be Pg 3,5diG > Cy 3,5diG $\approx$ Cy 3MG5G $\gg$ Pg 3MG5G. The C^* and L^* of each anthocyanin showed similar tendencies between pH 5.0 and pH 5.5 (Fig. 1), which corresponded to the petal pH of relatively deeper cyanic (purple, red, and black) cultivars (pH 4.9–5.5, Deguchi et al., 2013; Ohno et al., 2013). These results suggested that the order of the contribution should be consistent, and the color of Pg 3MG5G was always the farthest from black among the four anthocyanins in this petal pH range. At pH 3.0 at which each anthocyanin retained a relatively stable color, Pg 3MG5G had significantly higher L^* and C^* than the other three anthocyanins, in particular at higher concentrations (Fig. 3). The higher C^* of Pg 3MG5G was due to the higher absolute values of a^* and b^* (Table 4). The color plots at 30 min after dissolution (Fig. 3E) indicated that the contribution to black flower coloring at pH 3.0 appeared to be Cy 3,5diG $\approx$ Cy 3MG5G $\geq$ Pg 3,5diG $\gg$ Pg 3MG5G.

The mol concentration ($\text{mol} \cdot \text{mL}^{-1}$) of each anthocyanin is different when they are at the same mass concentration ($\text{mg} \cdot \text{mL}^{-1}$), because molecular weight of each anthocyanin is different. Therefore, it may be difficult to make a rigorous comparison of the color of anthocyanins from these results, especially between 3,5diG- and 3MG5G-type. However, the color of Pg 3MG5G at certain concentration was consistently brighter than the other anthocyanins at lower concentration [ex. comparing Pg 3MG5G at $3.0 \text{ mg} \cdot \text{mL}^{-1}$ ($= 5.0 \cdot 10^{-6} \text{ mol} \cdot \text{mL}^{-1}$)

and the other anthocyanins at $2.0 \text{ mg} \cdot \text{mL}^{-1}$ ($= 2.9\text{--}3.3 \cdot 10^{-6} \text{ mol} \cdot \text{mL}^{-1}$)], suggesting that Pg 3MG5G is the brightest among four anthocyanins even when compared at mol concentration. Therefore, it was suggested that the contribution of Pg 3MG5G to black flower coloring was by far the lowest among the four anthocyanins at both pH 5.0 and 3.0. Although the colors of anthocyanins in cellular condition are affected by several elements such as self-association (Goto and Kondo, 1991; Goto et al., 1986; Hoshino et al., 1980, 1982) and co-pigmentation (Asen et al., 1972; Brouillard, 1983; Goto et al., 1986), the lowest contribution of Pg 3MG5G to lowering petal L^* and C^* *in vitro* should be applicable *in vivo*.

Stock [*Matthiola incana* (L.) W.T.Aiton] ‘Vintage Burgundy’, which has a higher proportion of Cy, has been shown to have lower L^* than ‘Vintage Red’, which has a lower proportion of Cy (higher proportion of Pg) (Tatsuzawa et al., 2012), as in dahlias. However, a *Hippeastrum* (*Hippeastrum hybridum*) cultivar, ‘Royal Velvet’, which has a higher proportion of Cy, showed almost the same L^* and higher C^* than ‘Liberty’, which has a lower proportion of Cy (Byamukama et al., 2006). A eustoma [*Eustoma grandiflorum* (Raf.) Shinn.], ‘Mickey Rose’, which has a higher proportion of Cy, has been shown to have higher C^* but lower or higher L^* than the other cultivars, which have a lower proportion of Cy (‘Azuma no Yosooi’, ‘Azuma no Ho-hoemi’, and ‘Pink Rose’) (Uddin et al., 2004). These findings suggest that a higher proportion of Cy-based anthocyanins does not always lower petal L^* and C^* . Differences in the contribution to lowering petal L^* and C^* between Pg- and Cy-based anthocyanins may be attributed in part to the modification of anthocyanidins. Cy 3,5diG showed nearly the same or lower contribution to lowering L^* and C^* than Pg 3,5diG (Figs. 2 and 3). On the other hand, Cy 3MG5G showed higher contribution than Pg 3MG5G (Figs. 2 and 3). When the proportion of Cy was increased in an anthocyanin mixture, decreases in L^* and C^* were observed only in the 3MG5G-type anthocyanin mixture (Figs. 4 and 5). These results demonstrated that the lower petal L^* and C^* caused by a higher proportion of Cy-based anthocyanin depended on the modification of the accumulating anthocyanins. Therefore, if 3,5diG-type anthocyanins were accumulated dominantly in dahlia petals, the flower color of a high Cy-level cultivar would not be blacker than a high Pg-level cultivar. In conclusion, because 3MG5G-type anthocyanins are predominant pigments and Cy 3MG5G shows lower L^* and C^* than Pg 3MG5G, higher accumulation of Cy-based anthocyanins may be critical for the black flower coloring of dahlias.

