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Phytochemistry 67 (2006) 992–998
PHYTOCHEMISTRY
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Ferric ions involved in the flower color development of the Himalayan blue poppy, *Meconopsis grandis*

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Received 21 October 2005; received in revised form 31 January 2006; accepted 1 February 2006
Available online 6 May 2006

Abstract

The Himalayan blue poppy, *Meconopsis grandis*, has sky blue-colored petals, although the anthocyanidin nucleus of the petal pigment is cyanidin. The blue color development in this blue poppy involving ferric ions was therefore studied. We analyzed the vacuolar pH, and the organic and inorganic components of the colored cells. A direct measurement by a proton-selective microelectrode revealed that the vacuolar pH value was 4.8. The concentrations of the total anthocyanins in the colored cells were around 5 mM, and ca. three times more concentrated flavonols were detected. Fe was detected by atomic analysis of the colored cells, and the ratio of Fe to anthocyanins was ca. 0.8 eq. By mixing the anthocyanin, flavonol and metal ion components in a buffered aq. solution at pH 5.0, we were able to reproduce the same blue color; the visible absorption spectrum and CD were identical to those in the petals, with Fe^{3+}, Mg^{2+} and flavonol being essential for the blue color. The blue pigment in *Meconopsis* should be a new type of metal complex pigment that is different from a stoichiometric supramolecular pigment such as commelinin or protocyanin.

Keywords: *Meconopsis grandis*; Papa veraceae; Blue flower color development; Metal complex anthocyanin; Circular dichroism; Vacuolar pH

1. Introduction

Blue flower colors are mostly due to anthocyanins (Goto and Kondo, 1991; Brouillard and Dangles, 1994). For blue flower coloration, metalloanthocyanin, a self-assembled stoichiometric supramolecular metal-complex pigment composed of six molecules each of anthocyanin and flavone and two metal ions, plays a very important role (Goto et al., 1986; Goto and Kondo, 1991). Until now, commelinin from the blue dayflower (Kondo et al., 1992), protodelphin from blue salvia (Takeda et al., 1994; Kondo et al., 2001) and protocyanin from blue cornflower (Kondo et al., 1994, 1998; Shiono et al., 2005) have been chemically clarified to be metalloanthocyanins. All show a typical exiton-type Cotton effect in the circular dichroism (CD) caused by chiral molecular stacking of anthocyanidin chromophores (Hoshino et al., 1981a,b). In metalloanthocyanins, only Mg^{2+} complexation with delphinidin-type anthocyanin (commelinin and protodelphin) is sufficient to develop the blue color, while in the case of cyanidin-type anthocyanin (protocyanin), Fe^{3+} is essential for bluing (Kondo et al., 1994, 1998; Shiono et al., 2005). However, in nature, there should be other mechanisms for blue flower color development. Indeed the blue color of the hydrangea involves Al^{3+} complexation consisting of non-flavonoid co-pigments (Takeda et al., 1990; Kondo et al., 2005), although neither its structure nor composition is yet known. The blue petals of *Phacelia campanularia* may also be developed by intra- and inter-molecular stackings and the existence of a very small amount of metal ions not involved with any co-pigments (Mori et al., 2006).

The Himalayan blue poppy is well-known for its beautiful petal color (Fig. 1 upper), and the blue petals can be seen higher than 4000 m above sea level. It has already been reported on in terms petal anthocyanin and flavonols

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doi:10.1016/j.phytochem.2006.03.013

