

## Determination of Flower Coloration in *Delphinium* spp. by Monitoring Sepal Anthocyanin *in Vivo*

Fumio HASHIMOTO<sup>†</sup>, Shoko FUKUDA, Hiroko MAEDA, Mika TANAKA, Keiichi SHIMIZU,  
Naoyuki CHISHAKI, Yusuke SAKATA and Etsuji ISHIGURO  
(Laboratory of Ornamental Horticulture and Floriculture)

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### Summary

The relationship between sepal hue and major sepal anthocyanins of cyanic *Delphinium* flowers, *i.e.*, violdelphin (2) and cyanodelphin (3), is discussed. The mode of change in flower color *in vivo* was derived as a numerical equation relating two kinds of sepal pigments, from violdelphin [VD] (2) to cyanodelphin [CD] (3) for bluish flowers or from tulipanin [TP] (1) to [VD] (2) for purplish flowers, during anthesis, for changing flower hue from purple to blue or from pink to purple, respectively. The molar concentration of CD (3) relative to the molar concentration of VD (2), and of VD (2) relative to TP (1), in bluish and purplish sepals, respectively, had a strong regression with its hue angle ( $h$ ) in a double reciprocal plot, which yielded a molar ratio constant ( $K_H$ ) and a maximal hue angle ( $H_{max}$ ). This study demonstrates for the first time a clear correlation between primary sepal pigments and flower color, specifically hue angle, and thus a primary determinant of coloration can be explained by the structural definition of the primary sepal anthocyanins for bluing and purpling in cyanic *Delphinium* flowers.

**Key words:** *Delphinium*, flower color, hue angle, Ranunculaceae

### Introduction

The mechanisms of flower coloration can be explained by many factors. Floral anthocyanins are not always the only influence on flower hue. There are several factors that affect flower color, but the constituents and the quantities of floral pigments are the primary dominating determinants [1]. Examples include intramolecular copigmentation by acylated anthocyanins [2], intermolecular copigmentation in flowers of *Pharbitis nil* [3] and metalloanthocyanins from *Commelina communis* [4]. Moreover, the shapes of epidermal cells affect the color intensity of pigmentation in *Antirrhinum majus* [5].

Yoshida et al. [6] presented a possible mechanism of color emission pattern in cyanic *Delphinium* flowers, and followed this by suggesting the formation of an insoluble chromophore in the cytosol consisting of cyanodelphin (3) and  $Al^{3+}$  ions in a 2:1 ratio in association with a fibrous mass. *In vitro* experiments were also conducted to regenerate the bluish color by mixing cyanodelphin (3) and  $Al^{3+}$  ions in a controlled solution at pH 5.0 [7].

<sup>†</sup> : Correspondence to: F. HASHIMOTO  
(Laboratory of Ornamental Horticulture and Floriculture, Kagoshima University)  
Tel/Fax: +81-99-285-8559; E-mail: omihshf@agri.kagoshima-u.ac.jp

We recently reported a possible cause of coloration in cyanic *Delphinium* flowers [8] and that cyanic *Delphinium* flowers change color during anthesis [9]. Specifically, the hue angle decrease *in vivo* seemed to be accompanied by conversion of the major acylated anthocyanin from viodelphin (2) [10] to cyanodelphin (3) [11], which promptly causes the change in sepal coloration from a purplish to a bluish hue.

In this paper, we focus on exploring the relationship between flower coloration and the major inherent sepal pigments in order to identify the primary determinant of colors in cyanic *Delphinium* flowers. For this, a model of changes in flower color during anthesis was developed to illustrate the correlation between the molar concentrations of the major sepal pigments and floral coloration as determined by the CIELab color scale [12].

## Materials and Methods

### *Plant materials*

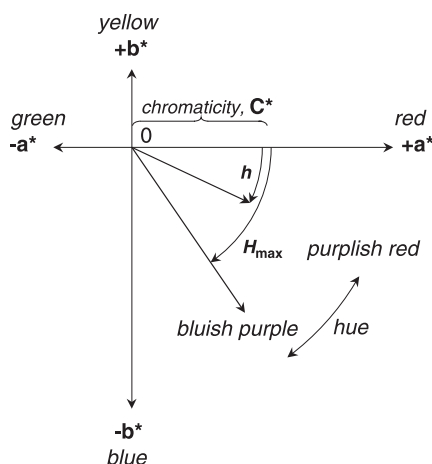
Delphiniums were cultivated as described in a previous report [8]. The S<sub>3</sub> seeds of *Delphinium* cultivars were regenerated from S<sub>2</sub> cultivars by self-pollination. The cultivars are as follows: blue flowered 'Blue Mirror', blue flowered 'Pacific Giant', light blue flowered 'Pacific Giant', purple flowered 'Pacific Giant'; and light purple flowered 'Pacific Giant'. They were germinated in petri dishes at 15°C, and seedlings were transplanted into a 128-plug tray in August 2000, 2002 and 2004, and kept in a greenhouse. Plants with five or six leaves were transferred to a greenhouse at the Kagoshima University Experimental Farm. At the beginning of January 2001, 2003 and 2005, the transferred plants were exposed to a 6 hr photoperiod (21:00-3:00) (3,000 lux light intensity, 1.5 m above the ground). The greenhouse temperature was kept at about 15°C during winter. Sepals were collected from among five similarly colored individuals of each variety with three replicates.

### *Measurements of sepal color, pH and concentrations of metal ions*

Wing sepals were collected at 3 hr intervals from anthesis until the 12 hr mark, at 6 hr intervals from 12 hr to 24 hr, and every 12 hr from 24 hr until the fifth day (120 hr). Colors were recorded for typical flowers (simple colored sepals) of 'Blue Mirror' and 'Pacific Giant' with three replicates during anthesis as described previously [9]. The collected sepals were analyzed for CIELab coordinates ( $a^*$  and  $b^*$ , the two Cartesian co-ordinates) using a color analyzer (NR-3000, Nippon Denshoku, Co.) (Fig. 1) [13]. Metric Chroma,  $C^*$ , and hue angle,  $h_{ab}$  (CIELCH notation), were calculated according to the following equations:  $C^* = (a^{*2} + b^{*2})^{0.5}$  and  $h_{ab} = \tan^{-1}(b^*/a^*)$  [12].

