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Mechanism of dusky reddish-brown "kaki" color development of Japanese morning glory, *Ipomoea nil* cv. Danjuro

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Abstract

The mechanism of dusky reddish-brown "kaki" color development of morning glory, *Ipomoea nil* cv. Danjuro, was studied. Three major known anthocyanins were isolated as glucosylated pelargonidin derivatives. Measurement of the vacuolar pH with proton-selective microelectrodes revealed the vacuolar pH of the colored cell of open flowers to be 6.8, while that of buds was 5.8. Mixing of the three anthocyanins according to the composition ratio in petals at pH 6.8 allowed the identical color to that of petals to be reproduced. The typical "kaki" color development was mostly caused by 5-OH free acylated anthocyanins, which have two λmax around 435 and 535 nm in the visible region.

Keywords: *Ipomoea nil* cv. Danjuro; Convolvulaceae; Proton-selective microelectrode; Micro-spectrophotometry; Vacuolar pH; Flower color development; Dusky kaki color

1. Introduction

Beautiful red, through purple to blue flower colors are mostly due to anthocyanins (Goto, 1987; Brouillard, 1988; Goto and Kondo, 1991; Brouillard and Dangles, 1994). Petal colors of Japanese morning glory, *Ipomoea nil*, vary greatly and in Japan there are special cultivars named "Susuke; dusky" or "Kaki" which have reddish-brown, or terra-cotta colored petals, prized by Japanese horticulturists for over a hundred years. For this kaki flower color development a *dusky* gene was reported (Hagiwara et al., 1956) and several anthocyanins have been isolated from maroon and reddish-brown petals (Saito et al. 1994, 1996, 1998). However, the mechanism of the typical kaki color development in flower petal cells has, to our knowledge, not been clarified. We report here the in vivo mechanism of kaki color development revealed by combination of a direct vacuolar pH measurement of living epidermal cells by using a proton-selective microelectrode and reproduction of colors by mixing petal components.

2. Results and discussion

2.1. Petal color of cv. Danjuro

As shown in Fig. 1A the open petals of morning glory, *Ipomoea nil* cv. Danjuro exhibit a typical kaki color. Fig. 1B shows a transverse section of an open flower petal. In the morning glory petals, colored cells are located in both the abaxicial and adaxicial epidermis, as in other flower petals. The shape of the adaxicial cells is conical and that of abaxicial ones is plain, as reported for *Ipomoea tricolor* cv. Heavenly blue (Yoshida et al., 1995).

The reflection spectrum of petals of cv. Danjuro showed two λvismax around 550 and 435 nm (Fig. 2). Since the spectrum recorded by an integral sphere apparatus is the average for many epidermal cells and affected by the cell shape (Noda et al., 1994), it is very broad and the λvismax wavelength varies. To obtain a visible absorption spectrum of individual cells, we

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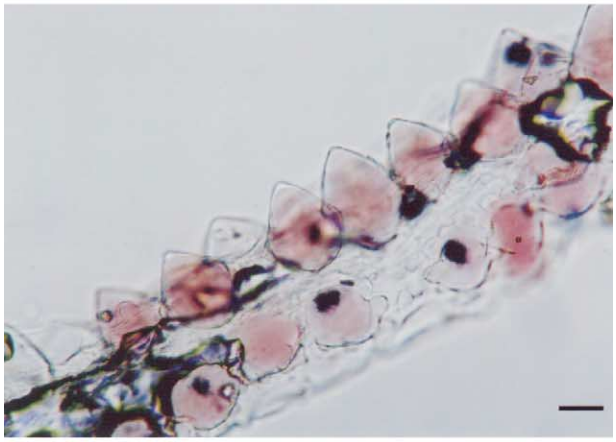
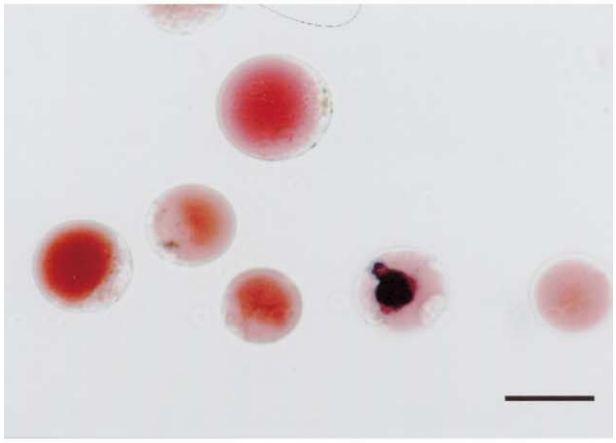
(A)



