Flavonoid Pigments and Color Expression in the Flowers of Black Hollyhock (Alcea rosea ‘Nigra’)

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Abstract Nine anthocyanins, delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, petunidin 3-O-glucoside, petunidin 3-O-rhamnosylglucoside, malvidin 3-O-glucoside, malvidin 3-O-rhamnosylglucoside and malvidin 3-O-malonylglucoside, three flavonols, myricetin 3-O-glucoside, kaempferol 3-O-rutinoside, kaempferol 3-O-glucoside, and a flavone, luteolin 4′-O-glucoside, were isolated from the black flowers of Alcea rosea ‘Nigra’. We also researched the color expression of these flowers using crude extract, which revealed one absorption maxima, showing that the black color of A. rosea ‘Nigra’ flowers occurs by high accumulation of anthocyanins alone, but not co-pigmentation between anthocyanins and other flavonoids.

Key words: Alcea rosea ‘Nigra’, anthocyanins, black flower, color expression, flavonoids.

Introduction

There are some plants with the so-called black flowers, but they are clearly dark purple or dark red rather than black (Davies, 2009). Major anthocyanins of two ornamental species, pansy (Viola tricolor L.) and tulip (Tulipa gesneriana L.) which put black flowers, have been characterized as delphinidin 3-O-[(4″-p-coumaroyl)lhamnosyl-(1→6)-glucoside]-5-O-glucoside (violalin) from pansy (Hayashi and Takeda, 1962; Takeda et al., 1963; Takeda and Hayashi, 1963a, 1963b, 1965; Goto et al., 1978), and delphinidin 3-O-rutinoside (tulipanin) from tulip (Shibata and Ishikura, 1960). In the case of pansy, it seems that the presence of yellow carotenoids in sub-epidermal cells as the background contributes considerably to the depth of color and the appearance of black (Davies, 2009).

The genus Alcea consists of ca. 60 species primarily of East Mediterranean region (Shaheen et al., 2010). Mostly erect annual, biennial or perennial herbs, sterile pubescent to glabrate (Naqushi et al., 1988). Black hollyhock (Alcea rosea L. ‘Nigra’) has been used as dye (Guinot et al., 2006), because of having dark purple flower such as painted lacquer. The pigments have been reported as the anthocyanins such as 3-O-glucosides and 3,5-di-O-glucosides of delphinidin, petunidin and malvidin (Kohlmunzer et al., 1983). However, further research is necessary to understand the black color expression of A. rosea ‘Nigra’. In this paper, we describe the isolation and identification of the anthocyanins and other flavonoids in the black flowers of A. rosea ‘Nigra’, and the expression of black color in vitro.

Materials and Methods

Plant Materials
The petals of *Alcea rosea* L. ‘Nigra’ were used as plant materials. They were cultivated in Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan (Fig. 1). The flowers with a grayed purple color [Grayed-Purple N186A by Royal Horticultural Society (R. H. S.) Color Chart] were collected in summer of 2005, 2006 and 2011. The samples collected in 2005 and 2006 were dried and preserved in freezer.

**Extraction and isolation of anthocyanins and other flavonoids**

The petals of *A. rosea* ‘Nigra’ were extracted with FM (HCOOH/MeOH = 8:92). The concentrated extracts were applied to preparative paper chromatography (PPC) using solvent systems, BAW (n-BuOH/HOAc/H₂O = 4:1:5, upper phase) and 15% HOAc. Other flavonoids were eluted with MeOH and applied to PPC using solvent system, BEW (n-BuOH/EtOH/H₂O, 4:1:2.2). The obtained compounds were purified by Sephadex LH-20 column chromatography using solvent systems, HOAc/MeOH/H₂O (5:70:25) for anthocyanins and 70% MeOH for other flavonoids. They were applied to preparative HPLC which was performed with Tosoh HPLC systems using Inertsil ODS-4 (I.D. 10 × 250 mm, GL Science Inc., Japan) or Senshu Pak PEGASIL ODS column (I.D. 1.0 × 250 mm, Senshu Scientific Co. Ltd., Japan), at a flow-rate of 3.0 ml min⁻¹; injection of 300–350 μl; detection wavelength of 350 (anthocyanins) or 350 nm (other flavonoids); solvent system, FMW (HCOOH/MeOH/H₂O = 5:15:80) or FMW2 (HCOOH/MeOH/H₂O = 2:20:78).