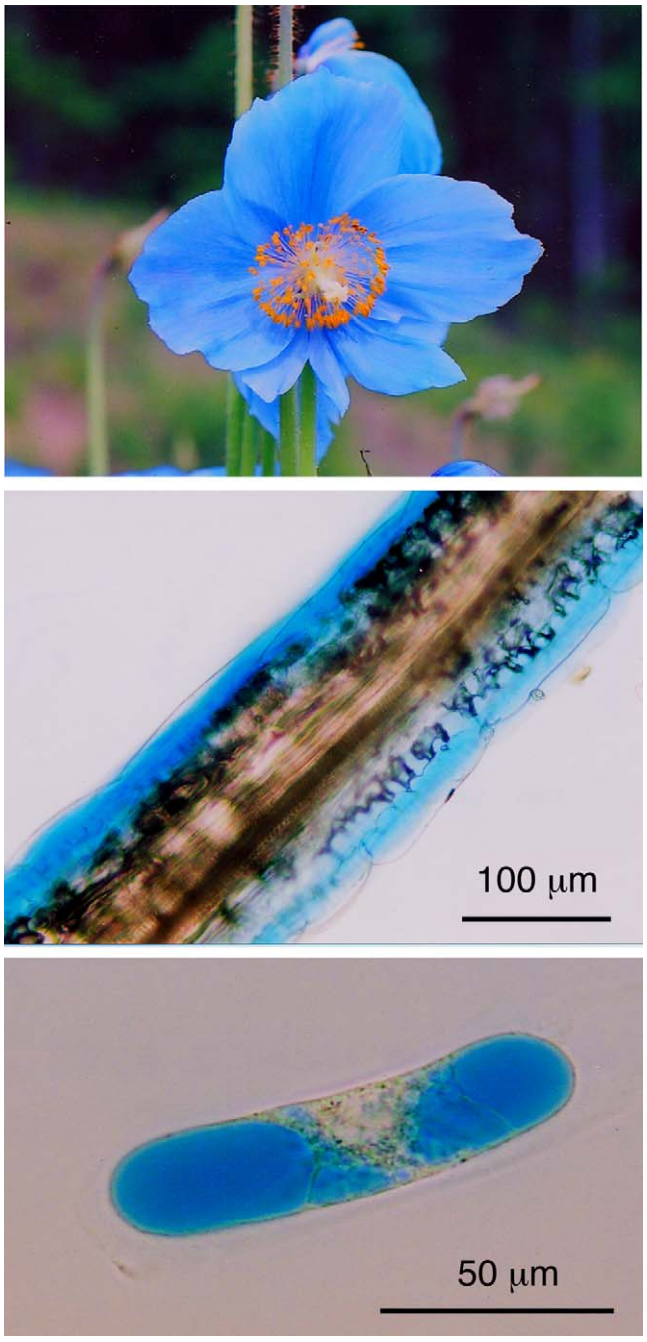


Fig. 1. Blue petals of *Meconopsis grandis* (upper), the transverse section of the petal (middle) and the colored protoplast (lower).

(Takeda et al., 1996; Tanaka et al., 2001). However, the anthocyanin has a cyanidin, not a delphinidin, nucleus like the blue cornflower pigment. (Scheme 1). In our research project on blue flower color development, we have found that the metalloanthocyanin is not responsible for the blue petal color of *Meconopsis grandis*. Moreover, it has no similarity to previously deduced mechanisms for blue color development. Herein, we report the measurement of vacuolar pH and a quantitative analysis of the organic and inorganic components of the colored cells. By the reproduction of the petal color by mixing the petal components, we identified the essential components of blue color development. The involvement of ferric ions in blue color development is discussed.

2. Results and discussion

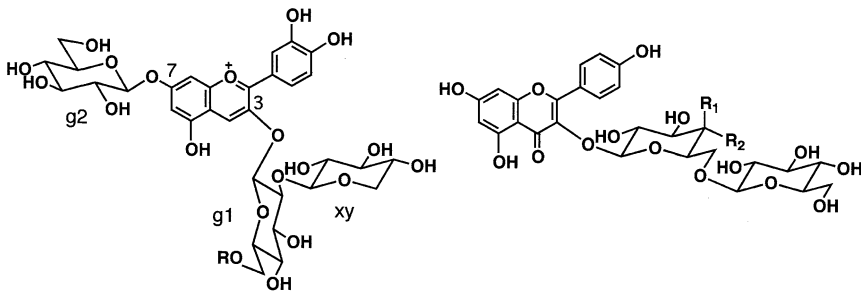
2.1. Visible absorption spectrum and CD of M. grandis petal in vivo

The visible absorption spectrum and CD of a *Meconopsis* petal in vivo were measured using a pre-evacuated petal (see Section 3). As shown in Fig. 2, the absorption spectrum of the blue petals has λ Vismax at 606 and 646 nm, with the λ Vismax being the longest among our recorded data of blue petals, and this spectrum was very different from previously reported metalloanthocyanins (Kondo et al., 1992, 1994, 2001). The petal showed a simple CD with a small negative peak at 552 nm; therefore, this could not be considered an exiton-type Cotton effect that is typical for metalloanthocyanin. These data strongly suggested that the blue pigment in *Meconopsis* petals is not due to metalloanthocyanin.