(B)



(C)

Fig. 1. Photo of open flower petal, petal tissue and colored protoplast of *Ipompea nil* cv. Danjuro. (A) Open flower. (B) Transverse section of open flower petal. (C) Protoplasts prepared from open flower. Scale bars: 10 μm.

prepared protoplasts from petals and measured absorption spectra by micro-spectrophotometry (Fig. 2). By enzyme treatment of fresh petals, only colored protoplasts could be obtained without any color change during the procedure (Fig. 1C). The visible absorption spectrum of a single kaki colored cell was sharp and showed two peaks (λvismax at 534 and 437 nm). Since simple anthocyanin solution (e.g., 3,5-diglycosyl anthocyanidin) which has one absorption peak in the visible region around 530 nm shows clear red color, the additional absorption peak at the shorter wavelength (λvismax 437 nm) might be responsible for development of the kaki color.

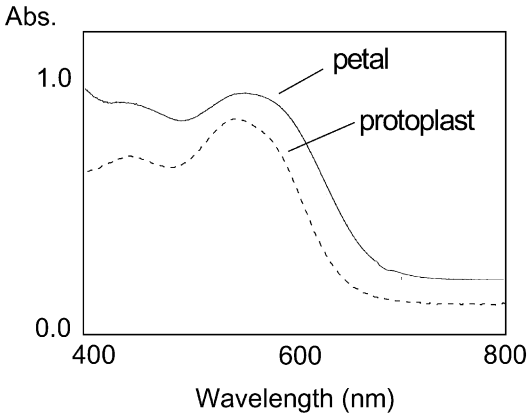


Fig. 2. Reflection spectrum of open flower petal and absorption spectrum of colored protoplast of *Pharbitis nil* cv. Danjuro. - - - - : petal, — : protoplast.

2.2. Vacuolar pH of colored epidermal cells

Vacuolar pH measurement of plant cells with microelectrodes (Felle and Bertl, 1986; Felle, 1987, 1993) has major advantages because data for single cells can be obtained directly. However, there have been very few reports for flower petal vacuolar pH because of technical difficulties. We applied two methods to record the vacuolar pH of kaki colored epidermal cells of morning glory cv. Danjuro. One procedure was to insert an electrode into a petal tissue cell directly (Yoshida et al., 1995) and the other was to insert an electrode into a protoplast under an inverted microscope (Yoshida et al., 2003).

A cut petal was set on a Plexiglas vessel with its abaxial side uppermost and a double-barreled proton-selective microelectrode was inserted into epidermal cells under microscopic observation (Table 1). Membrane potential value was monitored simultaneously to confirm the location of the tip of the electrode. The membrane potential value for open flower epidermis was found to be −57 mV and that of bud epidermis to be −50 mV, indicating that the tip was certainly inserted into a vacuole. The vacuolar pH value of open flower cells was 6.8 and that of buds was 5.8. The difference being highly significant ($P<0.001$) between the two flowering stages.

Micro-spectrophotomeric analysis of colored protoplasts was carried out with insertion and localization of

Table 1
Vacuolar pH of kaki colored epidermal cells of *Ipomoea nil* cv. Danjuro

Stage	Vacuolar pH[a]	(No. of experiments)	Membrane potential (mV)[b]
Open flower	6.8±0.44*	(19)	−57±14
Bud	5.8±0.37**	(15)	−50±21
Protoplast[c]	6.6±0.25*	(7)	−5.3±3.3[d]

Significant ($P<0.001$) difference between * and ** by Student's t test.
[a] Mean±S.D. (no. of experiments) vacuolar pH values.
[b] Mean±S.D. membrane potential values.
[c] Prepared from open flower petals.
[d] Membrane potential values of protoplast are known to be very low (Pantoja and Willmer 1986; Bouteau et al., 1999; Duijn and Heimovaara-Dijkstra, 1994; Shabala et al., 1998, Yoshida et al., 2003).

the electrode tip proven directly by microscopy. The vacuolar pH value of colored protoplasts prepared from open flower petals was 6.6 (Table 1). There was no significant difference between results with two methods using petal tissue and protoplasts.

