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Acylated peonidin glycosides from *duskish* mutant flowers of *Ipomoea nil*

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Abstract

Five acylated peonidin glycosides were isolated from the pale gray-purple flowers of a *duskish* mutant in the Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*) as major pigments, along with a known anthocyanin, Heavenly Blue Anthocyanin (HBA). Three of these were based on peonidin 3-sophoroside and two on peonidin 3-sophoroside-5-glucoside as their deacylanthocyanins; both deacylanthocyanins were acylated with caffeic acid and/or glucosylcaffeic acids. By spectroscopic and chemical methods, the structures of the former three pigments were determined to be 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-β-D-glucopyranoside], 3-*O*-[2-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-glucopyranoside], and 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranoside] of peonidin. The structures of the latter two pigments were also confirmed as 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside, and 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside of peonidin. The mutation affecting glycosylation and acylation in anthocyanin biosynthesis of Japanese morning glory was discussed.

Keywords: *Ipomoea nil*; Convolvulaceae; Japanese morning glory; Flower coloration; Acylated anthocyanins; Peonidin 3-sophoroside; Peonidin 3-sophoroside-5-glucoside; Caffeic acid; Glucosylcaffeic acid

1. Introduction

The Japanese morning glory (*Ipomoea nil* or *Pharbitis nil*) has been domesticated as an ornamental plant in Japan and its various spontaneous mutations exhibit a wide variety of different flower pigmentation (Iida et al., 2004). Among them, four recessive mutations, *dusky*, *duskish-1*, *duskish-2* and *dingy* designated by Hagiwara (1954, 1956), confer dull-colored flowers in these spontaneous mutants.

In our continuing work on flower color variation due to the production and accumulation of anthocyanins in the dull flower color mutants of the Japanese morning glory, we have reported the occurrence of eight new anthocyanins in the flowers of mutants affecting the enzymatic activities of glycosylation and acylation in their anthocyanin biosynthesis pathways (Saito et al., 1994, 1996; Toki et al., 2001, 2004). Particularly, in the *dusky* mutants, anthocyanin 3-glucosides and 3,5-

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diglucosides were found as the main deacylanthocyanins (Saito et al., 1994, 1996). Only pelargonidin 3-sophoroside was found as a major deacylanthocyanin pigment in the flowers of the *duskish-1* and *-2* mutants characterized previously (Toki et al., 2001, 2004), whereas the deacylanthocyanins detected in the *dingy* mutants were similar to those of wild-type, or to the normal types of the plant whose deacylanthocyanins are 3-sophoroside-5-glucosides (Toki et al., 2001; Lu et al., 1992a,b). However, the acylation with caffeic acid was incomplete in the *dingy* mutants compared with wild-type: anthocyanins were acylated with only one molecule of caffeic acid.

In this paper, we wish to report the structure elucidation of five new acylated peonidin glycosides isolated from the pale gray-purple flowers of a *duskish* mutant, whose deacylanthocyanins are mixture of peonidin 3-sophoroside and 3-sophoroside-5-glucoside.

2. Results and discussion

The flowers of a *duskish* mutant exhibiting gray-violet coloration at the freshly open flowers were immersed in 5% AcOH for 24 h at room temperature, and 17 anthocyanin peaks were observed by HPLC analysis. Their relative frequency of occurrence was pigment **1** (2.8%, retention time (RT: min) 12.4), **2** (1.1%, RT 14.8), **3** (1.5%, RT 15.9), **4** (1.7%, RT 16.2), **5** (9.8%, RT 17.7), **6** (2.3%, RT 18.7), **7** (6.6%, RT 20.9), **8** (1.1%, RT 23.3), **9** (1.1%, RT 25.1), **10** (2.6%, RT 26.2), **11** (1.2%, RT 26.8), **12** (4.9%, RT 27.2), **13** (19.8%, RT 27.8), **14** (22.0%, RT 28.7), **15** (6.0%, RT 20.9), **16** (7.4%, RT 30.7), and **17** (6.4%, RT 31.7). From the anthocyanin extract, pigments **5**, **7**, **13**, **14**, **15**, and **17**, were obtained by using the process described previously (Saito et al., 1994, 1996; Toki et al., 2001, 2004).

Upon acid hydrolysis, all six pigments (**5**, **7**, **13**, **14**, **15**, and **17**) gave peonidin, glucose, and caffeic acid. Upon alkaline hydrolysis, pigments (**5**, **13**, **14**) resulted in peonidin 3-sophoroside-5-glucoside, and pigments (**7**, **15**, **17**) yielded peonidin 3-sophoroside as their deacylanthocyanins. Both structures of deacylanthocyanins were confirmed by analysis of TLC and HPLC with authentic samples of peonidin 3-sophoroside and 3-sophoroside-5-glucoside, which were obtained previously (Lu et al., 1992b). Pigment **13** was identified based on the analysis of its NMR spectra, to be a known anthocyanin, HBA (Lu et al., 1992b).

The other five pigments (**5**, **7**, **14**, **15**, and **17**) were new acylated anthocyanins and their TLC, HPLC, and spectroscopic properties are shown in Table 1. The structures of the five anthocyanins were elucidated as follows.