2.2. Isolation and structural determination of anthocyanin and flavonol components

The blue petals of *M. grandis* were extracted with aqueous acetonitrile (CH_3CN) containing trifluoroacetic acid (TFA) and the extract was analyzed by HPLC. As shown in Fig. 3, the extract contained two anthocyanin (**1** and **2**) and two flavonol peaks (**3** and **4**). Purification of components from the petals (200 g) was conducted according to our general procedure (Yoshida et al., 1996, 1997, 2002, 2003b) to give **1** (5 mg), **2** (25 mg), **3** (45 mg) and **4** (142 mg), respectively.

The MALDI-TOF MS of **1** gave a molecular ion peak at $m/z = 743$ which was 86 mass units smaller than that of **2**, corresponding to a loss of a malonyl residue. The results of 3-D detection-HPLC indicate that **1** and **2** may have the same chromophores. Furthermore, HCl-MeOH treatment of **2** gave **1** via the methyl ester of **2** being very similar in behavior to malonylated anthocyanins (Yoshida et al., 1997). Those results strongly suggested that **1** was a demalonylated compound of **2**. The ^{1}H NMR spectrum showed that **1** has a cyanidin nucleus and three sugars. By 1D-TOCSY experiments irradiating at the anomeric protons clarified the existence of two β-glucopyranosyl residues and one β-xylopyranosyl moiety. The linkages of the sugars were determined by ROESY and HMBC experiments, then the structure of **1** was determined to be 3-*O*-(2-*O*-D-β-xylopyranosyl)-β-D-glucopyranosyl-7-*O*-β-D-glucopyranosylcyanidin as a newly isolated pigment. The structures of **2**–**4** were identified by similar MS and ID and 2D NMR spectroscopic analyses to be the same as previously reported by Takeda et al. in 2001 (Scheme 1).



Scheme 1. Structure of the pigment components in the petals.

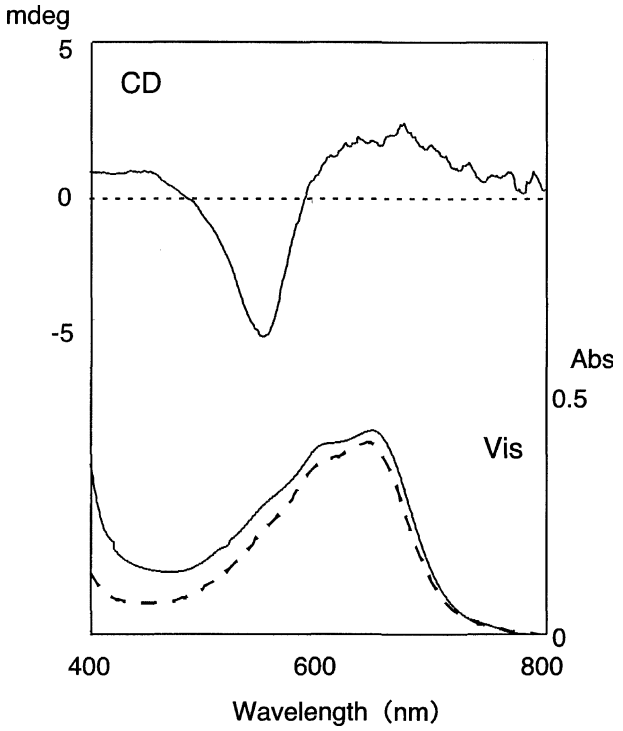


Fig. 2. Vis absorption spectra (lower) and CD (upper) of a blue poppy petal. ——— : petal, ------ : colored protoplast.

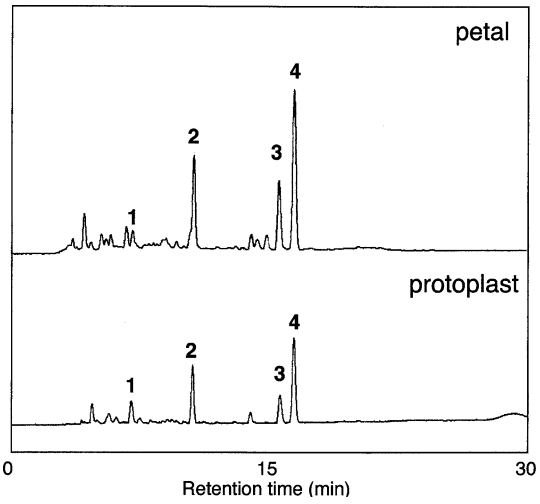


Fig. 3. HPLC chromatograms of the extract of the blue petals of *Meconopsis grandis* (upper) **1** and colored protoplast (lower).

