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A NOVEL ACYLATED PELARGONIDIN 3-SOPHORO-SIDE-5-GLUCOSIDE FROM GREYISH-PURPLE FLOWERS OF THE JAPANESE MORNING GLORY

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Abstract — A novel acylated anthocyanin was isolated from greyish-purple flowers of a *dingy* mutant in the Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*) with three known pigments as main pigments. This novel pigment was determined to be pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)caffeoyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside (**3**), and the other three pigments (**1**, **2**, and **4**) were 3-sophoroside-5-glucosides of pelargonidin and peonidin, and peonidin 3-*O*-[2-*O*-β-D-glucopyranosyl-(6-*O*-(β-D-glucopyranosylcaffeoyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside. In addition, pelargonidin 3-sophoroside and pelargonidin 3-glucoside were also detected as main pigments in two other mutants, *duskish-1* and *duskish-2*, displaying pale-purple hue flowers.

Recessive mutations of the Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*) conferring dull colors can be classified into four alleles, *dusky*, *duskish-1*, *duskish-2*, and *dingy*.[1] In our continuing efforts to elucidate flower color variation due to the production and accumulation of anthocyanins in the petals of *Pharbitis nil* cultivars bearing various flower colors,[1-9] we have reported the anthocyanin constituents of pigments in the dull color flowers, such as the maroon and slate flowers, of *Pharbitis nil* lines which are

believed to carry the *dusky* mutations.[6,8] The dull color flowers in these apparent *dusky* mutations contained anthocyanin 3-monoglucosides or 3,5-diglucosides as their deacylanthocyanins,[6,8] whereas the corresponding deacylanthocyanins in the bright color flowers of several cultivars examined were anthocyanidin 3-sophoroside-5-glucosides.[3,4] Indeed, the components of anthocyanin pigments in the flowers of an authentic *dusky* mutant[10] were found to be similar to those in the previously characterized cultivar 'Danjuro'.[6]

In this communication, we wish to report the structure of a novel acylated pelargonidin 3-sophoroside-5-glucoside, along with three known anthocyanins, in the grayish-purple flowers of a *dingy* mutant,[1,10] and also the presence of pelargonidin 3-sophoroside and pelargonidin 3-glucoside in the pale-purple hue flowers of the mutants carrying either the *duskish-1* or *duskish-2* allele.[1,10]

Dry flowers (15 g) of a *dingy* strain of *Pharbitis nil* were immersed in 5% AcOH for 24 h at room temperature to leave the red extract, in which six anthocyanin peaks were observed as main anthocyanins by HPLC analysis.[11] Their relative frequency of occurrence (the average of 5 HPLC analyses) was pigment-1 (**1**) (18%),[12] –2 (**2**) (7%),[13] –6 (**3**) (58%),[14] and -7 (**4**) (6%).[15] (Figure 1) From these mixed anthocyanins, pigment-6 (**3**) (*ca.* 20 mg), pigment-1 (**1**) (*ca.* 8 mg) and pigment-7 (**4**) (*ca.* 1 mg) were obtained as single compounds, respectively, by the procedure described previously.[9,16,17] The pigment-1 (**1**), -2 (**2**), and -7 (**4**) were elucidated to be pelargonidin 3-sophoroside-5-glucoside, peonidin 3-sophoroside-5-glucoside, and peonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(β-D-glucopyranosyl)-caffeoyl-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside (Pharbitis Blue anthocyanin 2),[4] respectively, by direct comparison of HPLC and TLC with authentic samples.[2-5]

Pigment-6 (**3**) was a novel anthocyanin, which on acid hydrolysis gave pelargonidin, glucose and caffeic acid. The FAB-MS spectra of pigment-6 (**3**) gave its molecular ion [M^+] at 1081 *m/z*, in good agreement with the mass calculated for $C_{48}H_{57}O_{28}$ (1081.304), which was composed of pelargonidin with four molecules of glucose and one molecule of caffeic acid. This mass value was confirmed by the measurement of its high resolution FAB-MS [Calcd for $C_{48}H_{57}O_{28}$ requires: 1081.3036. Found: 1081.3014].[14] In order to determine the structure of this pigment (Figure 2), its ^{1}H NMR measurements including ^{1}H-^{1}H COSY and negative NOE difference (DIFNOE)[2] spectra were carried out in CF_3CO_2D-DMSO-d_6 (1:9). The signals for ten aromatic protons of pelargonidin and caffeic acid moieties were assigned as shown in Table 1. Two olefinic protons of caffeic acid showed a large coupling constant (J = 15.6 Hz) supporting a *trans*