2.1. Pigments 5

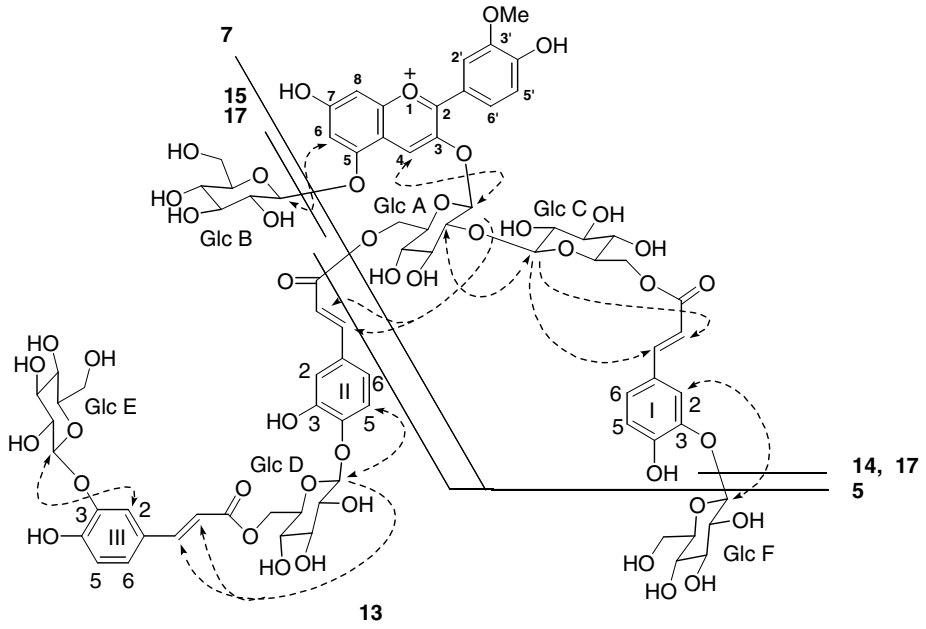
The FAB mass spectrum of pigment **5** gave its molecular ion $[M]^+$ at *m/z* 949 in good agreement with the mass calculated for $C_{43}H_{49}O_{24}$, which was composed of peonidin with three molecules of glucose and one molecule of caffeic acid. The elemental components of pigment **5** were further confirmed by high resolution FAB MS [Calc. for $C_{43}H_{49}O_{24}$ requires 949.2614. Found 949.2596]. Structural analysis of pigment **5**, involves determination of the ^1H and ^{13}C NMR spectra in CF_3COOD-DMSO-d_6 (1:9), whose proton and carbon chemical shifts were assigned as shown in Table 2 and Section 3.5. Nine aromatic proton signals of peonidin and caffeic acid moieties, and the proton signals corresponding to the methoxy group of peonidin were readily assigned (Table 2). Two olefinic protons of caffeic acid had large coupling constants ($J = 15.9$ Hz) indicating that it was in the *trans* configuration. The proton chemical shifts of the three glucose units (Glc A, Glc B, and Glc C) of this pigment were observed between δ 5.56–3.12, with the anomeric proton signals assigned at δ 5.56 (*d*, $J = 7.3$ Hz, Glc A), δ 5.16 (*d*, $J = 7.6$ Hz, Glc B), and δ 4.84 (*d*, $J = 7.9$ Hz, Glc C), respectively. These three glucose proton signals assigned had coupling constants in the range of 4.0–11.0 Hz, suggesting that the glucose units were in the β-glucopyranose form (Fig. 1).

Table 1
Chromatographic and spectroscopic data for *duskish-m* mutant anthocyanins *ipomoea nil*

Anthocyanins[b]	R_f values (×100)[a]				HPLC (Rt) (min)	Spectral data in 0.1% HCl–MeOH			FAB-MS $[M]^+$
	BAW	BuH	1% HCl	HOAc–HCl		λ_{max} (nm)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)	
Pigment **5**	54	26	37	63	17.7	281, 294, 329, 524	78	15	949
Pigment **7**	61	48	22	49	20.9	286, 324, 526	129	26	787
Pigment **13**	34	07	15	23	26.8	294, 316, 533	155	14	1759
Pigment **14**	45	18	10	28	28.1	293, 318, 533	140	14	1597
Pigment **15**	44	15	08	24	28.6	290, 316, 532	197	24	1597
Pigment **17**	57	36	04	19	31.8	290, 318, 533	193	25	1435

[a,b] For key to solvent mixtures, see Section 3.

Pigment **5**: peonidin3-[2-(caffeoylglucosyl)-glucoside]-5-glucoside. Pigment **7**: peonidin3-[2-(caffeoylglucosyl)-glucoside]. Pigment **13**: Heavenly Blue Anthocyanin, peonidin 3-[2-(glucosylcaffeoylglucosyl)-6-(glucosylcaffeoylglucosylcaffeoyl)-glucoside]-5-glucoside. Pigment **14**: peonidin3-[2-(caffeoylglucosyl)-6-(glucosylcaffeoylglucosylcaffeoyl)-glucoside]-5-glucoside. Pigment **15**: peonidin3-[2-(glucosylcaffeoylglucosyl)-6-(glucosylcaffeoylglucosylcaffeoyl)- glucoside]. Pigment **17**: peonidin3-[2-(caffeoylglucosyl)-6-(glucosylcaffeoylglucosylcaffeoyl)-glucoside].



13: Heavenly Blue Anthocyanin

Fig. 1. *Duskish-m* mutant anthocyanins of *Ipomoea nil*. Observed major NOE's are indicated by dashed arrows. Anthocyanin contents by HPLC analysis: **5**, **7**, **13**, **15**, **17** = 70.6% (total main anthocyanins); **7**, **15**, **17** = 34.6% (acylated peonidin 3-glucoside); **5**, **14**, **17** = 38.2% (anthocyanina are glucose free at 3-OH of caffeic acid); **5**, **7** = 16.4% (anthocyanin deacylated at 6″-OH of 3-glucose residue); **13**, **14**, **15**, **17** = 54.2% (di- or triacylated anthocyanins).