Possible approaches for the production of black flowers

We have previously proposed two mechanisms to produce black flower cultivars in dahlias. One mecha-

nism is PTGS of *DvFNS*, which causes a high accumulation of total and Cy-based anthocyanins, and the other mechanism is associated with the high ability of Cy synthesis to induce a high proportion of Cy-based anthocyanins (Deguchi et al., 2013, 2015). Both mechanisms are based on the fact that certain Cy-based anthocyanins (Cy 3MG5G) show higher contributions to lowering petal L^* and C^* than Pg-based anthocyanins (Pg 3MG5G) in dahlias. However, the contribution is suggested to depend on the structure of anthocyanin, including the absence or presence of hydroxylation, methylation, acylation, and the kind of sugar and acyl moiety. Thus, it is suggested that the accumulation of anthocyanins that contribute highly to lowering L^* and C^* rather than the accumulation of Cy-based anthocyanins is essential for the black flower coloring of various species. Further *in vitro* color evaluation of other anthocyanins accumulated in various species may provide information useful for the identification of a blackish pigment, namely anthocyanin with the highest contribution to lowering L^* and C^* .

Moreover, the amount of anthocyanins is also important for the black flower coloring. Color plots of the four anthocyanins at 30 min after dissolution (Figs. 2E and 3E) exhibit down-turned curves with an inflection on the right side, except for Pg 3MG5G at pH 5.0, which were similar to the plots of the flower color of dahlia cultivars showing various color intensities (Deguchi et al., 2013; Ohno et al., 2013). Two phases in the variation of C^* were observed as the anthocyanin concentration increases: an increasing phase and a subsequent decreasing phase (Figs. 2D, E and 3B, D, E). This variation of C^* suggested that a high anthocyanin concentration, at least above the inflection of C^* , is necessary for lowering both L^* and C^* and for the black flower coloring. Some *Thymus* species in the family *Labiatae* predominantly accumulate Cy 3MG5G in their flowers, but those flower colors are not black but purple (Saito and Harborne, 1992), which may be because of low concentrations. Our HPLC analysis showed that 2.3–3.4 mg of total anthocyanins are accumulated in 100 mg of fresh petals of black dahlia cultivars (Table 2). Because anthocyanins accumulate only in the vacuoles of epidermal cells of petals, the concentration is expected to be substantially higher than 2.0 mg·mL⁻¹.

A purple cultivar, ‘Yukino’, had a similar amount of total anthocyanins to a black cultivar, ‘Black Cat’, but never looked black because Pg 3MG5G accounted for the major portion of the total anthocyanins. If a flower predominantly accumulates anthocyanins with low contributions to lowering L^* and C^* , including Pg 3MG5G, an exceedingly high amount would be required to appear black, which may be impossible. Therefore, the most efficient way to produce black flowers in various species might be the induction of the highest possible accumulation of anthocyanin that can contribute highly

to lowering L^* and C^* .

In the measurement of solution color *in vitro*, recrystallization of 3,5diG-type anthocyanins, which was an interesting phenomenon, was observed. In roses, which accumulate 3,5diG-type anthocyanins in their petals, condensed anthocyanins have been observed in vacuoles when petal bluing occurred (Yasuda, 1974, 1976; Yasuda and Yoneda, 1985). A similar phenomenon has also been observed in *Eustoma* although accumulated anthocyanins were not 3,5diG-type, and the condensed anthocyanins were called anthocyanic vacuolar inclusions (AVIs) (Markham et al., 2000; Zhang et al., 2006). In carnations (*Dianthus caryophyllus* L.), which normally accumulate acylated anthocyanins modified with a malyl group, knockdown of 3-malyltransferase induced the accumulation of 3,5diG-type anthocyanins following the formation of AVIs (Okamura et al., 2013; Sasaki et al., 2013). The carnation in which AVIs was formed showed a dusky and metallic flower color (Okamura et al., 2013; Sasaki et al., 2013). In the present study, we observed that the L^* and C^* of 3,5diG-type anthocyanins were almost the same or lower than those of Cy 3MG5G (Figs. 2 and 3). Furthermore, the solution colors of 3,5diG-type anthocyanins after recrystallization were also as deep as that of Cy 3MG5G at the same point (data not shown). Therefore, another possible way to produce black dahlia cultivars is by the suppression of 3MT expression to induce the accumulation of only 3,5diG-type anthocyanins. In dahlias, AVIs were not observed in any cultivars including black cultivars (data not shown), however, the cultivars produced by the suppression of 3MT have possible to form AVIs, and have a novel black color, namely dusty black or metallic black.

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