2.3. Vacuolar pH of colored cells

To clarify the intra-vacuolar condition, we measured vacuolar pH using a proton-selective microelectrode. As shown in the transverse section of the petal (Fig. 1, middle), the adaxial and abaxial epidermal layers are colored and the size of the blue cell was ca. 150–200 μm in axis and 30–40 μm in depth. The segmented petal tissue was set on the vessel and a double-barreled electrode was inserted under microscope observation. A total of 18 experiments were succeeded to measure vacuolar pH with active membrane potential. The vacuolar pH was 4.8 ± 0.28 (average ± SD) with an active membrane potential of -71 ± 13.6 mV. These data suggested that the blue color of the poppy is not developed by a vacuolar pH increase to an alkaline range as for blue morning glory (Yoshida et al., 1995, 2005).

2.4. Quantitative analysis of components in colored cells

To analyze the components and their concentrations in colored cells, we first prepared colored protoplasts by enzyme treatment (Yoshida et al., 2003a,b, 2005). Fresh petals were cut and the pieces were added to an incubation medium (0.6 M mannitol, 1% cellulase, 0.1% pectinase, pH 6.3). The mixture was allowed to stand at 5 °C for 13 h, during which time, the cell walls were not completely removed but bean-shaped cells were obtained (Fig. 1, lower). However, further reaction gave no satisfactory results, but the yield of blue protoplasts became lower; therefore, we stopped the reaction at 13 h and the cells were collected. The colored cells were obtained in >95% purity; the rest were colorless protoplasts. The absorption spectrum of the colored cells measured by microspectrophotometry (Yoshida et al., 2003a,b) was identical to that of the petals and no color change occurred during the enzyme treatment (Fig. 2).

Table 1
Contents of anthocyanins, flavonols, and metals in the blue-colored cells[a]

	1	2	3	4	Mg	Fe
(mM)	1.3	3.4	2.8	12.6	14.3	3.6
eq.[b]	1.0		0.6	2.7	3.0	0.8

[a] The data were the average of three samples.
[b] Ratio of each component to total anthocyanin content.

HPLC analysis of the colored cell extract showed a somewhat simple chromatogram compared with that of the petal extract (Fig. 3). However, the composition of **1**–**4** was similar between the petal extract and the colored protoplasts, indicating that most of these flavonoid components were within the epidermal colored cells. The content of each component was quantified by comparison with the peak area of the authentic sample and their molar concentration in the cells was estimated by the calculation of the cell volume and the cell number (Table 1). The concentration of total anthocyanins, **1** and **2**, was ca. 4.7 mM and the molar equivalent of the flavonol, **3** and **4**, to total anthocyanin content was 0.6 and 2.7 eq., respectively.

Preliminary atomic analysis suggested that in the colored cells, Mg and Fe were present, whereas Al, Zn and Mn were not. Since the blue color of protocyanin is developed by a ligand to metal charge transfer (LMCT) between the cyanidin nucleus and Fe^{3+}, the involvement of ferric ions was strongly expected in the blue color development of *M. grandis*. Atomic analysis of the colored cells by induced coupled plasma-atomic absorption spectrometry (ICP-AES) clarified that Fe was contained in the cells at a ratio of 0.8 eq. to total anthocyanins, **1** and **2**.