configuration (Figure 2). The proton signals of sugar part (glucose was abbreviated as Glc throughout this paper) were observed in the region of 5.60 - 3.00 ppm (Table 1). The signals of four anomeric protons appeared at 5.66 ppm [d, J = 7.2 Hz, Glc A], 5.11 ppm (d, J = 7.2 Hz, Glc B), 4.75 ppm (d, J = 7.6 Hz, Glc C), and 4.80 ppm (d, J = 6.4 Hz, Glc D), indicating that all the glucose units are β-D-glucopyranosides. The two characteristic protons at 4.34 and 4.42 ppm with a geminal coupling constant (J = 11.3 Hz) were assigned to the methylene (C-6) of Glc A, supporting the notion that the caffeoyl moiety is attached to 6-OH of Glc A. Based on the analysis of its ^{1}H-^{1}H COSY spectrum, the anomeric proton (5.66 ppm, Glc A) was finally correlated with both protons (4.34 and 4.42 ppm, H-6a and 6b of Glc A). The proton (4.08 ppm, t, J = 7.6 Hz) at the 2-position of Glc A was also shifted in the lower magnetic field, indicating 1-OH of Glc C was bonded with 2-OH of Glc A through a glucosidic bond. This linkage was confirmed by the observation of a strong negative NOE signal at H-2 of Glc A by irradiation of H-1 of Glc C in its DIFNOE spectrum[2] (Figure 2).

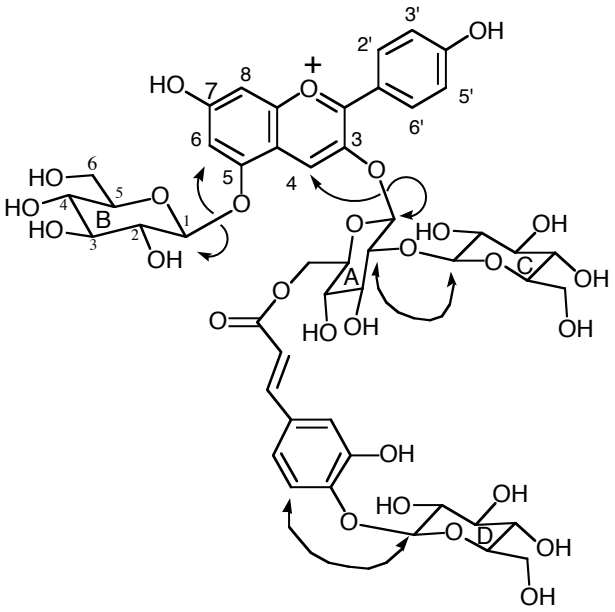

Figure 1 An acylated anthocyanin (Pigments-6) (**3**) from the dingy strain of *Pharbitis nil*. Observed NOE's are indicated by arrows.

Furthermore, the appearance of a negative NOE signal at H-4 of pelargonidin by irradiation at H-1 of Glc A showed Glc A to be attached to 3-OH of pelargonidin through a glycosidic bond, and Glc B was bonded to 5-OH of pelargonidin due to the occurrence of the strong NOEs between H-6 of pelargonidin and H-1 of Glc B. Similarly, Glc D was confirmed to attach to 4-OH of caffeic acid through a glycosidic bond by the presence of NOEs between H-5 of caffeic acid and H-1 of Glc D (Figure 2). Consequently, the structure of pigment-6 (**3**) was unambiguously determined to be pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-

(*trans*-4-*O*-(β-D-glucopyranosyl)caffeoyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside, which has not been reported previously in plants.[18,19]

Table 1 ^{1}H NMR spectral data of a dingy anthocyanin (pigment-6) (**3**) of *Pharbitis nil* .*