**High performance liquid chromatography (HPLC)**

HPLC analysis was performed with Shimadzu HPLC system using Inertsil ODS-4 (I.D. 6.0 × 150 mm, GL Science Inc.), at a flow-rate of 1.0 ml min⁻¹, detection wavelength of 350 and 530 nm, column temperature at 40°C with a solvent system comprised of mobile phase A (MeCN/HOAc/H₂O/H₃PO₄ = 6:8:83:3) and...
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B (MeCN/HOAc/H2O/H3PO4 = 10 : 8 : 79 : 3). The samples were eluted with a linear gradient of 0 to 100% of B in A for 10 to 30 min at a flow rate of 0.2 ml/min and then with 100% of B for 30 min.

Liquid chromatograph-mass spectra (LC-MS)
Molecular weight of the compounds was measured by LC-MS using a Senshu Pak PEGASIL ODS column (I.D. 2.0 × 150 mm, Senshu Scientific Co. Ltd.), at a flow-rate of 0.2 ml min⁻¹, detection wavelength of 280 and 350 or 530 and 500 nm, and the eluents was FMW2 or FMW3 (HCOOH/MeCN/H2O = 5 : 15 : 80), ESI⁺ 4.5 kV, ESI⁻ 3.5 kV, 250°C.

UV-Vis absorption spectra
UV-Vis absorption spectra of the isolated flavonoids were measured by a Shimadzu MPS-2000 multipurpose recording spectrophotometer (Shimadzu, Kyoto, Japan) (anthocyanins: 220–700 nm, other flavonoids: 220–500 nm) according to Mabry et al. (1970).

Acid hydrolysis
Acid hydrolysis of the isolated flavonoids was performed in 12% HCl for 30 min at 100°C. After cooling in water, the solution was shaken with diethyl ether. The aglycones (organic layer) and glycosidic sugars (aqueous layer) were applied to HPLC (aglycones) and paper chromatography (sugars) using solvent systems: BBPW (n-BuOH/ benzene/pyridine/H2O = 5 : 15 : 3 : 3) and BTPW (n-BuOH/toluene/pyridine/H2O = 5 : 1 : 3 : 3).

Identification of anthocyanins
The isolated anthocyanins were identified by UV-visible spectra, LC-MS, acid hydrolysis, direct thin layer chromatography (TLC) using solvent systems, BAW, BuHCl (n-BuOH/HCl = 1 : 1), 1% HCl, AHW (HOAc/HCl/H2O = 15 : 3 : 82) and Forestal (HOAc/HCl/H2O = 30 : 3 : 10) and HPLC comparisons with authentic specimens.

Chromatographic and spectroscopic properties of anthocyanins from the flowers of black hollyhock are shown in Table 1.

Identification of other flavonoids
Other flavonoids were identified by UV spectra, LC-MS, acid hydrolysis, direct TLC (BAW, 15% HOAc and BEW) and HPLC comparisons with authentic specimens. TLC, UV and LC-MS data of flavonols and flavone are as follows.

Table 1. Chromatographic and spectroscopic properties of anthocyanins from the black petals of *Alcea rosea* ‘Nigra’

<table>
<thead>
<tr>
<th>Anthocyanins</th>
<th>λ_max (nm)</th>
<th>E440/E_max (%)</th>
<th>Rf</th>
<th>LC-MS</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BAW</td>
<td>BuHCl</td>
<td>AHW</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[M]⁺</td>
<td>Rt (min)</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>277, 541</td>
<td>+</td>
<td>18</td>
<td>0.13</td>
<td>0.05</td>
</tr>
<tr>
<td>A2</td>
<td>278, 542</td>
<td>+</td>
<td>16</td>
<td>0.14</td>
<td>0.05</td>
</tr>
<tr>
<td>A3</td>
<td>281, 531</td>
<td>+</td>
<td>23</td>
<td>0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>A4</td>
<td>282, 536</td>
<td>+</td>
<td>20</td>
<td>0.23</td>
<td>0.11</td>
</tr>
<tr>
<td>A5</td>
<td>278, 539</td>
<td>+</td>
<td>19</td>
<td>0.20</td>
<td>0.04</td>
</tr>
<tr>
<td>A6</td>
<td>278, 540</td>
<td>+</td>
<td>18</td>
<td>0.16</td>
<td>0.03</td>
</tr>
<tr>
<td>A7</td>
<td>279, 539</td>
<td>–</td>
<td>22</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>A8</td>
<td>278, 540</td>
<td>–</td>
<td>20</td>
<td>0.26</td>
<td>0.08</td>
</tr>
<tr>
<td>A9</td>
<td>280, 539</td>
<td>–</td>
<td>22</td>
<td>0.29</td>
<td>0.17</td>
</tr>
<tr>
<td>Deacylated A9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.21</td>
<td>0.09</td>
</tr>
</tbody>
</table>