The linkages among peonidin, caffeic acid, and glucose units were elucidated from a series of NMR experiments including the analyses of 2D COSY, DIFNOE and HMBC spectra. The signals of H-1 (δ 5.56) of Glc A, H-1 (δ 5.16) of Glc B, and H-1 (δ 4.84) of Glc C could be correlated to H-4 (δ 9.03) of peonidin, H-6 (δ 7.00) of peonidin, and H-2 (δ 3.92) of Glc A, respectively, by the DIFNOE experiments, indicating that OH-3 and OH-5 of peonidin were bonded to Glc A and Glc B, respectively, and O-2 of Glc A was bonded to Glc C. Moreover, the down-field shifts of the signals for H-2 (δ 3.92) and C-2 (δ 81.1) of Glc A supported presence of a sophorose unit with Glc C. Similarly, the down-field shifts of the methylene protons and the carbon of Glc C were observed at δ 4.11, 4.04 and δ 63.2, indicating that the O-6 of Glc C is acylated with caffeic acid. From long-range correlations in HMBC spectrum, these linkages were also confirmed. Therefore, pigment **5** is peonidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside (Fig. 1), a new natural product. This structure was further confirmed by the analysis of the ^{13}C NMR (see Section 3.5), HMQC and HMBC spectra.

2.2. Pigment 7

The FAB mass spectrum of pigment **7** gave a molecular ion $[M]^+$ at *m*/*z* 787, in good agreement with the mass calculated for $C_{37}H_{39}O_{19}$, which was one molecule of glucose less than that of pigment **5**. Thus, the composition of pigment **7** was considered to be a peonidin with two molecules of glucose and one molecule of caffeic acid. The elemental components of **7** were further confirmed by the high resolution FAB MS [Calc. for $C_{37}H_{39}O_{19}$ requires 787.2086. Found 787.2097]. The ^{1}H NMR spectrum of pigment **7** was similar to that of pigment **5** except for the signals of the 5-glucose moiety as shown in Table 2. The chemical shifts of the protons and carbons were assigned by the same procedure as described for the structural determination of pigment **5** (Table 2 and Section 3.5). The linkages between peonidin and Glc A, and Glc A and Glc C, were determined by DIFNOE experiments. Since NOEs were observed between the H-4 of peonidin and H-1 of Glc A in the DIFNOE spectra, the peonidin was glycosylated with Glc A at OH-3 of peonidin. NOEs were also observed between H-2 of Glc A and H-1 of Glc C, indicating Glc C bonded with Glc A at O-2 of Glc A. Since the chemical shifts of the methylene protons of Glc C were shifted to a lower magnetic field at δ 4.10 and 3.91, Glc C was acylated with caffeic acid at O-6 of Glc C. Thus, pigment **7** was peonidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-β-D-glucopyranoside] (Fig. 1), which is also a new anthocyanin. This structure was unambiguously confirmed by the analysis of ^{13}C NMR (see Section 3.5), HMQC and HMBC spectra.

2.3. Pigment **14**

The FAB mass spectrum of pigment **14** gave a molecular ion $[M]^+$ at *m/z* 1597, in good agreement with the mass calculated for $C_{73}H_{81}O_{40}$, which was composed of peonidin with five molecules of glucose and three molecules of caffeic acid. The elemental components of this pigment were further confirmed by measurement of the high resolution FAB MS (see Section 3.6). In order to elucidate the structure, its ^{1}H NMR spectra including 2D COSY, NOESY and DIFNOE spectra were measured in CF_3COOD-DMSO-d_6 (1:9). The ^{1}H NMR spectrum of pigment **14** could be superimposed on that of HBA (pigment **13**, see Fig. 1) except for the signals of the Glc F moiety (Lu et al., 1992b; Kondo et al., 1987). The chemical shifts of the 15 aromatic protons including three caffeic acids (I–III) and the methoxy protons on peonidin were assigned and are shown in Table 2. Three pairs of olefinic proton signals in three caffeic acid moieties have large coupling constants (J = 15.9 Hz), indicating those caffeic acids to be *trans* configuration. Five anomeric proton resonances appeared at δ 5.64 (*d*, J = 7.0 Hz, Glc A), δ 5.12 (*d*, J = 7.7 Hz, Glc B), δ 4.78 (*d*, J = 7.7 Hz, Glc C), δ 4.91 (*d*, J = 7.4 Hz, Glc D), and δ 4.81 (*d*, J = 7.0 Hz, Glc E). Since all of the observed vicinal coupling constants of the five glucose units were 7.0–12.0 Hz, these five glucose units were considered to be in the β-glucopyranose form. The six methylene proton signals shifted to a lower magnetic field were assigned at δ 4.28 and 4.43 (Glc A), δ 4.02 and 4.09 (Glc C), and δ 4.32 and 4.48 (Glc D) by the analysis of 2D COSY and NOESY spectra, indicating these three glucose units were acylated with caffeic acids (I–III) at their O-6 groups, respectively. Upon measurement of the DIFNOE spectra by irradiation at H-1 of Glc A, Glc B, Glc D and Glc E, strong NOEs were observed between the H-4 of peonidin and the H-1 of Glc A, H-6 of peonidin and H-1 of Glc B, H-3 of caffeic acid (II) and H-1 of Glc D, and H-2 of caffeic acid (III) and H-1 of Glc E, respectively. Therefore, peonidin is glycosylated with Glc A at O-3 and Glc B at O-5, caffeic acid (II) is glycosylated with Glc D at O-4, and also caffeic acid (III) is glycosylated with Glc E at O-3. From analysis of the 2D COSY and DIFNOE spectra,Glc A is confirmed to be glycosylated with Glc C at O-2 forming a sophorose unit. Moreover, on the DIFNOE experiments, rather weak NOEs were observed between H-1 of Glc C and H-α or -β of caffeic acid (I), between H-1 of Glc A and H-α and/or -β of caffeic acid (II), and between H-1 of Glc D and H-α and/or -β of caffeic acid (III). Therefore, pigment **14** is peonidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside (Fig. 1), a new anthocyanin in plants.