2.5. Reproduction of blue color by mixing petal components

We attempted to reproduce the same blue color of *Meconopsis* petals by mixing the components in the colored cells. To a buffered solution at pH 5.0 (100 mM acetic acid–sodium acetate buffer), **2** and **4** and various metal ions were added, following which, visible absorption spectrum and CD of the mixtures were measured. By the addition of **4** (2 eq.), Mg^{2+} (5 eq.) and Fe^{3+} (1/6 eq.) to **2** (5 mM), the solution had the same blue color as that of the petals; the visible absorption spectrum and CD were also identical to those of the petals (Fig. 4). However, 1 eq. of **4** to **2** gave a different spectrum, even if Mg^{2+} (5 eq.) and Fe^{3+} (1/6 eq.) co-existed (Fig. 4). Four equivalents of **4** gave the similar blue solution with the same visible absorption spectrum and CD as those of the mixture of 2 eq. of **4**. Without Fe^{3+}, the solution was very unstable and quickly decolorized (Fig. 5). Excess Fe^{3+} (>1/3 eq. to **1**) gave a different blue-black-colored solution (Fig. 5). The mixture of the minor anthocyanin, **1**, with **4** (2 eq. to **1**), Mg^{2+} (5 eq. to **1**) and Fe^{3+} (1/6 eq. to **1**) showed the same blue color with a similar UV/Vis and CD; furthermore, the minor flavonol, **3**, also gave the same result. These data indicate that the malonyl residue of **2** and the structural difference in the sugar residues of flavonols did not affect blue color development. Finally, we dissolved **1**, **2**, **3**, and **4** (2.5 mM: 2.5 mM: 5 mM: 5 mM) with Mg^{2+} (25 mM, 5 eq. to anthocyanin) and Fe^{3+} (0.83 mM, 1/6 eq. to anthocyanin) in the acetate buffer (100 mM, pH 5.0) and were able to reproduce the same color. These results strongly indicate that all the components, 2 eq. of 3-*O*-glycosylflavonol (**3** and/or **4**), 1/6 eq. of Fe^{3+} and an excess of Mg^{2+} are necessary for blue color development of the poppy petals. The difference in Fe^{3+} concentration between the reproduction experiment and the analytical data of the blue cells might be due to the latter representing the combined concentration of the vacuole and the cytoplasm.

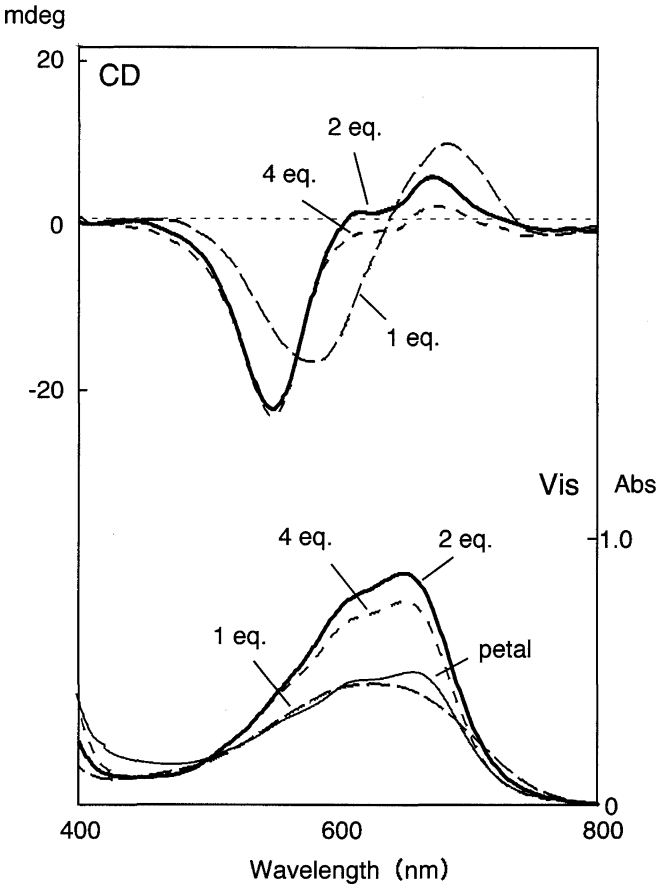


Fig. 4. Reproduction of the blue petal color of *Meconopsis grandis* by mixing **2** and various equivalents of **4** in the presence of Mg^{2+} (5 eq. to **2**) and Fe^{3+} (1/6 eq. to **2**) in a buffered solution (**2**: 5 mM, 100 mM acetate buffer, pH 5.0, path length: 0.1 mm). —— : petal, —— : 1 eq. of **4**, — : 2 eq. of **4**, – – – : 4 eq. of **4**.