These data showed that the vacuolar pH of colored epidermal cells of Japanese morning glory, *Ipomoea nil* cv. Danjuro, increased by one pH unit during blooming, as earlier found for the blue morning glory, *Ipomoea tricolor* cv. Heavenly blue (Yoshida et al., 1995). Therefore, the vacuolar pH increasing during flower opening could be a general phenomenon in morning glories. It might be physiologically essential for flower blooming, water absorption and cell expansion. The one pH unit less than *Ipomoea tricolor* might be caused by difference of species. The color change of anthocyanins from reddish-purple to blue is responsible for the structural change from anhydrobase to anhydrobase anion form. This chemical transformation occurs around pH 7.0; therefore, *Ipomoea tricolor* changes its flower color remarkably during blooming and *Ipomoea nil*, cv. Danjuro, does not.

2.3. Isolation and structural identification of petal components

Photo-diode array detection HPLC of extracts of kaki colored flowers, *Pharbitis nil* cv. Danjuro, suggested that the petals contain three anthocyanins and one UV absorbing component (Fig. 3). Those components were isolated according to our general procedures for anthocyanins (Yoshida et al., 2000, 2002). Fresh petals (650 g) were extracted with 50% acetonitrile (CH_3CN) containing 3% trifluoroacetic acid (TFA), and the extracts chromatographed repeatedly with Amberlite XAD-7 and preparative ODS columns to give the three anthocyanins (**1**, **2** and **3**) and one UV absorbing component (**4**). The structure of **1**, **2** and **3** was studied by 1D- and 2D-NMR and MS analysis. The linkage of glucosyl and caffeoyl residues was determined by NOE experiments and acylation shift. **1** was identified to be 3-*O*-*β*-D-glu-

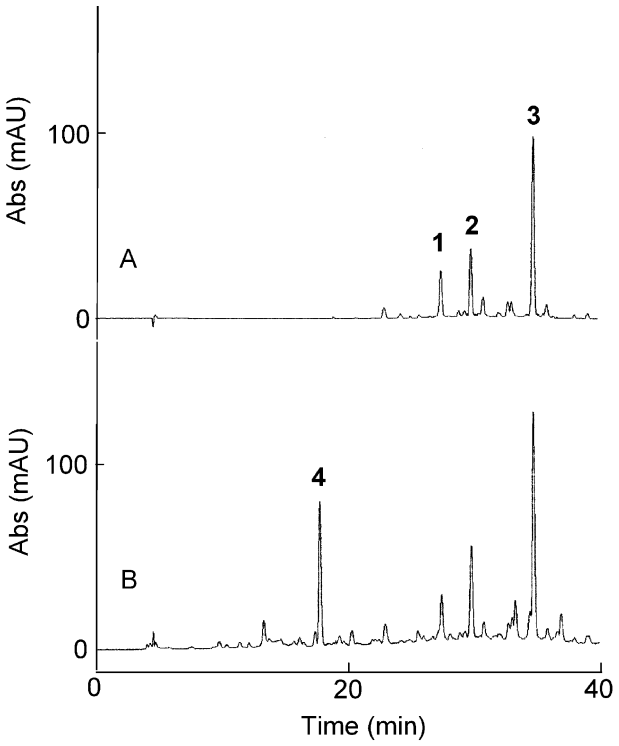
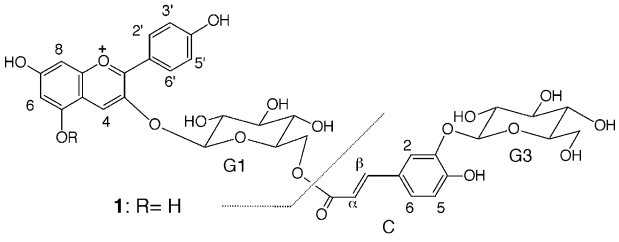


Fig. 3. HPLC chromatogram of the extract from open petals of *Ipomoea nil* cv. Danjuro. Upper chromatogram (A): detection at 530 nm, lower chromatogram (B): detection at 280 nm.