2.4. Pigment **15**

The FAB mass spectrum of pigment **15** gave same molecular ion $[M]^+$ at *m/z* 1597 as that of pigment **14** (calculated for $C_{73}H_{81}O_{40}$; 1597.43), which was composed of peonidin with five molecules of glucose and three molecules of caffeic acid. The elemental components of this pigment were confirmed by measurement of the high resolution FAB MS (see Section 3.6). From the UV and VIS spectral data (E_{440}/vis = 24%, Table 1), the structure of pigment **15** is presumed to be an acylated peonidin 3-sophoroside and is free from the glycosyl group at OH-5 of peonidin. The ^{1}H NMR spectrum of this pigment was very similar to that of HBA except for the signals of the Glc B (5-glucoside) moiety. The structure of pigment **15** was elucidated based on the same process as described for pigment **14**. The chemical shifts of its protons are shown in Table 2. The signals of five anomeric protons appeared at δ 5.56 (*d*, J = 7.0 Hz, Glc A), δ 4.75 (*d*, J = 7.0 Hz, Glc C), δ 4.92 (*d*, J = 6.7 Hz, Glc D), δ 4.86 (*d*, J = 7.0 Hz, Glc E), and δ 4.86 (*d*, J = 7.0 Hz, Glc F), and all of the observed vicinal coupling constants of the five glucose units are in the β-glucopyranose form. The six methylene proton signals shifted to a lower magnetic field were assigned at δ 4.19 and 4.46 (Glc A), δ 3.89 and 4.06 (Glc C), and δ 4.29 and 4.51 (Glc D) following analysis of the 2D COSY and NOESY spectra; hence Glc A, C, and D were confirmed to be acylated with caffeic acids at their O-6 groups. Upon the DIFNOE experiments by irradiation of each anomeric proton of Glc A, D, E, and F, strong NOEs between H-4 of peonidin and H-1 of Glc A, H-5 of caffeic acid (II) and H-1 of Glc D, H-2 of caffeic acid (III) and H-1 of Glc E, and H-2 of caffeic acid (I) and H-1 of Glc F, were observed, indicating that peonidin was glycosylated with Glc A at O-3, caffeic acid (II) was glycosylated with Glc D at O-4, caffeic acid (III) was glycosylated with Glc E at O-3, and caffeic acid (I) was glycosylated with Glc F at O-3, respectively. Furthermore, from the DIFNOE experiments, the acylation linkages between O-6 of Glc A and caffeic acid (II), O-6 of Glc C and caffeic acid (I), and O-6 of Glc D and caffeic acid (III) were confirmed by the observation of their NOEs. Consequently, pigment **15** was determined to be peonidin 3-*O*-[2-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-glucopyranoside] (Fig. 1), a new anthocyanin in plants.

2.5. Pigment **17**

The FAB mass spectrum of pigment **17** gave a molecular ion $[M]^+$ at *m/z* 1435 corresponding to the mass calculated for $C_{67}H_{71}O_{35}$; 1435.38, which was composed of peonidin with four molecules of glucose and three mol-

Table 2
NMR spectroscopic data of *duskish-m* mutant anthocyanins of *Ipomoeanu* (500 MHz in DMSO-d_6-CF_3CO_2D, TMS as an internal standard)