Pelargonidin		Glucose A (3-Glc)		Glucose C (2"-Glc)	
4	8.85	1	5.66 d (7.2)	1	4.75 d (7.6)
6	7.00 br s	2	4.08 t like (7.6)	2	3.00 t like (8.4)
8	7.12 br s	3	3.75 t like (8.8)	3	3.14 t like (8.7)
2'	8.61 d (7.6)	4	3.53 t like (8.2)	4	3.05 t like (9.2)
3'	7.10 d (7.6)	5	3.97 m	5	2.86 m
5'	7.10 d (7.6)	6a	4.34 m	6a	3.28 m
6'	8.61 d (7.6)	6b	4.42 d (11.4)	6b	3.33 m
Caffeic acid		**Glucose B (5-Glc)**		**Glucose D**	
2	7.00 br s	1	5.11 d (7.2)	1	4.80 d (6.4)
5	7.11 d (9.2)	2	3.53 m	2	3.33 m
6	6.99 m	3	3.39 m	3	3.55 - 3.18
α	6.33 d (15.7)	4	3.28 m	4	
β	7.39 d (15.7)	5	3.51 m	5	
		6a	3.55 m	6a	
		6b	3.80 d (11.3)	6b	3.75 m

* ^{1}H NMR (500 MHz) (CF_3CO_2D-DMSO-d_6, 1:9), at 25 °C, an internal standard of TMS; Coupling constants (*J* in Hz) in paretheses.

Except for their aglycones, the anthocyanins for acylation and glycosidation in the *dingy* mutant were similar in structure to those in the cultivars with light-blue flowers characterized before.[4] The *dingy* mutant contained pelargonidin and peonidin, while the light-blue cultivars peonidin and cyanidin. It is thus likely that the light-blue cultivars also carry *dingy* mutant(s) and that the *Dingy* gene product is responsible for the acylations of caffeic acids at both 6-OH residues of Glc C and D, since the pigments in both the light-blue cultivars and the *dingy* mutant displaying grayish-purple flowers were lacking the acylations.

In other two pale grayish-purple flower mutants, *duskish-1* and *duskish-2*, the anthocyanin extracts were obtained from their flowers with the same solvents, and their anthocyanin components were analyzed by HPLC with the authentic anthocyanins. As Figure 2 shows, pelargonidin 3-sophoroside (pigment-3)[20] and pelargonidin 3-glucoside (pigment-4)[21] were detected in the extract of *duskish-1* as main anthocyanins. The extract of *duskish-2* contained pelargonidin 3-[tri(glucosylcaffeoyl)sophoroside]-5-glucoside (Pharbitis red anthocyanin 1, pigment-8)[22] and pelargonidin 3-[2-glucosylcaffeoylglucosyl-6-(caffeoylglucosyl-caffeoyl)glucoside]-5-glucoside (Ipomoea red anthocyanin 1, pigment-9)[23] as its main anthocyanins as well as pelargonidin 3-sophoroside and 3-glucoside. Interestingly, total anthocyanin contents of both flowers of the *duskish-1* and *duskish–2* mutants were found to be reduced to less than 10% of that in the flower extract

of the *dingy* mutant. Since the content of anthocyanins in the *dingy* flowers appears to be normal, the results imply that both *duskish* mutations affect either the biosynthesis of anthocyanin pigments or their transport into the vacuoles of the floral epidermal tissue. Identification of the *Duskish* gene would help to clarify its role in pigmentation processes and would provide an explanation for the pale pigmentation of the *duskish* mutants.