Sepal vacuolar pH was recorded according to Fukui et al. [14]. Fresh sepals of delphiniums (ca. 2g) were collected and frozen below -30°C for 1 hr. The frozen sepals were compressed with a hand-type compressor to yield sepal juice. The obtained juice was stored in an assist-tube. The juice was subsequently applied to a pH electrode (Horiba 6069-10C with thermo-adjusted electrode 4163-10T) conducted with a Horiba pH meter F-12.

Sepal concentrations of metal ions (Al, Fe, and Mg) were recorded as follows. Fresh sepals (ca. 2 g) were collected in a tall beaker, and concentrated nitric acid (10 ml) was added. The beaker was then covered with a glass, followed by heating at 180°C, and stood for two or three days to complete the degradation of organic composition. After complete degradation and cooling, perchloric acid (1 ml) was added to the degraded acidic solution, followed by heating at 180°C. After cooling the mixture, the degraded solution was filled to 50 ml with H<sub>2</sub>O, and the solution was tested by atomic adsorption spectroscopy to measure the concentrations of metal ions.

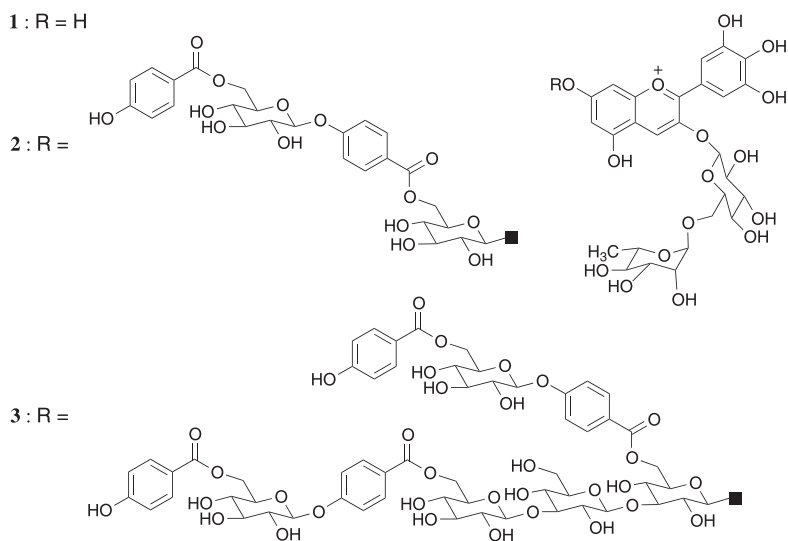


**Figure 1.** A hue diagram showing the change in hue angle as a function of the conversion of the major sepal pigments from [VD (2)] to [CD (3)] during anthesis in *Delphinium* flowers. The chromatic component,  $a^*$ , is a positive or negative coordinate defining a locus relative to a purplish red to bluish green axis, whereas  $b^*$  is a positive or negative coordinate defining a locus relative to a yellow to blue axis on the hue diagram. The chromaticity,  $C^*$ , refers to a Euclidian distance,  $C^* = (a^{*2} + b^{*2})^{1/2}$ . The hue angle represents the angle from axis  $+a^*$  to display a locus of color on the diagram; a direction of color ( $h = \tan^{-1}[b^*/a^*]$ ).

#### HPLC analysis of anthocyanins

The extraction and analytical procedure for anthocyanins 1-3 with their HPLC conditions were done as described in a previous report [8, 9] (Fig. 2). Anthocyanins in the calyx were extracted with 5 ml of a modified acidic methanolic solution (methanol,  $H_2O$ , formic acid, and trifluoroacetic acid, 70:27:2:1, v/v %), and the extracts were subjected to millipore disk filtration (5  $\mu$ m). The linear flow-gradient and conditions for qualitative and quantitative analyses were: solution A, 1.5% phosphoric acid and formic acid, 8:2 (v/v %); solution B, phosphoric acid, formic acid, acetonitrile, tetrahydrofuran (THF) and  $H_2O$ , 1.43:19:23.75:5:50.82 (v/v %); A:B = 100:0 (initial) to 54:46 (v/v %), 65 min.; column, TSKgel ODS-80Ts QA 4.5mm  $\phi$  x 150 mm, Toso Co.; column temperature, 40  $^{\circ}C$ ; flow rate, 0.8 ml/min.; wavelength, 525 nm and injection volume, 10  $\mu$ l/sample. The system used for HPLC was comprised of an Intelligent Sampler AS 950-10, a 3-Line Degasser DG-980-02, an Intelligent HPLC pump PU-980, an Intelligent Column Thermostat CO-966, a Ternary Gradient Unit LG-980-02 and a UV Spectrophotometer UVDEC-100-III FP-920 (JASCO, Co.). Concentrations of anthocyanins in the calyx were measured quantitatively from a simple linear regression using the respective isolated authentic anthocyanins 1-3 (Fig. 2) and are expressed as nmol/g (nmol in g fresh calyces). The retention times on HPLC are as follows: 1,  $15.9 \pm 0.5$  min; 2,  $31.6 \pm 0.6$  min; and 3,  $58.5 \pm 0.8$  min (three replicates).

The known anthocyanins were identified as: tulipanin (1), delphinidin 3-*O*-rutinoside [15]; violdelphin (2), 7-*O*-(6-*O*-(4-(6-*O*-(4-hydroxybenzoyl)- $\beta$ -D-glucopyranosyl)-oxybenzoyl)- $\beta$ -D-glucopyranosyl)-3-*O*-(6-*O*- $\alpha$ -L-rhamnopyranosyl)- $\beta$ -D-glucopyranosyl)-delphinidin [10]; and cyanodelphin (3), 3-*O*-(6-*O*-( $\alpha$ -L-rhamnosyl)- $\beta$ -D-glucopyranosyl)-7-*O*-(3-*O*-(3-*O*-(6-*O*-(4-*O*-(6-*O*-*p*-hydroxybenzoyl)- $\beta$ -D-glucopyranosyl)-*p*-hydroxybenzoyl)- $\beta$ -D-glucopyranosyl)- $\beta$ -D-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-*p*-hydroxybenzoyl)- $\beta$ -D-glucopyranosyl)-*p*-hydroxybenzoyl)- $\beta$ -D-glucopyranosyl)-delphinidin [11] (Fig. 2).