The blue colored solution was applied to a gel-filtration column chromatography for purification. However, sephadex G-10 and cellurofine GC-15 m gel column chromatography, which were useful for the purification of metalloanthocyanin (Kondo et al., 1992, 1994, 1998, 2001) were both unsuccessful. The reproduced blue pigment could not be eluted from the column using H_2O as eluant; only a purple-colored fraction was obtained indicating that dissociation of the blue pigment had occurred in the column. These characteristics of blue poppy pigment

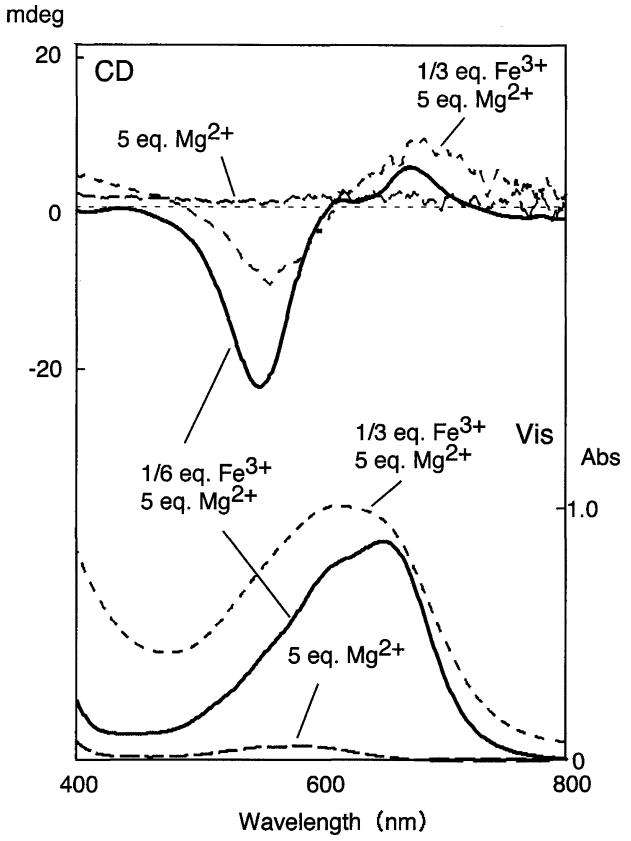


Fig. 5. Visible spectrum and CD of a mixture of **2** and **4** with various metal ions in a buffered solution (**2**: 5 mM, **4**: 10 mM, 100 mM acetate buffer, pH 5.0, path length; 0.1 mm). ——: 1/6 Fe^{3+} and 5 eq. of Mg^{2+} to **2**, – – –: 1/3 Fe^{3+} to **2**, ——: 5 eq. of Mg^{2+} to **2**.

are thus very similar to those of the pigment of blue hydrangea sepals. The blue color of the hydrangea could be developed by anthocyanin, 3-*O*-glucosyldelphinidin, with two or more equivalent of co-pigment, 5-*O*-acylated-quinic acid and Al^{3+} (Takeda et al., 1990; Yoshida et al., 2003b; Kondo et al., 2005). The CD of the hydrangea did not show an exciton-type Cotton effect indicating that there is no self-association between anthocyanidin chromophores, but there is co-pigmentation between the aromatic rings of the anthocyanidin chromophores and that of the 5-*O*-acyl moiety in co-pigments (Kondo et al., 2005). The reproduced hydrangea pigment could not be purified using any kind of chromatography or with a precipitation method (Kondo et al., 2005).

2.6. Concluding remarks

In conclusion, the blue petal color of *M. grandis* is due to a new type of metal complex anthocyanin and the pigment is composed of two or more equivalents of kaempferol derivatives (**3** and **4**), 1/6 eq. of Fe^{3+} and excess Mg^{2+}. Ferric ions (strictly controlled as 1/6 eq. to anthocyanin) are essential for blue color development and chelate the *ortho*-dihydroxy group of the B-ring of the anthocyanin nucleus. Flavonols may stack on both sides of the anthocyanidin nucleus and are stabilized by a co-pigmentation effect. Excess Fe^{3+} gave a different blue-black-colored complex, which was the same in the case of protocyanin (Kondo et al., 1994, 1998). The blue pigment might be composed of a very weak molecular interaction and is only realized in an aqueous solution like that of hydrangea. The similarity of chemical characteristics of the blue pigment of *Meconopsis* and the hydrangea is of interest, and further research is in progress.