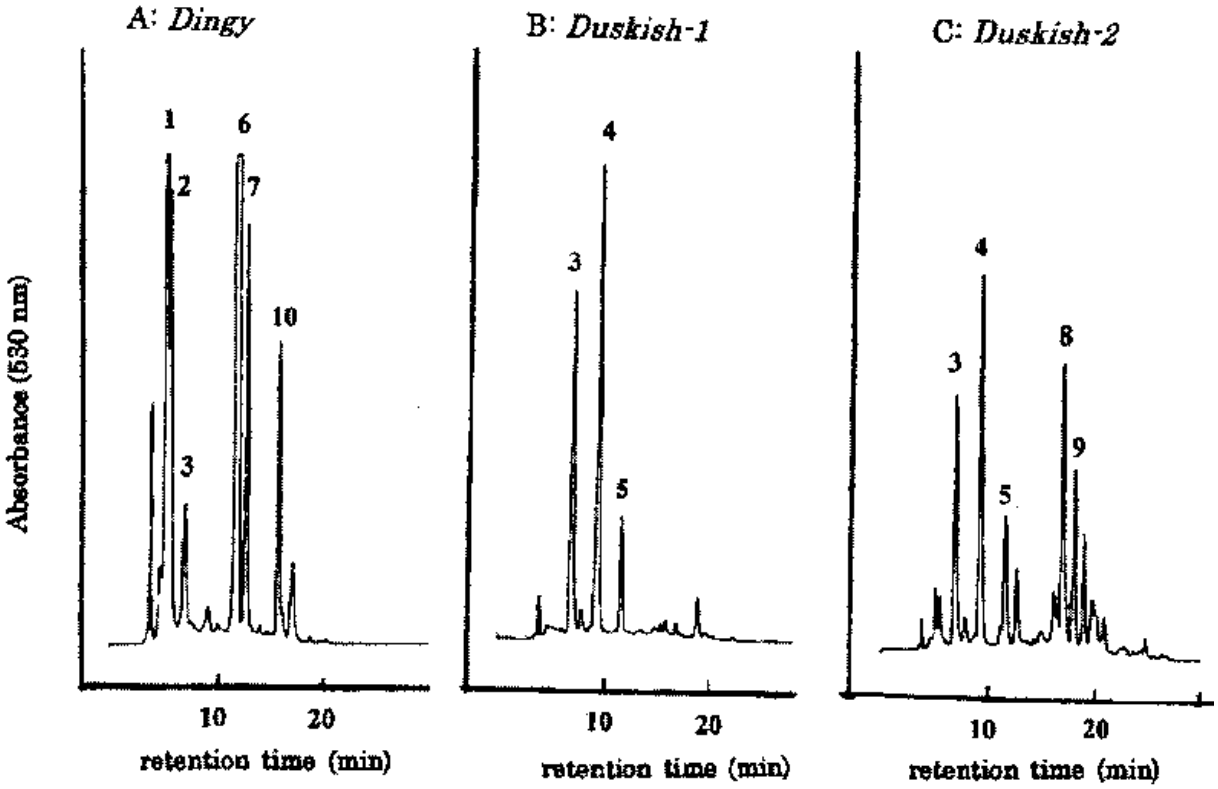

Figure 2. HPLC Analysis of the pigments in the *dingy* (A), *duskish-1* (B), and *duslish-2* (C) flowers *Pharbitis nil* mutants. The piments identified are: pigment-1 (peak 1) = pelargonidin 3-sophoroside-5-glucoside; pigment-2 (peak 2) = peonidin 3-sophoroside-5-glucoside; pigment-3 (peak 3) = pelargoniddin 3-sophoroside; pigment-4 (peak 4) = pelargonidin 3-glucoside; pigment-5 (peak 5) = pelargonidin; pigment-6 (peak 6) = pelargonidin 3-[2-glucosyl-6-(4-glucosylcaffeoyl)glucoside]-5-glucoside; pigment-7 (peak 7) = peonidin 3-[2-glucosyl-6-(4-glucosylcaffeoyl)glucoside]-5-glucoside; pigment-8 (peak 8) = pelargonidin 3-[2-glucosylcaffeoylglucosyl-6-(glucosylcaffeoylglucosylcaffeoyl)glucoside]-5-glucoside (Pharbitis Red anthocycanin 5); pigment-9 (peak 9) = pelargonidin 3-[2-glucosylcaffeoylglucosyl-6-(caffeoylglucosyl-caffeoyl)glucoside]-5-glucoside (Ipomoea Red anthocyanin 1); pigment-10 (peak 10) = unidentified anthocyanin.