2 : R= *β*-Glc (G2)

3 : R= H

copyranosylpelargonidin by comparison with authentic sample (Yoshida et al., 1996). The structures of **2** and **3** were identified to be 3-*O*-(6-*O*-((*E*)-3-*O*-(*β*-D-glucopyranosyl)caffeoyl)-*β*-D-glucopyranosyl)-5-*O*-*β*-D-glucopyranosylpelargonidin and 3-*O*-(6-*O*-((*E*)-3-*O*-(*β*-D-glucopyranosyl) caffeoyl)-*β*-D-glucopyranosyl)pelargonidin, respectively (Saito et al., 1994). Component **4** was identified to be 3-*O*-caffeoylquinic acid (chlorogenic acid) with reference to an authentic sample.

2.4. Reprodution of kaki color with combined petal components

Based on the results of vacuolar pH measurement and HPLC analysis we tried to reproduce the same kaki color by mixing isolated components. As shown in Fig. 4, the VIS spectra of the individual anthocyanins in

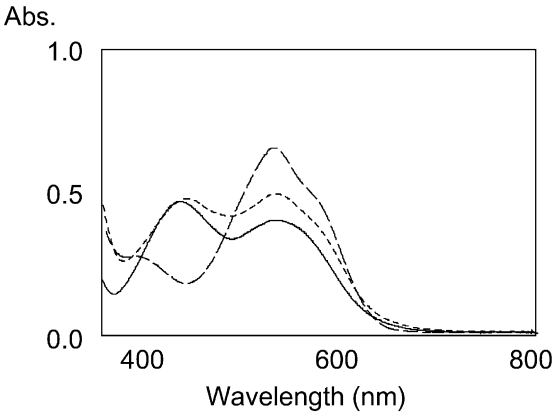


Fig. 4. Visible absorption spectra of **1**–**3** (5×10^{-4} M) in 100 mM phosphate buffer (pH 6.8, path length; 1.0 mm). - - - : **1**, – – – : **2**, — : **3**. The λvismax (nm); **1**: 531,433, **2**: 527, **3**: 531, 439.

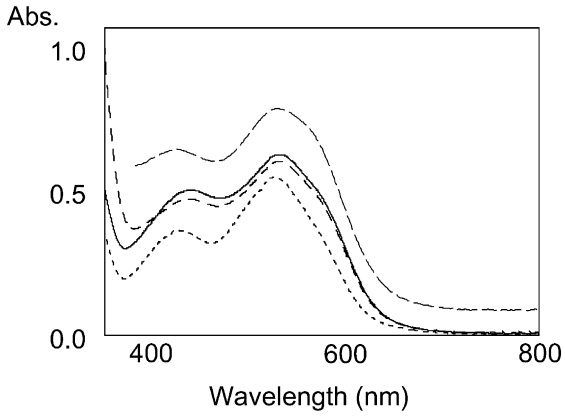


Fig. 5. Reproduction of the color of the protoplast of *Ipomoea nil* cv. Danjuro by mixing components, **1**, **2**, **3** and **4** in 100 mM phosphate buffer, path length; 1.0 mm. — — : Visible absorption spectrum of colored protoplast, — : the mixture of **1** (1×10^{-4} M), **2** (1×10^{-4} M), and **3** (3×10^{-4} M) at pH 6.8, - - - : the mixture of **1** (1×10^{-4} M), **2** (1×10^{-4} M), **3** (3×10^{-4} M), **4** (5×10^{-4} M) at pH 6.8, : · · · · the mixture of **1** (1×10^{-4} M), **2** (1×10^{-4} M), and **3** (3×10^{-4} M) at pH 5.8.

aqueous solutions (phosphate buffer and diluted aqueous NaOH solution) at the same pH as the vacuole, pH 6.8, were not the same as the spectra in petals. Interestingly, 5-OH free anthocyanins (**1** and **3**) showed an absorption peak at 430 nm, but 5-*O*-glucosylated anthocyanin (**2**) did not. A difference in the visible spectrum between 3-*O*-monoglycoside and 3,5-*O*-diglycoside in acidic methanol solution was reported by Harborne (1958), so that becomes clear that the difference around 440 nm is more distinct in neutral solutions. When the three anthocyanins **1**, **2** and **3** were mixed at the ratio of 1:1:3, which is the same ratio as in fresh petals at pH 6.8, the same spectrum as that of protoplasts could be obtained (Fig. 5). Addition of **4** had no effect on the color or stability. The mixture of **1**, **2** and **3** (1: 1: 3) in pH 5.8 showed redder color than that in pH 6.8; the λvismax was shifted to a shorter wavelength at 525 nm. This color difference can well explain the finding that the petal color of the buds appeared a little redder than that of the open flower petals. Therefore, the typical kaki color of morning glory petals cv. Danjuro was reproduced with a 1:1:3 mixture of **1**, **2** and **3** at pH 6.8.