3. Experimental

3.1. General

The visible absorption spectra were recorded on a JASCO V-560 spectrometer. The absorption spectra of the colored cells were measured with an inverted microscope (IX70, OLYMPUS) equipped with a micro-spectrophotometer (MCPD-7000, Photal). Circular dichroism (CD) was measured with a JASCO J-720 spectrometer. FABMS data were recorded with a JEOL JMS-700 using glycerol-HCl as a matrix. MALDI-TOF MS data were recorded on a Voyager-DE PRO (PerSeptive Biosystems) using α-cyano-4-hydroxy-cinnamic acid as a matrix. NMR spectra were obtained with a JEOL ECA-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) and JNM-A600 spectrometer (^{1}H: 600 MHz, ^{13}C: 150 MHz) in a 5-mm∅ tube at variable temperatures using 5–10% TFA*d*-CD_3OD as a solvent. Chemical shifts were recorded as parts per million (ppm) with the CD_2HOD resonance as a standard. Analytical and preparative HPLC were conducted according to our procedures (Yoshida et al., 1996, 1997, 2002, 2003b) using an ODS-column (Develosil ODS-HG-5 2.0 mm∅ × 250 mm, 4.6 mm∅ × 250 mm and 20 mm∅ × 250 mm, Nomura Chemical). Analytical HPLC was performed at 40 °C with a 30-min linear gradient elution from 10% to 30% aq. CH_3CN solution containing 0.5% TEA, or an isocratic elution with various concentrations of CH_3CN aq. solution containing 0.5% TFA. Elemental analysis was conducted with a Perkin–Elmer Optima 2100 DV ICP-AES instrument.

3.2. Plant materials

Blue petals of *M. grandis* were kindly provided by Nakamura Farm in Nagano Prefecture.

3.3. Visible absorption spectrum and CD of M. grandis petals in vivo

The absorption spectrum and CD of the petals were recorded with a pre-evacuated tissue (Mori et al., 2006). A fresh petal was cut into squares of ca. 10 mm × 10 mm, and the pieces of petal placed into a flask with water. The flask was evacuated several times under reduced pressure, in order to fill the intercellular space of the petal tissue

with water, thus results in the transparency of the petal segments being increased, following which the petal was fixed on a quartz plate and both the visible absorption spectrum and CD were measured.

3.4. Isolation of anthocyanin and flavonol components

Blue petals (200 g) of *M. grandis* were extracted with 2.0 L of 50% aq. CH_3CN solution containing 2.0% TFA. The extract was evaporated under reduced pressure to one-tenth volume, and the condensed extract was poured into an Amberlite XAD-7 column. The column was eluted with a stepwise gradient elution from 0.5% TFA-H_2O to 50% aq. CH_3CN solution containing 0.5% TFA. The 5–20% aq. CH_3CN fraction was evaporated to give a crude anthocyanin product, whereas the 20–30% aq. CH_3CN fraction gave a crude flavonol fraction. Further purification was conducted by a preparative ODS-HPLC eluted with 8–50% aq. CH_3CN solution containing 0.5% TFA to give **1** (5 mg) and **2** (25 mg) was obtained as dark-red amorphous TFA salts with **3** (45 mg) and **4** (142 mg) as a yellow mass.

3.5. Structure of anthocyanins

3.5.1. 3-O-(2-O-D-β-xylopyranosyl)-β-D-glucopyranosyl-7-O-β-D-glucopyranosylcyanidin (*1*)

UV/VIS (0.1% HCl-MeOH) λmax nm (ε): 524 (19,100), 284 nm (13,500); MALDI-TOF MS: $m/z = 743$ $[M]^+$. ^1H NMR (500 MHz, 5% TFA*d*-CD_3OD): 3.04 (1H, brt, $J = 11.0$, xy-5a), 3.14 (1H, *dd*, $J = 9.0$, 7.5, xy-2), 3.29 (1H, *t*, $J = 9.0$, xy-3), 3.38 (1H, *ddd*, $J = 11.0$, 9.0, 5.0, xy-4), 3.40 (1H, *t*, $J = 9.0$, g2-4), 3.48 (1H, *t*, $J = 9.0$, g1-4), 3.53 (1H, *t*, $J = 9.0$, g2-3), 3.55 (1H, *dd*, $J = 9.0$, 7.5, g2-2), 3.60 (1H, *ddd*, $J = 9.0$, 3.0, 2.0, g1-5), 3.61 (1H, *ddd*, $J = 9.0$, 5.0, 1.5, g2-5), 3.66 (1H, *dd*, $J = 11.0$, 5.0, xy-5b), 3.7 (1H, *dd*, $J = 12.0$, 3.0, g1-6a), 3.71 (1H, *dd*, $J = 12.0$, 5.0, g2-6a), 3.78 (1H, *t*, $J = 9.0$, g1-3), 3.91 (1H, *dd*, $J = 12.0$, 2.0, g1-6b), 3.94 (1H, *dd*, $J = 12.0$, 1.5, g2-6b), 3.970 (1H, *dd*, $J = 9.0$, 7.5, g1-2), 4.75 (1H, *d*, $J = 7.5$, xy-1), 5.19 (1H, *d*, $J = 7.5$, g2-1), 5.50 (1H, *d*, $J = 7.5$, g1-1), 6.84 (1H, *brs*, H-6), 7.02 (1H, *d*, $J = 8.6$, H-5′), 7.26 (1H, *d*, $J = 2.3$, H-8), 8.09 (1H, *d*, $J = 2.3$, H-2′), 8.38 (1H, *dd*, $J = 8.6$, 2.3, H-6′), 8.93 (1H, *brs*, H-4). ROE correlation; H-4 and g1-1, H-6 and g2-1, H-8 and g2-1, g1-2 and xy-1, g1-3 and xy-1. HMBC correlation; g1-2 and xy-1.