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10. All of *Pharbitis nil* the mutants used were from Dr. Nitasaka's collection at Kyusyu University [see: "The Japanese Morning Glory (=Asagao) Home Page"; http://mg.biology.kyyusyu-u.ac.jp/index.html]. His collection numbers of the mutants used here are: *duskish-1*, 853; *duskish-2*, 854; *dusky*, 856; *dingy*, 857.
11. HPLC was run on Inertsil ODS-2 column (4.6×250 mm for analysis, and 20×250 mm for prep.) at 35°C, and monitoring at 530 nm. Solvent systems for analysis were as follows: linear gradient elution for 40 min from 40 – 85% solvent B (1.5% H_3PO_4, 20% AcOH, 25% MeCN) in solvent A (1.5% H_3PO_4).
12. Pigment-1 (**1**) = pelargonidin 3-sophoroside-5-glucoside: UV λmax (0.1% HCl-MeOH) 507, 268 nm; HPLC Rt (min) 5.1.
13. Pigment-2 (**2**) = peonidin 3-sophoroside-5-glucoside: UV λmax (0.1% HCl-MeOH) 522, 277 nm; HPLC Rt (min) 5.5.
14. Pigment-6 (**3**) = pelargonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside: UV λmax (0.1% HCl-MeOH) 510, 322, 281 nm, Eacyl/Emax = 0.68; HPLC Rt (min) 11.8. ^{13}C NMR (125.65 MHz) (CF_3CO_2D-DMSO-d_6,

1:9): δ pelargonidin: 165.3 (C-2), 146.8 (C-3), 133.1 (C-4), 111.8 (C-4a), 155.4 (C-5), 105.0 (C-6), 167.5 (C-7), 96.4 (C-8), 162.8 (C-8a), 121.0 (C-1'), 135.2 (C-2'), 117.1 (C-3'), 155.4 (C-4'), 116.3 (C-5'), 128.8 (C-6'); caffeic acid: 125.6 (C-1), 116.7 (C-2), 145.2 (C-3), 147.3 (C-4), 118.6 (C-5), 121.0 (C-6), 121.0 (C-α), 144.2 (C-β), 166.3 (C=O); Glc A: 99.3 (C-1), 79.5 (C-2), 69.9 (C-4), 62.9 (C-6); Glc B: 103.1 (C-1), 69.9 (C-4), 60.8 (C-6); Glc C: 101.7 (C-1), 69.9 (C-4), 60.8 (C-6); Glc D: 101.7 (C-1), 69.9 (C-4), 60.8 (C-6), 77.5-77.1 (4×C), 76.3-75.8 (4×C), 74.4 (2×C), 73.4 (2×C). HRFAB-MS [Calcd for $C_{48}H_{57}O_{28}$ requires: 1081.3036. Found: 1081.3014].

15. Pigment-7 (**4**) = peonidin 3-*O*-[2-*O*-(β-D-glucopyranosyl)-6-*O*-(β-D-glucopyranosyl)caffeoyl-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside (Pharbitis Blue anthocyanin 2): UV λmax (0.1% HCl-MeOH) 525, 324, 280 nm, Eacyl/Emax = 0.40; HPLC Rt (min) 12.7.
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20. Pigment-3 = pelargonidin 3-sophoroside: UV λmax (0.1% HCl-MeOH) 509, 270 nm; HPLC Rt (min) 6.9.
21. Pigment-4 = pelargonidin 3-glucoside: UV λmax (0.1% HCl-MeOH) 509, 270 nm; HPLC Rt (min) 9.3.
22. Pigment-8 = pelargonidin 3-[tri(glucosylcaffeoyl)sophoroside]-5-glucoside (Pharbitis Red anthocyanin 1: UV λmax (0.1% HCl-MeOH) 515, 318, 288 nm; Eacyl/Emax = 1.16; HPLC Rt (min) 16.9.
23. Pigment-9 = pelargonidin 3-[2-glucosylcaffeoylglucosyl-6-(caffeoylglucosylcaffeoyl)glucoside]-5-glucoside (Ipomoea Red anthocyanin 1): UV λmax (0.1% HCl-MeOH) 515, 318, 288 nm; Eacyl/Emax = 1.06; HPLC Rt (min) 18.1.