**Figure 2.** Structures of known anthocyanins, tulipanin (1), viodelphin (2) and cyanodelphin (3), in pink, purplish and bluish *Delphinium* flowers, respectively.

## Results

### *Relationship between petal coloration and anthocyanin accumulation*

The values of hue angle ( $h$ ) in flowers of the purple flowered ‘Pacific Giant’ and the blue flowered ‘Blue Mirror’ gradually decreased to some extent during anthesis (Table 1). This implies that the colors changed from pink to purple and to bluish purple, respectively. The major anthocyanins, viodelphin (2) and cyanodelphin (3), were reported to be identified in purple and blue flowers, respectively, presumably through the conversion of their respective precursors, tulipanin (1) and viodelphin (2) (Table 2).

Since we had not previously identified changes in colors and anthocyanins prior to one day after anthesis [9], and this is considered to be the best way to understand the rapid change in their development, we performed an investigation of colors and anthocyanin development at 3 hr intervals from anthesis until the 12 hr mark, and every 6 hr from 12 hr to 24 hr after anthesis. The data obtained between 0 and 24 hr showed a dramatic change in colors and anthocyanin concentrations in every cultivar flower (Tables 1 and 2, and Fig. 3).

The hue angle,  $h$ , was plotted against the change in the molar ratio of the concentrations of tulipanin (1) and 2 [VD/TP] and 2 and 3 [CD/VD] after anthesis in purplish and bluish sepals, respectively (Fig. 4). The hue angle decreases with the increase of the ratio of [CD] against [VD]. When the ratio was small, the change of hue angle was linearly proportional to the molar ratio. The hue angle initially decreased with changes in molar ratio, but at higher molar ratios the hue angle was nearly independent.

Subsequently, the two inverse variables,  $1/h$  and  $1/[VD/TP]$  or  $1/[CD/VD]$ , were regressed to give a significant reciprocal straight line, given in equation (a) (Fig. 5), where the slope is  $K_H/H_{\max}$ , the intercept on the vertical axis is  $1/H_{\max}$  and the intercept on the horizontal axis is  $-1/K_H$ .  $K_H$  represents a molar ratio constant and  $H_{\max}$  represents the maximal hue angle. The regression can also be expressed as the following equation (b):

**Table 1. Sepal coloration of *Delphinium* flowers after flower opening.<sup>a</sup>**

Cv (individual)	Hours after anthesis							
	Opening	3	6	9	12	18	24	
<b>Blue flowered</b>								
Blue Mirror (n=3)	L*	62.2 ± 5.2	57.6 ± 4.2	55.4 ± 4.4	57.0 ± 3.3	53.3 ± 1.8	49.1 ± 5.9	44.2 ± 5.0
	C*	13.8 ± 9.8	20.4 ± 5.1	25.5 ± 8.3	20.3 ± 6.0	28.9 ± 1.3	37.0 ± 13.1	45.3 ± 12.5
	b	-19.3 ± 18.0°	-33.6 ± 11.5°	-42.1 ± 7.1°	-35.4 ± 5.0°	-43.6 ± 2.5°	-46.2 ± 6.0°	-49.3 ± 0.8°
Pacific Giant (n=3)	L*	58.4 ± 4.2	56.6 ± 2.7	54.5 ± 4.3	53.5 ± 2.1	50.3 ± 2.5	47.4 ± 3.3	46.3 ± 5.3
	C*	16.6 ± 3.4	19.0 ± 3.5	22.6 ± 4.1	24.9 ± 2.8	31.2 ± 5.5	31.8 ± 3.4	34.9 ± 8.9
	b	-30.8 ± 15.6°	-38.4 ± 13.8°	-37.8 ± 8.2°	-40.1 ± 6.5°	-42.6 ± 9.8°	-45.1 ± 8.2°	-46.0 ± 8.3°
<b>Light blue flowered</b>								
Pacific Giant (n=3)	L*	64.4 ± 0.9	63.0 ± 1.2	60.7 ± 0.3	59.7 ± 2.2	61.2 ± 0.7	59.3 ± 0.9	57.9 ± 1.4
	C*	8.2 ± 3.0	8.6 ± 3.7	14.2 ± 3.2	14.2 ± 3.5	14.0 ± 3.6	19.3 ± 4.3	20.6 ± 2.4
	b	-28.2 ± 18.5°	-36.7 ± 12.8°	-39.6 ± 11.5°	-44.9 ± 5.9°	-44.7 ± 9.7°	-58.9 ± 4.2°	-59.2 ± 2.5°
<b>Purple flowered</b>								
Pacific Giant (n=3)	L*	54.8 ± 5.3	55.8 ± 0.5	49.6 ± 8.5	49.5 ± 4.4	46.6 ± 7.0	43.0 ± 5.7	44.8 ± 4.5
	C*	24.9 ± 7.5	24.5 ± 1.7	32.3 ± 10.5	33.6 ± 5.1	33.2 ± 8.9	41.8 ± 9.6	40.9 ± 8.6
	b	-34.1 ± 12.0°	-35.2 ± 7.1°	-37.8 ± 5.4°	-38.0 ± 7.6°	-38.6 ± 8.9°	-42.4 ± 3.4°	-40.9 ± 4.4°
<b>Light purple flowered</b>								
Pacific Giant (n=3)	L*	67.5 ± 1.4	64.7 ± 2.8	63.4 ± 3.5	63.0 ± 4.9	64.5 ± 4.1	63.3 ± 3.0	61.3 ± 3.6
	C*	14.7 ± 2.5	18.7 ± 5.1	20.3 ± 6.1	22.4 ± 7.2	18.6 ± 4.4	23.1 ± 3.5	28.5 ± 5.3
	b	-34.8 ± 4.0°	-39.9 ± 7.4°	-40.8 ± 3.4°	-40.6 ± 0.8°	-41.5 ± 3.7°	-42.7 ± 5.8°	-44.6 ± 2.9°