3.5.2. 3-O-(6-O-malonyl-2-O-D-β-xylopyranosyl)-β-D-glucopyranosyl-7-O-β-D-glucopyranosylcyanid in (*2*)

UV/VIS (0.1% HCl-MeOH) λmax nm (ε): 524 (19,700), 283 nm (13,300); FABMS: $m/z = 829$ $[M]^+$.

3.5.3. Acid hydrolysis of *2*

2 (TFA salt, 1.0 mg) was dissolved in 1% HCl-methanol (1 mL) and allowed to stand at 40 °C. The reaction mixture was analyzed using a photodiode array detection HPLC repeatedly with a linear gradient elution from 10% aq. CH_3CN to 30% aq. CH_3CN containing 0.5% TFA.

3.6. Preparation of protoplasts

The preparation of colored protoplasts was carried out using a modified method (Yoshida et al., 2003a,b, 2005). Fresh petals (0.5 g) were excised at 3-mm thickness and the pieces added to 10 mL of a reaction medium (0.6 M mannitol, 20 mM MES/Tris, pH 6.3) containing 0.1% (W/V) Macerozyme R-10 (Yakult) and 1.0% (W/V) Cellulase ONOZUKA R-10 (Yakult). The mixture was allowed to stand at 5 °C for 13 h, then filtered through nylon mesh (108 μm) with the filtrate so obtained was centrifuged (100 g × 5 min, 4 °C). The precipitated blue cells were washed three times and suspended in 0.6 M mannitol, with cell numbers counted using a hemocytometer.

3.7. Vacuolar pH measurement

The preparation of proton-selective microelectrodes and the measurement of vacuolar pH were conducted according to the previous reports (Yoshida et al., 2003a,b). All data having a drift of less than 5 mV/5 s and a membrane potential of over −50 mV were adopted.

3.8. Analysis of components in colored protoplasts

An aliquot of the suspension of colored protoplasts was diluted with water containing 0.5% TFA and analyzed by HPLC. The amounts of **1**, **2**, **3** and **4** were calculated directly by a calibration curve obtained by HPLC analysis of the authentic standards. Another aliquot of the suspension of colored protoplasts was diluted with 0.5% (w/v) aq. HNO_3 and Al, Mg and Fe were analyzed by ICP-AES under standard operating conditions.

3.9. Reproduction of petal color

To a buffer solution (100 mM acetic acid–sodium acetate, pH 5.0) was added an aq. solution of flavonol (**3** and/or **4**), $Mg(OAc)_2$ (500 mM) and **1** and/or **2** (neutralized with aq. NH_4OH) and ferric ammonium alum (17 mM) in order at a final concentration of anthocyanin of 5 mM. The UV–Vis spectrum and CD were measured in a quartz cell (path length: 0.1 mm) at 25 °C.

Acknowledgements

We are grateful to Mr. and Mrs. Nakamura of Nakamura Farm for providing the flower petals and Dr. K. Oyama of Research Center for Materials Science, Nagoya University for MS measurement. This work was financially supported by The Ministry of Education, Culture, Sports, Science and Technology, Japan ((B)

No.16370021, The 21st Century COE Program No.14-COEB01-00, Creative Scientific Research No.16GS0206, and Priority Areas No. 17035041).

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