In conclusion, we could simultaneously assess the color and the vacuolar pH of living petal epidermal cells by proton-selective microelectrode. Color-reproduction by mixing isolated components of petals revealed the in vivo mechanism of kaki color development.

3. Experimental

3.1. General

UV/VIS spectra were recorded on a JASCO Ubest-55 spectrometer. NMR spectra were obtained with Jeol ECA-500 (^{1}H: 500 MHz) or GX600 spectrometers (^{1}H: 600 MHz) in a 5 mm ∅ tube at variable temperatures using 10% TFA*d*-CD_3OD as solvent. Chemical shifts were recorded as parts per million (ppm) with the CD_2HOD resonance set at 3.30 ppm as a standard. ESI TOF MS data were recorded on a PEBiosystem QSTAR. Analytical HPLC was carried out using an ODS-column (Develosil ODS-HG5 4.6 mm ∅ ×250 mm Nomura Chemical). HPLC was carried at 40 °C with linear gradient elution from 10 to 30% aq. CH_3CN solution containing 0.5% TFA in 30 min.

3.2. Plant material

Seeds of *Ipomoea nil* cv. Danjuro (Q114 line) were kindly provided by Dr. Eiji Nitasaka of Kyushu University. They were soaked in concentrated H_2SO_4 for 5 min, then washed with tap water for 12 h and sown on a nursery bed. After a few weeks, seedlings were replanted into pots or at the experimental farm of Nagoya University, then grown.

3.3. Reflection spectrum measurement

Fresh sepals were cut into ca. 10×10 mm squares and fixed on a cover glass, then placed on a white plate of barium oxide (BaO) and set in the integral sphere apparatus of a JASCO Ubest-55 spectrometer for recording of the reflection spectrum from 400 to 800 nm.

3.4. Preparation of protoplasts and measurement of the visible absorption spectrum

Fresh sepals (ca. 1.0 g) were cut at 1 mm thickness with a razor blade. To the fragments was added 0.6 M mannitol (20 mM MES-Tris, pH 6.3, 10 ml) containing 0.2% (W/V) Macerozyme R-200 and 2.0% (W/V) Cellulase Y-C. The mixture was treated in diminished

pressure for 1 min, incubated at 25 °C for 1 h, then filtered through Miracloth (Calbiochem). The filtrate was washed 3 times with the buffer and centrifuged (70 *g*, 5 min) under 4 °C. The obtained protoplasts were suspended in ca. 1 ml of the buffer and a 200 μl aliquot of protoplast suspension was poured onto a poly-L-lysine pre-coated cover glass (18×18 mm). The cover glass was placed in a plastic dish (35 mm ∅) and 2 ml of the buffer was poured into the dish. It was then set on an inverted microscope (IX70, OLYMPUS), equipped with a micro-spectrophotometer (MCPD-7000, Photal). The absorption spectrum of colored protoplasts was measured in the visible region (400–800 nm) with a 10 μm diameter optical light beam.

3.5. Preparation of proton-selective microelectrodes and measurement of vacuolar pH

Proton-selective microelectrodes were prepared according to the methods of Okazaki et al. (1994) and Yoshida et al. (1995, 2003), with slight modification. One glass tube of a triple-barreled pipette (IB 100 F-4, World Precision Instruments. Inc.) was broken at the tip, another in the middle, and the remaining longest tube was treated with chlorotrimethylsilane. Hydrogen Ionophore II-Cocktail A (Fluka) containing nitrocellulose was then introduced into the longest pipette. The pipette was stored in a dry box before measurement. The longest pipette was filled with the proton-ionophore cocktail, then filled with the reference solution (100 mM MES-Tris, 500 mM KCl, pH 6.0). The next longest pipette was filled with 500 mM KCl. Calibration of the electrode was made before and after measurement using buffers (10 mM DMG–Tris (pH 4.0, 5.0), 10 mM MES–Tris (pH 6.0), 10 mM HEPES–Tris (pH 7.0) containing 100 mM KCl). Electrodes with a slope more than 45 mV per pH unit were selected for measurement.