Cv (individual)	Hours after anthesis							
	36	48	60	72	84	96	120	
<b>Blue flowered</b>								
Blue Mirror (n=3)	L*	41.6 ± 4.1	41.9 ± 1.8	40.6 ± 2.0	40.7 ± 0.1	39.9 ± 2.6	41.9 ± 2.7	41.0 ± 4.7
	C*	52.6 ± 9.5	57.1 ± 11.4	61.9 ± 12.3	67.3 ± 5.0	70.4 ± 4.2	66.1 ± 3.3	70.0 ± 4.4
	b	-49.1 ± 1.1°	-51.4 ± 0.3°	-49.9 ± 1.1°	-50.2 ± 0.5°	-50.9 ± 1.3°	-51.3 ± 1.8°	-51.2 ± 2.4°
Pacific Giant (n=3)	L*	41.6 ± 6.3	38.7 ± 3.6	36.6 ± 3.5	36.7 ± 4.7	37.7 ± 5.9	37.2 ± 4.0	38.3 ± 3.6
	C*	46.4 ± 12.7	56.2 ± 15.5	68.8 ± 9.1	70.0 ± 6.8	71.5 ± 13.4	72.2 ± 9.1	71.4 ± 9.3
	b	-47.6 ± 4.4°	-47.5 ± 3.0°	-48.3 ± 3.2°	-48.9 ± 3.3°	-49.3 ± 4.3°	-49.2 ± 2.8°	-49.6 ± 2.2°
<b>Light blue flowered</b>								
Pacific Giant (n=3)	L*	59.4 ± 2.9	58.0 ± 0.5	58.4 ± 2.0	58.0 ± 1.3	60.4 ± 1.1	59.9 ± 1.7	60.5 ± 0.7
	C*	23.1 ± 4.2	24.8 ± 2.8	27.6 ± 7.9	28.5 ± 2.8	30.2 ± 5.3	32.8 ± 4.1	33.0 ± 1.8
	b	-57.6 ± 5.6°	-59.4 ± 2.5°	-62.4 ± 3.2°	-62.2 ± 1.3°	-63.7 ± 1.5°	-63.0 ± 1.5°	-63.5 ± 0.2°
<b>Purple flowered</b>								
Pacific Giant (n=3)	L*	42.5 ± 6.5	37.7 ± 3.9	36.1 ± 2.3	35.9 ± 3.6	35.6 ± 2.6	36.5 ± 2.0	36.4 ± 1.5
	C*	44.4 ± 10.6	55.3 ± 6.4	56.4 ± 5.2	59.8 ± 8.2	60.2 ± 5.7	60.1 ± 2.4	61.5 ± 4.6
	b	-42.0 ± 2.7°	-42.3 ± 1.5°	-42.4 ± 1.8°	-42.4 ± 0.7°	-43.6 ± 1.1°	-43.7 ± 2.3°	-44.2 ± 1.2°
<b>Light purple flowered</b>								
Pacific Giant (n=3)	L*	61.2 ± 3.0	59.5 ± 3.9	59.8 ± 3.1	60.8 ± 1.9	61.8 ± 3.1	63.1 ± 3.1	62.7 ± 1.2
	C*	29.6 ± 4.4	36.7 ± 7.4	36.3 ± 5.2	35.4 ± 4.1	34.0 ± 5.5	31.9 ± 6.4	32.7 ± 3.9
	b	-46.2 ± 2.9°	-46.3 ± 1.2°	-45.7 ± 1.1°	-46.3 ± 0.8°	-46.6 ± 1.5°	-46.5 ± 1.6°	-46.1 ± 0.6°

<sup>a</sup>A wing sepal was collected after flower opening (mean ± SD). L\*, lightness; C\*, chroma (brightness); b, hue angle (degree, °) = arctan (b\*/a\*)

Table 2. Contents of anthocyanins in sepals of *Delphinium* cvs after flower opening.<sup>a</sup>

Cv (individual)	Pigments <sup>b</sup>	Hours after anthesis						
		Opening	3	6	9	12	18	24
Blue flowered								
Blue Mirror (n=3)	2	377.2 ± 256.9	549.8 ± 279.7	466.3 ± 250.5	585.3 ± 339.6	467.4 ± 141.0	733.3 ± 358.6	654.6 ± 205.1
	3	4.3 ± 7.5	24.1 ± 17.5	29.8 ± 23.9	22.2 ± 10.6	29.3 ± 10.0	99.3 ± 62.9	128.3 ± 44.9
Pacific Giant (n=3)	2	146.4 ± 43.3	224.0 ± 122.2	213.9 ± 70.9	189.8 ± 95.4	250.4 ± 56.7	288.0 ± 88.1	245.8 ± 50.6
	3	39.2 ± 16.0	66.8 ± 30.1	56.8 ± 21.3	49.8 ± 6.6	57.8 ± 10.3	168.2 ± 99.9	204.6 ± 154.3
Light blue flowered								
Pacific Giant (n=3)	2	92.7 ± 30.3	80.9 ± 30.4	107.0 ± 34.9	111.3 ± 50.1	130.4 ± 69.8	122.3 ± 66.9	131.2 ± 57.5
	3	29.5 ± 2.2	32.5 ± 15.3	51.9 ± 14.0	45.8 ± 11.6	47.2 ± 21.3	88.3 ± 24.4	113.9 ± 28.8
Purple flowered								
Pacific Giant (n=3)	1	95.2 ± 43.9	81.1 ± 33.8	80.4 ± 40.1	80.6 ± 32.4	90.1 ± 35.6	102.0 ± 33.8	89.2 ± 30.3
	2	1707.0 ± 438.7	1741.7 ± 583.4	1689.5 ± 76.4	2226.2 ± 210.5	2319.5 ± 493.1	3218.3 ± 1401.5	3122.7 ± 907.7
Light purple flowered								
Pacific Giant (n=3)	1	9.0 ± 4.2	8.2 ± 3.0	7.5 ± 2.9	5.4 ± 0.9	9.8 ± 3.7	10.1 ± 6.6	7.1 ± 2.5
	2	149.6 ± 48.4	150.3 ± 83.0	147.4 ± 70.6	136.1 ± 61.3	160.7 ± 59.4	164.8 ± 73.6	163.0 ± 85.8