	Pigment **5**	Pigment **7**	Pigment **14**	Pigment **15**	Pigment **17**
Peonidin					
4	9.03 *s*	9.03 *s*	8.95 *s*	8.94 *s*	8.94 *s*
6	7.00 *d* (1.8)	6.71 *d* (1.8)	6.98 *d* (1.6)	6.66 *d* (1.5)	6.66 *d* (2.1)
8	7.19 *d* (1.8)	6.91 *d* (1.8)	7.06 *d* (1.6)	6.80 *d br s*	6.77 *d* (2.1)
2′	8.03 *d* (2.1)	8.08 *d* (2.1)	7.98 *d* (2.2)	7.94 *d* (1.8)	7.94 *d* (2.2)
5′	7.15 *d* (8.5)	7.09 *d* (8.1)	7.09 *d* (8.7)	7.05 *d* (8.9)	7.04 *d* (8.6)
6′	8.45 *dd* (2.1, 8.5)	8.26 *dd* (2.1, 8.1)	8.40 *dd* (2.2, 8.7)	8.29 *dd* (1.8, 8.9)	8.25 *dd* (2.2, 8.6)
OMe	3.95 *s*	3.94 *s*	3.90 *s*	3.89 *s*	3.88 *s*
Caffeic acid (I)					
2	6.92 *d* (2.1)	6.89 *d brs*	6.87 *brs*	7.30 *d* (1.0)	6.32 *d* (1.6)
5	6.77 *d* (8.2)	6.77 *m*	6.75 *m*	6.78 *d* (8.6)	6.69 *m*
6	6.82 *dd* (2.1, 8.2)	6.77 *m*	6.75 *m*	6.91 *brd* (7.4)	6.69 *m*
α	5.96 *d* (15.9)	5.83 *d* (15.9)	5.87 *d* (15.9)	5.98 *d* (16.2)	5.79 *d* (15.9)
β	7.23 *d* (15.9)	7.15 *d* (15.9)	7.16 *d* (15.9)	7.33 *d* (16.2)	7.10 *d* (15.9)
Caffeic acid (II)					
2			7.07 *d* (1.5)	7.04 *brs*	7.04 *d* (1.8)
5			7.09 *m*	7.08 *d* (8.6)	7.08 *d* (8.2)
6			6.89 *brd* (8.3)	6.86 *brd* (8.6)	6.86 *brd* (8.3)
α			6.22 *d* (15.9)	6.16 *d* (16.2)	6.15 *d* (15.9)
β			7.33 *d* (15.9)	7.33 *d* (16.2)	7.33 *d* (15.9)
Caffeic acid (III)					
2			7.50 *brs*	7.49 *d* (1.2)	7.49 *d* (1.6)
5			6.81 *d* (8.3)	6.76 *d* (8.6)	6.77 *d* (8.2)
6			7.15 *dd* (1.6, 8.3)	7.11 *brd* (8.0)	7.10 *brd* (8.6)
α			6.44 *d* (15.9)	6.43 *d* (16.2)	6.43 *d* (15.9)
β			7.53 *d* (15.9)	7.52 *d* (16.2)	7.52 *d* (15.9)
Glucose A					
1	5.56 *d* (7.3)	5.51 *d* (7.3)	5.64 *d* (7.0)	5.56 *d* (7.0)	5.55 *d* (7.3)
2	3.92 *t* (8.6)	3.82 *t* (8.5)	3.96 1 (8.0)	3.89 *m*	3.88 *m*
3	3.69 *t* (8.9)	3.67 *t* (8.9)	3.76 *m*	3.71 *m*	3.71 *m*
4	3.56 − 3.51	3.34 *t* (9.2)	3.50 *m*	3.48 *m*	3.44 *m*
5		3.47 *m*	3.90 *m*	3.85 *m*	3.82 *m*
6a	3.60 *m*	3.50 *dd* (6.4, 11.0)	4.28 *m*	4.19 *m*	4.20 *dd* (7.3, 11.6)
6b	74 *m*	3.70 *d* (11.0)	4.43 *d* (11.0)	4.46 *d* (10.4)	4.45 *d* (1 1.6)
Glucose B					
1	5.16 *d* (7.6)		5.12 *d* (7.7)		
2	3.52 *m*		3.58 *t* (8.5)		
3	3.43 *t* (8.9)		3.42 *m*		
4	3.53 *m*		3.28 *m*		
5	3.56 − 3.49		3.50 *m*		
6a	3.57 *m*		3.60 − 3.50		
6b	3.79 *d* (10.1)		3.81 *d* (11.5)		
Glucose C					
1	4.84 *d* (7.9)	4.76 *d* (7.6)	4.78 *d* (7.7)	4.75 *d* (7.0)	4.75 *d* (7.6)
2	3.32 *t* (9.4)	3.14 *t* (9.2)	3.20 *m*	3.17 *m*	3.16 *m*
3	3.12 *t* (7.9)	3.27 *t* (8.9)	3.50 − 3.25	3.50 − 3.20	3.40 − 3.20
4	3.27 *m*	3.21 *t* (9.2)			
5	3.27 *m*	3.50 *dd* (6.4, 9.2)	3.27 *m*	3.31 *m*	3.35 *m*
6a	4.04 *dd* (4.0, 11.0)	3.96 *dd* (6.4, 10.4)	4.02 *m*	3.89 *m*	4.02 *dd* (6.1, 11.6)
6b	4.11 *d* (11.0)	4.10 *d* (10.4)	4.09 *d* (12.0)	4.06 *m*	4.12 *d* (11.6)
Glucose D					
1			4.91 *d* (7.4)	4.92 *d* (6.7)	4.92 *d* (6.7)
2			3.42 *m*	3.40 *m*	3.42 *m*
3			3.45 − 3.41	3.50 − 3.30	3.50 − 3.30
4					
5			3.77 *m*	3.77 *m*	3.74 *m*
6a			4.32 *m*	4.29 *m*	4.28 *dd* (6.6, 11.9)
6b			4.48 *d* (10.4)	4.51 *d* (10.7)	4.52 *d* (1 1.9)

Table 2 (*continued*)

	Pigment **5**	Pigment **7**	Pigment **14**	Pigment **15**	Pigment **17**
Glucose E					
1			4.81 *d* (7.0)	4.86 *d* (7.0)	4.78 *d* (7. 3)
2			3.35 *m*	3.35 *m*	3.35 *m*
3			}	}	}
4			} 3.80 – 3.16	} 3.55 – 3.20	} 3.85 – 3.20
5			}	}	}
6a			}	}	}
6b					
Glucose F					
1				4.86 *d* (7.0)	
2				3.35 *m*	
3				}	
4				}	
5				} 3.55 – 3.20	
6a				}	
6b					