A cut petal (ca. 10×10 mm) was placed on the vessel, and a reference electrode (FLEXREF, World Precision Instruments. Inc.) was placed in a hole on the vessel, which led to the opposite side of the petals with 10 mM KCl. A microelectrode was inserted into the epidermal colored cell under microscopic observation (BX50WI, OLYMPUS) using a mechanical micromanipulator. Electric signals were collected into a personal computer with Chart software (AD Instruments Inc.) with a high input impedance electrometer (FD223, World Precision Instruments Inc.) via a salt bridge (500 mM KCl, 2% W/V agarose) and a microelectrode holder (MEH3SF, World Precision Instruments. Inc.). The collected data were processed with reference to the calibration data recorded after vacuolar pH measurement each time.

Vacuolar pH measurement of protoplasts was carried out as above except for using an inverted microscope (IX70, OLYMPUS), with cells fixed on a cover glass by poly-L-lysine in storage buffer (Yoshida et al., 2003).

3.6. Isolation of pigments

Fresh petals of *Pharbitus nil* cv. Danjuro (650 g) were extracted with H_2O–CH_3CN (1:1) containing 3% TFA (3×3.0 l). The combined extract was evaporated under reduced pressure, and applied to an Amberlite XAD-7 column (60 ∅ ×400 mm), eluted with 5–60% aq. CH_3CN containing 0.5% TFA and the major pigments were found in the 15–35% fractions. After evaporation in vacuo, they were dissolved in 0.5% aq. TFA solution, then re-applied an Amberlite XAD-7 column followed by preparative ODS-HPLC and elution with 0–20% CH_3CN containing 0.5% TFA. Three anthocyanins, **1**, **2** and **3** were obtained at 18, 38, and 80 mg, respectively, as dark-red amorphous TFA salts. Simultaneously, an amorphous colorless compound, **4** (20 mg), was isolated.

3.6.1. 3-O-β-D-Glucopyranosylpelargonidin (*1*)

UV/VIS (0.1% HCl–MeOH) nm (ϵ: absorption coefficient): 334 (4050), 432 (8370), 510 (21200); ESI TOF MS: $m/z=433$ $[M]^+$; ^{1}H NMR (10% TFA*d*-CD_3OD, 600 MHz, 25 °C): δ (ppm) 3.34 (*t*, 9.0, G1-4), 3.45 (*t*, 9.0 Hz, G1-3), 3.47 (*ddd*, 9.0, 6.0, 2.0 Hz, G1-5), 3.56 (*dd*, 9.0, 7.5 Hz, G1-2), 3.62 (*dd*, 12.0, 6.0 Hz, G1-6a), 3.83 (*dd*, 12.0, 2.0 Hz, G1-6b), 5.19 (*d*, 7.5 Hz, G1-1), 6.59 (*d*, 2.0 Hz, H-6), 6.84 (*d*, 2.0 Hz, H-8), 6.97 (*d*, 9.0 Hz, H-3′,5′), 8.51 (*d*, 9.0 Hz, H-2′,6′), 9.00 (*s*, H-4), NOE irr G1-1 to H-4.

3.6.2. 3-O-(6-O-(E)-3-O-(β-D-Glucopyranosyl) caffeoyl)-β-D-glucopyranosyl-5-O-β-D-glucopyranosylpelargonidin (*2*)