Cv (individual)	Pigments <sup>b</sup>	Hours after anthesis						
		36	48	60	72	84	96	120
Blue flowered								
Blue Mirror (n=3)	2	715.2 ± 559.6	584.7 ± 362.4	772.3 ± 556.7	606.3 ± 648.9	555.2 ± 255.9	488.3 ± 374.8	368.3 ± 230.2
	3	136.6 ± 51.0	294.5 ± 132.4	317.2 ± 70.5	399.8 ± 91.8	585.6 ± 134.0	639.5 ± 55.4	723.2 ± 131.3
Pacific Giant (n=3)	2	375.7 ± 89.2	422.3 ± 111.5	529.3 ± 271.9	463.1 ± 307.9	416.6 ± 244.3	437.7 ± 252.8	346.0 ± 134.2
	3	254.2 ± 173.8	348.0 ± 233.1	431.7 ± 153.4	469.6 ± 157.8	650.0 ± 95.0	576.3 ± 140.0	572.9 ± 127.1
Light blue flowered								
Pacific Giant (n=3)	2	151.2 ± 83.9	145.1 ± 86.9	98.7 ± 45.3	96.1 ± 59.6	38.3 ± 11.8	62.8 ± 19.3	51.5 ± 8.6
	3	116.8 ± 59.5	154.9 ± 49.4	164.7 ± 12.0	162.3 ± 39.1	192.8 ± 41.1	206.7 ± 12.2	211.2 ± 24.8
Purple flowered								
Pacific Giant (n=3)	1	77.6 ± 35.1	62.3 ± 16.9	83.8 ± 35.6	41.1 ± 21.4	37.8 ± 19.1	27.4 ± 14.0	24.6 ± 10.0
	2	3339.7 ± 726.3	4683.0 ± 1157.3	5282.3 ± 1097.1	5318.5 ± 877.8	6065.7 ± 740.0	6071.9 ± 1423.2	6652.8 ± 1168.2
Light purple flowered								
Pacific Giant (n=3)	1	7.3 ± 1.1	5.3 ± 0.4	3.9 ± 3.7	2.9 ± 2.9	0.8 ± 1.3	n.d.	n.d.
	2	219.4 ± 98.2	273.8 ± 183.8	221.6 ± 62.2	216.7 ± 115.1	195.6 ± 99.5	193.1 ± 116.9	203.3 ± 106.2

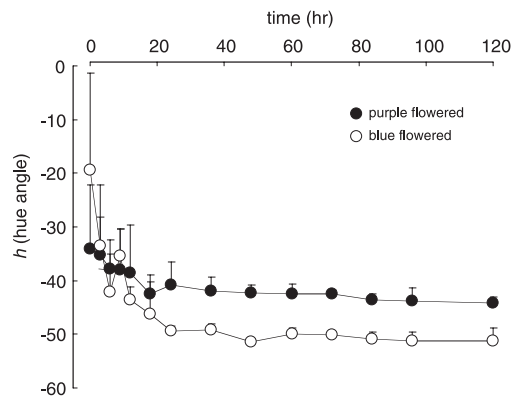
<sup>a</sup>A wing sepal was collected after flower opening (mean ±SD, nmol/g of fresh calyces). N.d., not detected.

<sup>b</sup>Pigments are summarized in Fig. 1.

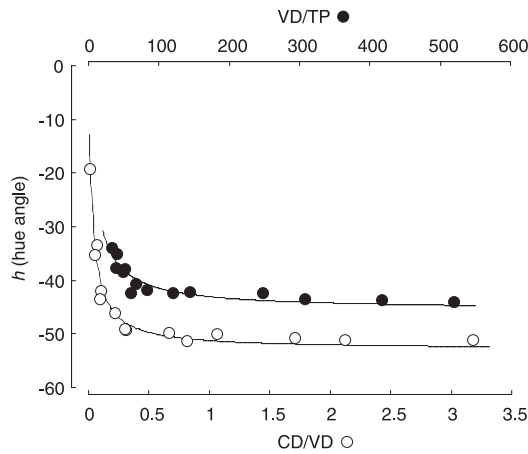
$$\frac{1}{H} = \frac{1}{H_{\max}} + \frac{K_H}{H_{\max}} \cdot \frac{1}{[\text{CD/VD}]} \quad (\text{a})$$

$$H = H_{\max} \frac{[\text{CD/VD}]}{[\text{CD/VD}] + K_H} = \text{tang}^{-1}(b^*/a^*) \quad (\text{b})$$

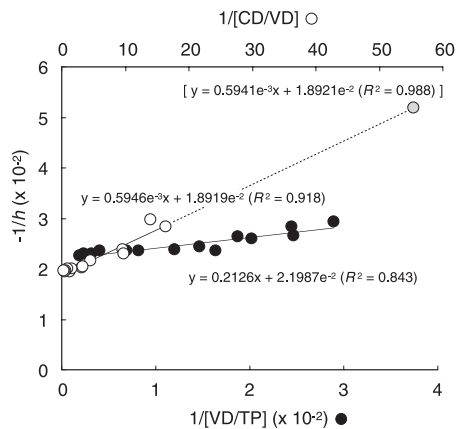
The maximal hue angles of the blue flowered ‘Blue Mirror’ and the ‘Pacific Giant’ were below ca. -53°, but the highest value, -73°, was found in the light blue flowered cv. ‘Pacific Giant’ (Table 3). The molar ratio constant was similar in the colored flowers.  $K_H$  values in the blue flowered cvs ‘Blue Mirror’ and in ‘Pacific Giant’ were 0.02 and 0.06, respectively, whereas they were 6.60 and 7.23 in the purple and the light purple flowered cv. ‘Pacific Giant’, respectively.



**Figure 3.** Time course of the change in hue angle,  $h$ , after anthesis in the sepals of purple flowered ‘Pacific Giant’ (closed circle) and blue flowered ‘Blue Mirror’ (open circle) (mean  $\pm$  SE).