A1 = Delphinidin 3-O-glucoside, A2 = Delphinidin 3-O-rutinoside, A3 = Cyanidin 3-O-glucoside, A4 = Cyanidin 3-O-rutinoside, A5 = Petunidin 3-O-glucoside, A6 = Petunidin 3-O-rhamnosylglucoside, A7 = Malvidin 3-O-glucoside, A8 = Malvidin 3-O-rhamnosylglucoside, A9 = Malvidin 3-O-malonylglucoside. + = bathochromic shift of Band I with AlCl₃, n.d. = no data.
Results and Discussion

Identification and relative amounts of anthocyanins

Nine anthocyanins from *Alcea rosea* 'Nigra' flowers were characterized as delphinidin 3-O-glucoside (A1), delphinidin 3-O-rutinoside (A2), cyanidin 3-O-glucoside (A3), cyanidin 3-O-rutinoside (A4), petunidin 3-O-glucoside (A5), petunidin 3-O-rhamnosylglucoside (A6), malvidin 3-O-glucoside (A7), malvidin 3-O-glucoside (A7), and delphinidin 3-O-glucoside (A8). The petunidin 3-O-glucoside (A5) concentration was measured using its characteristic UV spectrum at 520 nm. The other anthocyanin concentrations were calculated based on their ratio to petunidin 3-O-glucoside (A5).

In vitro expression of black color

The crude extract was obtained from 0.2 g fresh petal using 4 ml FMWV (HCOOH/MeCN/H2O = 8:20:72) to survey the chromaticity and spectra of black flowers. The extract (0.5 ml) was evaporated and dissolved to 3, 4, 5 and 6 ml McIlvaine’s citrate-phosphate buffer (pH 4.5). The colorimetric value of pigments were measured in vitro by digital single-lens reflex camera Nikon D200 (Nikon Co., Japan) using Mini Color Checker (X-Rite Inc.). The data were then calibrated and calculated using Color Checker Passport software 1.0.2 (X-Rite Inc., Michigan, USA) and Adobe Photoshop (Adobe Systems Inc., California, USA). The data were expressed as \( L^* \), \( a^* \) and \( b^* \) values. The \( L^* \) shows the lightness of the color, ranging from black (\( L^* = 0 \)) to white (\( L^* = 100 \)). The \( a^* \) negative and positive are for green and red, respectively. We also calculated the chroma (C*) and hue-angle (h) values were calculated based on the equations: \( C^* = (a^{*2} + b^{*2})^{1/2} \) and \( h = \tan^{-1}(b^*/a^*) \) (Gonnet, 1998).

UV-visible spectra were also measured on a Shimadzu MPS-2000 multipurpose recording spectrophotometer using 10 mm quartz cell.
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3-O-rhamnosylglucoside (A8) and malvidin 3-O-malonylglucoside (A9) (Fig. 2). Relative amount of each anthocyanin which was measured by HPLC were 17.1% (A1), 18.1% (A2), 1.2% (A3), 1.5% (A4), 5.3% (A5), 15.3% (A6), 6.4% (A7), 16.5% (A8) and 5.8% (A9), and that of each anthocyanidin was cyanidin (2.7%), delphinidin (35.2%), petunidin (20.6%) and malvidin (26.7%). All anthocyanins are glycosylated at 3-position by glucose or rhamnosylglucose. It has been reported that the anthocyanins in A. rosea ‘Nigra’ are 3-O-glucosides and 3,5-di-O-glucosides of delphinidin, petunidin and malvidin (Kohlmunzer et al., 1983; Takeda et al., 1989). However, 3,5-di-O-glucosides of anthocyanidins could not be detected in this experiment. Although the major anthocyanins of black flowers are generally delphinidin glycosides (Davies, 2009), those of A. rosea ‘Nigra’ were constituted with petunidin and malvidin, together with delphinidin. Thus, it was presumed that black flower color of this cultivar is due to co-occurrence of many anthocyanins, because the absorption maxima of each anthocyanin is slightly different and anthocyanins are highly accumulated in petals, so that its absorption region spread in all visible range and the petals are visualized as black color.