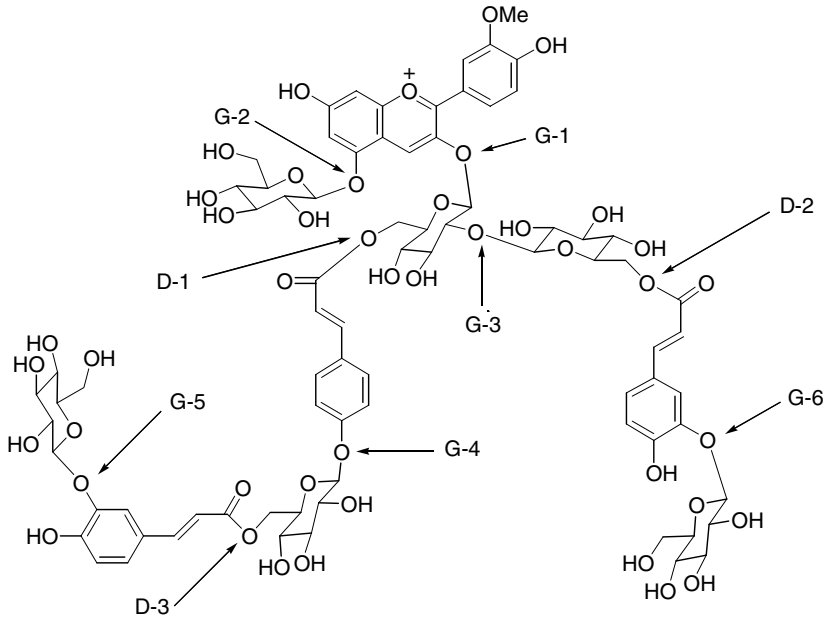
ecules of caffeic acid. The elemental components of this pigment were confirmed by measurement of the high resolution FAB MS (see Section 3.6). The structure elucidation was carried out in accordance with the process for pigment **14** by the measurement of ^{1}H NMR spectra including the 2D COSY, NOESY and DIFNOE spectra. The ^{1}H NMR spectrum of pigment **17** could be superimposed with that of pigment **14** without the resonances for the Glc B moiety. Therefore, its structure was presumed to be a triacylated peonidin 3-sophoroside. The proton chemical shifts of pigment **17** are shown in Table 2. Four molecules of glucose observed were confirmed to be in the β-glucopyranose form. Three caffeic acids were also confirmed to have a *trans* configuration as widened by the large coupling constants (J = 15.9, 15.9 and 15.9 Hz) of the olefinic proton signals. Six characteristic methylene protons shifted to a lower magnetic field were assigned at δ4.20 and 4.45 (H-6a and -6b of Glc A), δ 4.02 and 4.12 (H-6a and -6b of Glc C), and δ 4.28 and 4.52 (H-6a and -6b of Glc D) following analyses of 2D COSY and DIFNOE spectra. Upon measurement of the DIFNOE spectra of pigment **17**, the linkages of peonidin, glucose and caffeic acid were confirmed to be identical with those of pigment **14**. Therefore, pigment **17** was determined to be peonidin 3-*O*-[2-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-6-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-glucopyranoside], (Fig. 1), a new anthocyanin in plants.

2.6. Conclusions

As mentioned before, four recessive mutations, *dusky*, *dingy*, *duskish-1*, and *duskish-2*, designated by Hagiwara (1954), confer dull-colored flowers. Due to the genetic defects in the *Dusky*, *Duskish* and *Dingy* genes, these mutant flowers contained various acylated anthocyanins whose acylated side chains at the 3-position of anthocyanins were imperfectly synthesized apparently because they were only partially glycosylated and/or acylated (Fig. 2).

In the *dusky* mutants, UDP-glucose: anthocyanidin 3-*O*-glucoside-2-*O*″-glucosyltransferase encoded by the *Dusky* gene was deficient (Morita et al., 2005). Consequently, the process G-3 in Fig. 2 mediated by the enzyme did not take place, and the glycosylation at 5-OH of anthocyanidin (Fig. 2, G-2) was also significantly reduced probably because proper amounts of appropriate precursors were not produced. Therefore, anthocyanidin 3,5-diglucosides and 3-glucosides accumulated in the mutant flowers as deacylanthocyanins (Saito et al., 1994, 1996; Morita et al., 2005). In the *dingy* mutants, anthocyanidin 3-[2-(glucosyl)-6-(glucosylcaffeoyl)-glucoside]-5-glucosides accumulated as major pigments in these flowers (Lu et al., 1991; Toki et al., 2001) probably due to reduced activities of both acyltransferases (Fig. 2, D-2 and D-3) at two glucose 6-OH groups of the side chain residues in the anthocyanins. On the other hand, very pale flowers in the *duskish-1* and *-2* mutants were reported to contain only small amount of anthocyanins, and their main pigments were elucidated to be pelargonidin 3-sophoroside and pelargonidin 3-glucosylcaffeoylsophoroside (Toki et al., 2001, 2004). While the *Duskish* gene was thought to encode UDP-glucose: flavonoid 3-*O*-glucosyltransferase or UF3GT (Morita et al., 2001), which catalyzes the reaction G-1 in Fig. 2, the anthocyanin biosynthesis pathways promoted by UDP-glucose: anthocyanin 5-*O*-glucosyltransferase or UA5GT (Fig. 2, G-2) and by acyltransferase (Fig. 2, D-2) appeared to be heavily affected probably due to failure of formation of proper amounts of appropriate precursors in the *duskish* mutants.

In this study, we showed that pale gray-purple or pale dull-blue flowers in another *duskish* mutant contained small amount of anthocyanins, the structures of which were elucidated as shown in Fig. 1. Molecular genetic analysis revealed that the *duskish* mutant is a leaky



Heavenly Blue Anthocyanin

Fig. 2. The enzymatic glycosylations and acylations, and some genes controlled in the anthocyanin biosynthesis of Japanese morning glory: G-1–6, the positions of glycosylation; D-1–3, the positions of acylation. The presumed block or inhibition positions of the mutants in Japanese morning glory. *Dusky*: G-2 and G-3; *Dingy*: D-2 and G-3; *Duskish-1* and *Duskish-2*: G-1, G-2, G-3 and D-1 (imperfectly); *Duskish-m*: G-1, G-2, G-6, and D-1 (imperfectly).