**Figure 4.** A plot of hue angle,  $h$ , against the change in the ratio of [VD (2)/TP (1)] and [CD (3)/VD (2)], in the sepals of purple flowered ‘Pacific Giant’ and blue flowered ‘Blue Mirror’, respectively.



**Figure 5.** A plot of  $-1/h$  against  $1/[VD (2)/TP (1)]$  or  $1/[CD (3)/VD (2)]$  is presented for the purple flowered ‘Pacific Giant’ and the blue flowered ‘Blue Mirror’. The two variables yield a monomial regressive straight line with a slope of  $K_H/H_{max}$ , an intercept of  $1/H_{max}$  on the vertical axis and an intercept of  $-1/K_H$  on the horizontal axis. Note that the one plot of the cv ‘Blue Mirror’ at day of flower opening was rejected due to the possible irregular variation.

**Table 3. The maximal hue angle,  $H_{\max}$  and molar ratio constant,  $K_H$ .<sup>a</sup>**

Cultivar	Flower color	Pigments <sup>b</sup>	$H_{\max}$ (°)	$K_H$
Blue Mirror	blue	CD / VD	-53.0 ± 2.1	0.02 ± 0.01
Pacific Giant	blue	CD / VD	-51.9 ± 1.4	0.06 ± 0.02
	light blue	CD / VD	-72.8 ± 5.9	0.37 ± 0.27
	purple	VD / TP	-45.3 ± 1.3	6.60 ± 8.97
	light purple	VD / TP	-55.3 ± 5.0	7.23 ± 4.70

<sup>a</sup>Values are presented as mean ± SD in triplicate.

<sup>b</sup>Major anthocyanins during flowering: TP, tulipanin (1); VD, viodelphin (2); and CD, cyanodelphin (3). Structures are summarized in Fig. 2.

**Table 4. Sepal pH of *Delphinium* flowers after flower opening.**

Cv (individual)	Hours after anthesis <sup>a</sup>														Total <sup>b</sup>
	Opening	3	6	9	12	18	24	36	48	60	72	84	96	120	
B. M	5.22	5.30	5.31	5.24	n.d. <sup>c</sup>	5.31	5.29	5.22	5.23	5.20	5.35	5.18	5.30	5.29	5.26 ± 0.05
P. G blue	5.14	5.10	5.03	5.19	5.22	5.22	5.24	5.27	5.26	5.21	5.28	5.21	5.28	5.33	5.21 ± 0.08
P. G lightblue	5.03	5.08	5.04	5.17	5.11	5.07	5.13	5.16	5.17	5.15	5.19	5.16	5.18	5.19	5.13 ± 0.06
P. G purple	5.20	5.15	5.23	5.08	5.18	5.17	5.36	5.21	5.28	5.35	5.38	5.46	5.46	5.43	5.28 ± 0.12
P. G lightpurple	5.25	5.31	5.24	5.26	5.32	5.25	5.24	5.35	5.27	5.26	5.25	5.35	5.36	5.38	5.29 ± 0.05

<sup>a</sup>The data were calculated by one-way analysis of variance (ANOVA) and no significant difference was recorded among hours after anthesis.

<sup>b</sup>The data were calculated by one-way analysis of variance (ANOVA) and a significant difference was found among the cultivars' pH ( $P < 0.001$ ).

<sup>c</sup>n.d., not detected.

### Sepal pH and $Al^{3+}$ concentration

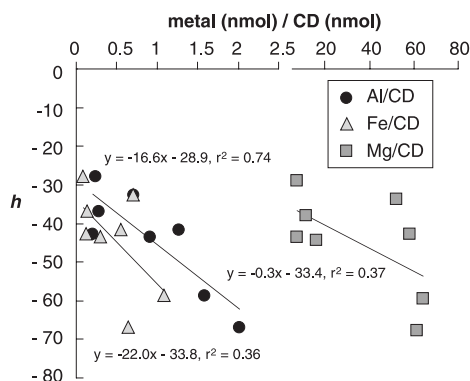
We determined the sepal pH (Table 4) and  $Al^{3+}$  concentration (data not shown) at every stage after anthesis. However, no changes were observed during flowering, although the sepal pH values of the individual cultivars were significantly different ( $P < 0.001$ ). It has been reported that the vacuolar pH of *Delphinium* flowers was ca. 5.00 [7]. Our measurement of pH was ca. 0.10–0.30 higher. It is therefore suggested that the difference in data is possibly due to the different equipment used for pH measurement [14]. We also determined the sepal  $Al^{3+}$  concentration for every day after anthesis, but no significant changes were observed during flowering (data not shown).

We preliminarily investigated the sepal concentrations of ions such as Fe, Mg as well as Al metals to find a relationship with the pigment 3 in the matured *Delphinium* flowers. As a matter of fact, a molar ratio between the  $Al^{3+}$  and cyanodelphin (3) gave a tendency to decrease the hue angle along with an increase of the ratio (Fig. 6).

## Discussion

Flower color can be specifically designated by using a colorimeter to define color as a numeric number on a continuous color diagram [16]. Because one fails to see actual flower color when one expresses color in colloquial names such as red, blue or yellow, a broad estimation of flower color has often resulted in misunderstandings of the minute details of floral coloration. It is a major scientific theme to breed for new flower colors by conventional methods, updated biotechnological techniques or genetic transformation, while the importance for color expression of copigmentation, vacuolar pH and metallo-complexes, as well as the synthesis of pigments, must be well understood in association with the three color attributes of flower color.





**Figure 6.** Distribution of floral hue angles changing sepal metal ions (nmol) and cyanodelphin (3) (nmol). The horizontal axis is expressed as the ratio between the individual sepal ion concentrations of metals and the concentration of cyanodelphin (3).