UV/VIS (0.1% HCl–MeOH) nm (ϵ): 287 (20900), 319 (19000), 509 (29900); ESI TOF MS: $m/z=919$ $[M]^+$; ^{1}H NMR (10% TFA*d*-CD_3OD, 600 MHz, 25 °C): δ (ppm) 3.37 (*m*, G3-4,5), 3.41 (*t*, 9.0 Hz, G2-4), 3.48 (*t*, 9.0 Hz, G3-3), 3.51 (*t*, 9.0 Hz, G1-4), 3.52 (*dd*, 9.0, 7.5 Hz, G3-2), 3.55 (*t*, 9.0 Hz, G2-3), 3.56 (*t*, 9.0 Hz, G1-3), 3.57 (*ddd*, 9.0, 5.0, 2.0 Hz, G2-5), 3.65 (*dd*, 12.0, 5.5 Hz, G3-6a), 3.68 (*dd*, 12.0, 5.0 Hz, G2-6a), 3.69 (*dd*, 9.0, 7.5 Hz, G1-2), 3.72 (*t*, 9.0 Hz, G2-2), 3.85 (*ddd*, 9.0, 6.5, 2.0 Hz, G1-5), 3.86 (*dd*, 12.0, 1.5 Hz, G3-6b), 3.96 (*dd*, 12.0, 2.0 Hz, G2-6b), 4.42 (*dd*, 12.0, 6.5 Hz, G1-6a), 4.52 (*dd*, 12.0, 2.0 Hz, G1-6b), 4.76 (*d*, 7.5 Hz, G3-1), 5.14 (*d*, 7.5 Hz, G2-1), 5.35 (*d*, 7.5 Hz, G1-1), 6.23 (*d*, 16.0, C-α), 6.83 (*d*, 8.0 Hz, C-5), 6.97 (*brs*, H-6, H-8), 7.04 (*d*, 8.0 Hz, H-3́,5́), 7.05 (*dd*, 8.0, 2.0 Hz, C-6), 7.29 (*d*, 2.0 Hz, C-2), 7.34 (*d*, 16.0 Hz, C-β), 8.57 (*d*, 9.0 Hz, H-2́,6́), 8.98 (*s*, H-4), NOEs irr G1-1 to H-4, irr G2-1 to H-6, irr G3-1 to C-2.

3.6.3. 3-O-(6-O-(E)-3-O-(β-D-Glucopyranosyl) caffeoyl)-β-D-glucopyranosylpelargonidin (*3*)

UV/VIS (0.1% HCl–MeOH) nm (ϵ): 284 (26400), 320 (19500), 437 (10800), 511 (27700); ESI TOF MS: $m/z=$

757 $[M]^+$; ^1H NMR (10% TFA*d*-CD_3OD, 500 MHz, 25 °C): δ (ppm) 3.37 (*t*, 9.0 Hz, G3-4), 3.43 (*ddd*, 9.0, 6.0, 2.0 Hz, G3-5), 3.45 (*t*, 9.0 Hz, G1-4), 3.48 (*t*, 9.0 Hz, G3-3), 3.53 (*dd*, 9.0, 7.5 Hz, G3-2), 3.55 (*t*, 9.0 Hz, G1-3), 3.67 (*dd*, 9.0, 7.5 Hz, G1-2), 3.69 (*dd*, 11.0, 6.0 Hz, G3-6a), 3.80 (*ddd*, 9.0, 8.0, 3.0 Hz, G1-5), 3.92 (*dd*, 11.0, 2.0 Hz, G3-6b), 4.39 (*dd*, 12.0, 8.0 Hz, G1-6a), 4.48 (*dd*, 12.0, 3.0 Hz, G1-6b), 4.77 (*d*, 7.5 Hz, G3-1), 5.28 (*d*, 7.5 Hz, G1-1), 6.22 (*d*, 16.0, C-α), 6.50 (*s*, H-6), 6.80 (*s*, H-8), 6.80 (*d*, 8.0 Hz, C-5), 6.97 (*dd*, 8.0, 2.0 Hz, C-6), 7.01 (*d*, 9.0 Hz, H-3′,5′), 7.33 (*d*, 2.0 Hz, C-2), 7.35 (*d*, 16.0 Hz, C-β), 8.53 (*d*, 9.0 Hz, H-2′,6′), 8.89 (*s*, H-4), NOEs irr G1-1 to H-4, irr G3-1 to C-2.

3.7. Absorption spectra

Each isolated anthocyanin (TFA salt) was dissolved in water at a concentration of 5×10^{-2} M. Individual solutions were diluted to 5×10^{-4} M with 0.1 M phosphate buffer (pH 6.8), with UV/Vis spectra subsequently measured in a quartz cell ($d=1$ mm) at 25 °C.

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