mutant of the *UF3GT* gene and its expression is reduced compared with the wild-type but remains significantly able to catalyze the reaction G-1 in Fig. 2 (Morita unpublished data). Indeed, the amounts of anthocyanins in the mutant were more abundant than those in the previously characterized *duskish-1* and *-2* mutants (Toki et al., 2001, 2004). As one of major anthocyanins in the leaky *duskish* mutant, HBA was accumulated as much as 19.8% at the ratio of its anthocyanin contents by HPLC analysis. For the deacylanthocyanins, the proportion of peonidin 3-sophoroside-5-glucoside and peonidin 3-sophoroside were roughly 50% and 20%, respectively, with the frequency of anthocyanins acylated at the 6-O of 3-glucose moiety in total anthocyanins being 54.2%. Thus not only the reaction catalyzed by UF3GT (Fig. 2, G-1) but also those by UA5GT (Fig. 2, G-2) and an acyltransferase (Fig. 2, D-1) were affected in the leaky *duskish* mutant. The leaky mutant described here may provide an example of conferring a novel flower hue that seems to result from a mixture of various anthocyanins.

3. Experimental

3.1. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using seven mobile phases: BAW (*n*-BuOH–HOAc–H_2O, 4:1:5), BuH (*n*-BuOH–2N HCl, 1:1), 1% HCl and HOAc–HCl (HOAc–HCl–H_2O, 15:3:82) for anthocyanins, and BAW, *i*-PrOH–*n*-BuOH–H_2O (7:1:2), and PhOH–H_2O (4:1) for sugars and BAW, EtOAc–HOAc–H_2O (3:1:1), and EtOH–H_2O–NH_4OH (16:3:1) for organic acids. Analytical HPLC was performed on a Hitachi 6200 system, using an Inertsil ODS-2 (4.6∅ · 250 mm) column at 35 °C with a flow rate of 0.8 mL/min and monitoring at 520 nm. The eluant was applied as a linear gradient for 40 min from 25% 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). UV–VIS spectra were recorded on a MPS-2400 (Shimadzu) in 0.1% HCl–MeOH (from 200 to 700 nm), whereas FAB mass spectra were obtained in the positive ion mode using the magic bullet. NMR spectra were acquired at 500 MHz for ^1H spectra and 125.78 MHz for ^{13}C spectra in DMSO-CF_3COOD (9:1). Chemical shifts are reported relative to a TMS internal standard, and coupling constants are in Hz.

3.2. Plant materials

The *duskish* line of *I. nil* used in this study is a rarely variegated derivative of a *duskish*-mutable line Q531 strain displaying variegated flowers in the collection of Dr. E. Nitasaka (Kyusyu University, Japan), and exhibits pale gray-purple flowers rarely with a few very fine spots. The plants were grown in the farm of Minami-Kyusyu University. The fresh corollas of this strain were collected in July to October, and dried at 45 °C.

3.3. Isolation of anthocyanins

The dried corollas (25 g) were extracted with 5% HOAc (10 L) at room temperature (ca. 20 °C) overnight. The extract was adsorbed on a Diaion HP-20 column, and the column was washed with H_2O. The pigments were eluted with MeOH–HOAc–H_2O (75:5:20). After concentration, the eluates were fractionated with Sephadex LH-20 CC using MeOH–HOAc–H_2O (6:1:12). The frs. were further purified with PC (*n*-BuOH–HOAc–H_2O, 4:1:2 and 15% HOAc) and prep. HPLC. Prep. HPLC was performed on a Hitachi 6200 system using an Inertsil ODS-2 column (20∅ · 250 mm) with HOAc solvent. Pigment **5** (5.0 mg), pigment **7** (4.0 mg), pigment **13** (21.6 mg), pigment **14** (17.8 mg), pigment **15** (6.2 mg), and pigment **17** (7.0 mg), were obtained by the process described previously (Lu et al., 1992b; Toki et al., 2001).

3.4. Analysis of anthocyanins

Characterization of pigments were carried out by using TLC and HPLC, and UV–VIS, FABMS and NMR spectroscopic data are shown in Tables 1 and 2. The procedures of deacylation with alkaline and acid hydrolyses were carried out to those pigments, and the products of deacylation and acid hydrolysis were detected and confirmed as described previously (Harborne, 1984; Lu et al., 1992a,b; Toki et al., 2001). The known pigments peonidin 3-sophoroside, peonidin 3-sophoroside-5-glucoside, and HBA were identified by the analysis of TLC and HPLC with the authentic samples obtained from the blue-violet flowers of the wild-type plants (Lu et al., 1992b). The structure of HBA was confirmed by measurements of FAB mass and ^{1}H NMR spectra.

3.5. ^{13}C NMR spectral data of pigments 5 and 7

Pigment **5**: Peonidin, 162.8 (C-2), 143.7 (C-3), 138.5 (C-4), 155.4 (C-5), 104.1 (C-6), 168.0 (C-7), 96.7 (C-8), 155.8 (C-9), 111.9 (C-10), 119.6 (C-1), 113.8 (C-2), 148.8 (C-3), 156.0 (C-4), 116.9 (C-5), 129.8 (C-6), 56.3 (OMe); caffeic acid (I), 125.8 (C-1), 115.2 (C-2), 145.3 (C-3), 148.6 (C-4), 116.0 (C-5), 121.5 (C-6), 113.8 (C-α), 145.7 (C-β), 166.6 (CO); Glc A, 100.8 (C-1), 81.1 (C-2), 76.6 (C-3), 77.8 (C-4), 69.5 (C-5), 60.8 (C-6); Glc B, 101.5 (C-1), 73.3 (C-2), 76.2 (C-3), 77.7 (C-4), 69.8 (C-5), 60.8 (C-6); Glc C, 104.1 (C-1), 74.7 (C-2), 74.3 (C-3), 76.4 (C-4), 69.7 (C-5), 63.2 (C-6).