Floral coloration is generally described by three color attributes: chromaticity, lightness, and hue (hue angle,  $h$ ) [13]. Light reflection might also be considered in cases where the petal or sepal cells have different shapes within the flower [5]. We formerly demonstrated that chromaticity ( $C^*$ ) and lightness ( $L^*$ ) exhibited an inverse correlation in *Delphinium* flowers after 3-4 days of anthesis, and that chroma is logarithmically dependent on the accumulation of sepal anthocyanins, as shown in equations (c) and (d), respectively, where  $k_1$ - $k_4$  are constants [8]. In this study, these strong correlations are also derived from the altered floral coloration and pigmentation during anthesis (Fig. 7). Thus, chromaticity ( $C^*$ ), lightness ( $L^*$ ) and anthocyanin concentration are numerically well associated.

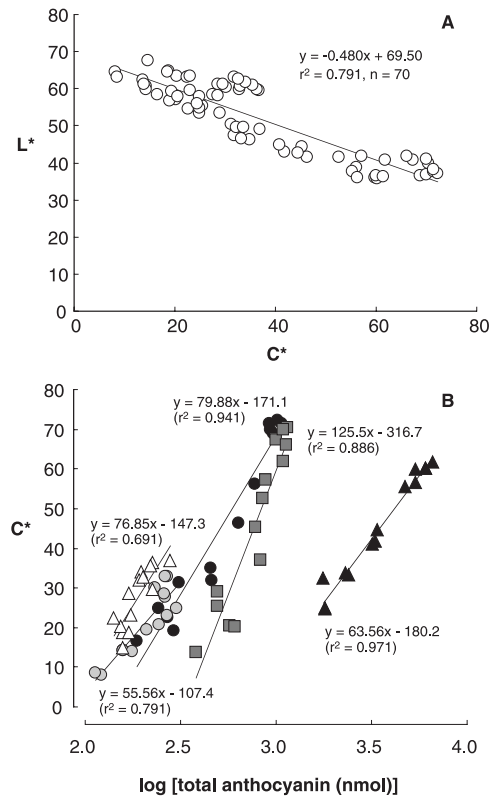
$$C^* = k_1 \log[\text{total anthocyanin}] + k_2 \quad (c)$$

$$L^* = k_3 C^* + k_4 \quad (d)$$

A rapid increase in violdelphin (2) and cyanodelphin (3) caused a change in the hue angle towards the negative end of the scale of a color wheel on the hue diagram (Fig. 1). As a result, the mix of acylated anthocyanin concentrations defined the flower hue (Fig. 4). Thus, the colors in *Delphinium* flowers are expressed by the constitution and accumulation of sepal anthocyanins, through which the direction of floral color ( $h$ ) and chromaticity ( $C^*$ ) are numerically controlled, as shown in equations (a) - (d) (Fig. 1).

The constant  $K_H$  represents the molar ratio at which the rate of change is half of its maximal value,  $H_{\max}$  (Table 3). We can find the maximal hue angle for each flower from the value  $H_{\max}$  since the value is thought to be important for understanding the final flower hue after anthesis. Moreover, it is possible that flowers will attain their final floral hue at the end of the enzymatic conversion of pigmentation.

We understand that  $K_H$  does not measure the rate of the enzymatic conversion of pigmentation, but is a constant representing the ratio between the two major pigments during anthesis. It has been suggested that similar values of  $K_H$  not only show similarities in the rate of change in coloration, but can also be used to classify colored flowers within *Delphinium* species as a phenotypic trait. The idea has been considered that as long as pigmentation changes with time, presumably as a result of enzymatic glycosylation and acylation, it results in changes in hue angle. However, the change in floral coloration can be explained kinetically based upon the change in inherent pigmentation due to the high statistical correlation coefficients ( $r^2 = 0.918$  and  $0.843$ , Fig. 5). When this is considered, the pigmentation is then one of the major factors for coloration.



**Figure 7.** Distribution of *Delphinium* flowers changing sepal coloration and total anthocyanins (log nmol). A The relationship between  $C^*$  and  $L^*$ , open circle; all five cultivars. B The relationship between total anthocyanin (log nmol) and  $C^*$ ; shaded rectangle, 'Blue Mirror'; closed circle; blue flowered 'Pacific Giant'; shaded circle; light blue flowered 'Pacific Giant'; closed triangle; purple flowered 'Pacific Giant'; and open triangle, light purple flowered 'Pacific Giant'.

Because the content of anthocyanins, specifically of cv 'Blue Mirror', are not stable at day of flower opening (Table 2), it is suggested that a rapid change from violdelphin (2) to cyanodelphin (3) occurred between the opening day and 3 hr after flower opening. The data could have a great influence on calculation for the reciprocal straight line of cv 'Blue Mirror' (Fig. 5) since it was considered that the lack of data of 3 hr interval within this 3 hr period might give the wrong calculation statistically. Under this consideration, the plots of the cv 'Blue Mirror' data were recalculated rejecting the one plot of the opening day, given in almost the similar equation with or without the data ( $r^2 = 0.988$  or  $0.917$ , respectively) (Fig. 5). This infers that the lacking data between the opening day and 3 hr after flower opening could possibly follow this dashed straight line, thus it suggests that this determination is also applicable for this specific calculation for cv 'Blue Mirror'.

We agree that the mechanism responsible for the color emission pattern is the formation of an insoluble chelate as a chromophore to express purplish and/or bluish colors in association with violdelphin (2) and/or cyanodelphin (3) [6, 7]. In 2003, Yoshida's group further updated the findings indicating that the mechanism of blue light emission was not based upon the formation of a chelate with several heavy metal ions, such as Mg, Al and Fe, in the cytosol when the blue sepal protoplast was cultured [17]. The variation in cyanic coloration of delphiniums results from the admixture of non-acylated

and/or acylated anthocyanins under conditions of unchanged vacuolar pH and Al<sup>3+</sup>.

We investigated the fact preliminarily and we obtained data that the hue angle decreased along with the increase of the ratio between Al<sup>3+</sup> and cyanodelphin (3) (Fig. 6). The result will likely be controversial because the ratios of the blue flowers at earlier stages after the day of flower opening are relatively high enough to become bluish color. However, the blue flowers contained an extremely large amount of violodelphin (2) when compared with that of cyanodelphin (3) at earlier stages. From this point of view, the ratio between major pigments such as 2 and 3 contribute largely to the expression of bluish flower coloration, with the co-factor of the formation of metal-complex between Al<sup>3+</sup> and cyanodelphin (3).