Pigment **7**: Peonidin, 162.2 (C-2), 143.9 (C-3), 138.3 (C-4), 156.3 (C-5), 102.5 (C-6), 169.2 (C-7), 94.7 (C-8), 158.1 (C-9), 112.1 (C-10), 119.6 (C-1), 114.5 (C-2), 148.4 (C-3), 155.2 (C-4), 116.8 (C-5), 128.6 (C-6), 56.2 (OMe); caffeic acid (I), 125.7 (C-1), 115.1 (C-2), 145.7 (C-3), 148.6 (C-4), 116.0 (C-5), 121.4 (C-6), 113.5 (C-α), 145.2 (C-β), 166.5 (CO); Glc A, 102.0 (C-1), 82.3 (C-2), 76.2 (C-3), 69.4 (C-4), 77.9 (C-5), 60.8 (C-6); Glc C, 104.7 (C-1), 74.8 (C-2), 76.2 (C-3), 70.0 (C-4), 74.3 (C-5), 63.5 (C-6).

3.6. High resolution FABMS

Pigment **5**: HR-FABMS Calc. for $C_{43}H_{49}O_{24}$: 949.2614. Found: 949.2596. Pigment **7**: HR-FABMS Calc. for $C_{37}H_{39}O_{19}$: 787.2086. Found: 787.2108. Pigment **14**: HR-FABMS Calc. for $C_{73}H_{81}O_{40}$: 1597.4304. Found: 1597.4358. Pigment **15**: HR-FABMS Calc. for $C_{73}H_{81}O_{40}$: 1597.4304. Found: 1597.4349. Pigment **17**: HR-FABMS Calc. for $C_{67}H_{71}O_{35}$: 1435.3776. Found: 1435.3816.

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References

Hagiwara, T., 1954. Recent genetics on the flower-colour of Japanese morning glory with reference to biochemical studies. Bull. Res. Coll. Agri. Vet. Sci., Nihon Univ. 3, 1–15.

Hagiwara, T., 1956. Genes and chromosome maps in the Japanese morning glory. Bull. Res. Coll. Agri. Vet. Sci., Nihon Univ. 5, 34–56.

Harborne, J.B., 1984. Phytochemical Methods, second ed. Chapman & Hall, London.

Iida, S., Morita, Y., Choi, J.-D., Park, K.-I., Hoshino, A., 2004. Genetics and epigenetics in flower pigmentation associated with transposable elements in morning glories. Adv. Biophys. 38, 141–159.

Kondo, T., Kawai, H., Tamura, H., Goto, T., 1987. Structure determination of Heavenly Blue Anthocyanin, a complex monomeric anthocyanin from the morning glory *Ipomoea tricolor*, by means of the negative NOE method. Tetrahedron Lett. 28, 2273–2276.

Lu, T.S., Saito, N., Yokoi, M., Shigihara, A., Honda, T., 1991. An acylated peonidin glycoside in the violet-blue flowers of *Pharbitis nil*. Phytochemistry 30, 2387–2390.

Lu, T.S., Saito, N., Yokoi, M., Shigihara, A., Honda, T., 1992a. Acylated pelargonidin glycoside in the red-purple flowers of *Pharbitis nil*. Phytochemistry 31, 289–295.

Lu, T.S., Saito, N., Yokoi, M., Shigihara, A., Honda, T., 1992b. Acylated peonidin glycosides in the violet-blue flowers of *Pharbitis nil*. Phytochemistry 31, 659–663.

Morita, Y., Hoshino, A., Kikuchi, Y., Okuhara, H., Ono, E., Tanaka, Y., Fukui, Y., Saito, N., Nitasaka, E., Noguchi, H., Iida, S., 2005. The Japanese morning glory *dusky* mutants displaying reddish-brown or purplish-gray flowers are deficient in a novel glycosylation enzyme for anthocyanin biosynthesis, UDP-glucose:anthocyanidin 3-*O*-glucoside-2-*O*″-glucosyltransferase, due to 4-bp insertions in the gene. Plant J. 42, 353–363.

Morita, Y., Saito, N., Iida, S., 2001. The *duskish-1* mutant deficient for flower pigmentation carries a mutation in the gene encoding UF3GT in the Japanese morning glory. Abstract paper, the 73rd Annual Meeting of the Genetics Society of Japan.

Saito, N., Lu, T.S., Akaizawa, M., Yokoi, M., Shigihara, A., Honda, T., 1994. Acylated pelargonidin glycosides in the maroon flowers of *Pharbitis nil*. Phytochemistry 35, 407–411.

Saito, N., Tatsuzawa, F., Kasahara, K., Yokoi, M., Iida, S., Shigihara, A., Honda, T., 1996. Acylated peonidin glycosides in the slate flowers of *Pharbitis nil*. Phytochemistry 41, 1607–1611.

Toki, K., Saito, N., Iida, S., Hoshino, A., Shigihara, A., Honda, T., 2001. A novel acylated pelargonidin 3-sophoroside-5-glucoside from grayish-purple flowers of Japanese morning glory. Heterocycles 55, 2261–2267.

Toki, K., Saito, N., Morita, Y., Hoshino, A., Iida, S., A, Shigihara, A., Honda, T., 2004. An acylated pelargonidin 3-sophoroside from the pale brownish-red flowers of *Ipomoea nil*. Heterocycles 63, 1449–1454.