Finally, these results are mathematical determinations that identify for the first time the relationship between anthocyanins and intact coloration. In conclusion, the primary determinant for color in cyanic *Delphinium* flowers is the type and mixture of acylated anthocyanins. Colors can be specified by enumeration on the plot, and the sepal anthocyanins immediately designate the direction of flower color (hue).

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### References

- [1] Goto, T. and Kondo, T.: Structure and molecular stacking of anthocyanins - flower color variation. *Angew. Chem. Int. Ed. Engl.*, 30, 17-33 (1991)
- [2] Figueiredo, P., George, F., Tatsuzawa, F., Toki, K., Saito N. and Brouillard. R.: New features of intramolecular copigmentation by acylated anthocyanins. *Phytochemistry*, 51, 125-132 (1999)
- [3] Dangles, O., Saito, N. and Brouillard. R.: Kinetic and thermodynamic control of flavylium hydration in the pelargonidin-cinnamic acid complexation. Origin of the extraordinary flower color diversity of *Pharbitis nil*. *J. Am. Chem. Soc.*, 115, 3125-3132 (1993)
- [4] Tamura, H., Kondo, T. and Goto. T.: The composition of commelinin, a highly associated metalloanthocyanin present in the blue flower petals of *Commelina communis*. *Tetrahedron Lett.*, 27, 1801-1804 (1986)
- [5] Noda, K., Glover, B. J., Linstead, P. and Martin. C.: Flower colour intensity depends on specialized cell shape controlled by a *Myb*-related transcription factor. *Nature*, 369, 661-664 (1994)
- [6] Yoshida, K., Terakura, K., Kawano, Y., Toyama, Y., Kameda, K. and Kondo. T.: Blue flower color development of *Delphinium hybridum*. *Plant Cell Physiol.*, Supplement 43, s226, 727 (3aJ08) (2002)
- [7] Yoshida, K., Kawano, Y., Kameda, K., Osanai, M. and Kondo. T.: Vacuolar pH measurement by microelectrode and flower color development. *Plant Cell Physiol.*, Supplement 42, s207, 686 (F801) (2001)
- [8] Hashimoto, F., Tanaka, M., Maeda, H., Shimizu, K. and Sakata. Y.: Characterization of cyanic flower color of *Delphinium* cultivars. *J. Jpn. Soc. Hortic. Sci.*, 69, 428-434 (2000)
- [9] Hashimoto, F., Tanaka, M., Maeda, H., Fukuda, S., Shimizu, K. and Sakata. Y.: Changes of

- flower coloration and sepal anthocyanins in cyanic *Delphinium* cultivars during flowering. *Biosci. Biotech. Biochem.*, 66, 1652-1659 (2002)
- [10] Kondo, T., Oki, K., Yoshida, K. and Goto. T.: Structure of violdelphin, an anthocyanin from violet flower of *Delphinium hybridum*. *Chem. Lett.*, 137-138 (1990)
- [11] Kondo, T., Suzuki, K., Yoshida, K., Oki, K., Ueda, M., Isobe, M. and Goto. T.: Structure of cyanodelphin, a tetra-*p*-hydroxybenzoated anthocyanin from blue flower of *Delphinium hybridum*. *Tetrahedron Lett.*, 44, 6375-6378 (1991)
- [12] CIE.: Recommendations on uniform color spaces, color difference evaluations and psychometric color terms. p. 1-74. In: CIE Central Bureau (ed) *Colorimetry* (2nd ed). Publication No 15.2. Commission Internationale de l'Éclairage Central Bureau, Vienna, Austria (1986)
- [13] Gonnet, J. F.: Colour effects of co-pigmentation of anthocyanins revisited-1. A colorimetric definition using the CIELAB scale. *Food Chem.*, 63, 409-415 (1998)
- [14] Fukui, Y., Kusumi, T., Yoshida, K., Kondo, T., Matsuda, C. and Nomoto. K.: Structures of two diacylated anthocyanins from *Petunia hybrida* cv. Surfinia Violet Mini. *Phytochemistry*, 47, 1409-1416 (1998)
- [15] Shibata, M.: Über das Anthocyanin in den dunkelpurpuren Blüten von *Tulipa gesneriana* L. *Bot. Mag. (Tokyo)*, 69, 462-468 (1956)
- [16] Gonnet, J. F.: Colour effects of co-pigmentation of anthocyanins revisited-2. A colorimetric look at the solutions of cyanin co-pigmented by rutin using the CIELAB scale. *Food Chem.*, 66, 387-394 (1999)
- [17] Yoshida, K., Shinkai, Y., Mano, S., Nishimura, M. and Kondo. T.: Flower color expression of cyanic *Delphinium* petals. Annual Meeting of the Japanese Society for Bioscience, Biotechnology, and Agrochemistry. Abstract, p263 (2003) (In Japanese)

## デルフィニウムの花（新鮮がく片）のアントシアニン色素と花色の数理解析法

橋本文雄<sup>†</sup>・福田祥子・前田寛子・田中見佳・  
清水圭一・樗木直也・坂田祐介・石黒悦爾

### 要 約

デルフィニウムの新鮮がく片の主要アントシアニンであるピオルデルフィンとシアノデルフィンなどを開花中に定量し、花色との関係を数理的に解析した。即ち、青色花ではピオルデルフィンからシアノデルフィンへの変換と花色変化を、紫色花ではチューリパニンからピオルデルフィンへの変換と花色変化を同時にモニターした。その結果、それぞれの色素量比の逆数と花色属性の一つである色相角 ( $b$ ) の逆数に非常に強い一次回帰の関係を見出し、その数式からモル比定数 ( $K_H$ ) と最大色相角 ( $H_{max}$ ) を得た。がく片の内生素と花色、特に色相角との関係を数理的に解析した初めての例であり、がく片の主要色素が青色と紫色の花色を決定する一次的要因であることを説明している。

キーワード：デルフィニウム, 花色, 色相角, キンボウゲ科

<sup>†</sup>：連絡責任者：橋本文雄（生物生産学科観賞園芸学研究室）

Tel 099-285-8559, E-mail: omihshf@agri.kagoshima-u.ac.jp