Identification of other flavonoids

Four other flavonoids were isolated from the black flowers of A. rosea ‘Nigra’. They were shown to be flavonols (F1-3) and flavone (F4) by UV spectral properties. Their UV spectral properties showed that three flavonols and a flavone are substituted at 3-position and 4′-position, respectively. The sugars were obtained by acid hydrolysis of their glycosides and identified as glucose, or glucose and rhamnose by PC comparison with authentic samples. Finally, these flavonoids were identified as myricetin 3-O-glucoside (F1), kaempferol 3-O-rutinoside (F2), kaempferol 3-O-glucoside (F3) and luteolin 4′-O-glucoside (F4) (Fig. 3) by direct TLC and HPLC comparisons with authentic samples from the fronds of Cyrtomium microindusium Sa. Kurata

<table>
<thead>
<tr>
<th>Anthocyanins</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>OH</td>
<td>OH</td>
<td>glucosyl</td>
</tr>
<tr>
<td>A2</td>
<td>OH</td>
<td>OH</td>
<td>rutinosyl</td>
</tr>
<tr>
<td>A3</td>
<td>OH</td>
<td>H</td>
<td>glucosyl</td>
</tr>
<tr>
<td>A4</td>
<td>OH</td>
<td>H</td>
<td>rutinosyl</td>
</tr>
<tr>
<td>A5</td>
<td>OCH₃</td>
<td>OH</td>
<td>glucosyl</td>
</tr>
<tr>
<td>A6</td>
<td>OCH₃</td>
<td>OH</td>
<td>rhamnoglycosyl</td>
</tr>
<tr>
<td>A7</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>glucosyl</td>
</tr>
<tr>
<td>A8</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>rhamnoglycosyl</td>
</tr>
<tr>
<td>A9</td>
<td>OCH₃</td>
<td>OCH₃</td>
<td>malonylglycosyl</td>
</tr>
</tbody>
</table>

Flavonoids R₁ R₂ R₃
F1 OH OH glucosyl
F2 H H rutinosyl
F3 H H glucosyl

Fig. 2. Chemical structures of anthocyanins (A1-9) isolated from the flowers of Alcea rosea ‘Nigra’.

Fig. 3. Chemical structures of flavonols (F1-3) and flavone (F4) isolated from the flowers of Alcea rosea ‘Nigra’.
(Dryopteridaceae) (Iwashina et al., 2006), the aerial parts of Osyris alba L. (Santalaceae) (Iwashina et al., 2008), the leaves of Phytolacca americana L. (Phytolaccaceae) (Iwashina and Kitajima, 2009), and the leaves of Cirsium confertissimum Nakai (Asteraceae) (Iwashina, unpublished data).

The flavone and flavonols of A. rosea 'Nigra' flowers has already been reported and characterized as kaempferol, quercetin, luteolin and myricetin derivatives (Matlawska, 1992; Dudek et al., 2006). In this experiment, their chemical structures were completely identified except for quercetin derivative which was detected by HPLC, but it could not be isolated for less amount of the compound.

**Flower color expression**

The pH value of the pressed juice from the petals of black hollyhock was estimated to be 4.5. The spectrum of crude extract in buffer solution (pH 4.5) was shown in Fig. 4 and has only one peak at 529.9 nm. On the other hand, isolated delphinidin 3-O-glucoside (A1), which is one of main anthocyanin in A. rosea 'Nigra' flowers, showed the absorption maxima at 524.1 nm. In wild black lisianthus (Lisianthus nigrescens R.E.Weaver), it had been presumed that flower color expression is due to higher concentration of anthocyanins and also their co-pigmentation with flavones such as apigenin and luteolin 8-C-glucosides and their 7-methyl ethers (Markham et al., 2004). On the other hand, we presumed that the petals of black hollyhock mainly occurs by higher accumulation of many anthocyanins, but not co-pigmentation between anthocyanins and other substances.

Chromaticity values of *in vitro* expression were shown in Table 2. The chromaticity value revealed the higher shift of lightness (\(L^*\)), redness (\(a^*\)) and chroma (\(C^*\)), together with decrease of concentration. The darkest and dull-est sample is 50 mg fresh weight/3 ml (\(L^* = 6.55, C^* = 1.9\)), and conversely the lightest and most vivid ones is 50 mg fresh weight/6 ml (\(L^* = 38.26, C^* = 32.0\)). These means that the black flowers depend on higher accumulation of the anthocyanins in petals.

In the black fruits and leaves of red pepper (Capsicum annuum L.), chlorophylls contribute to their colors, in addition to high concentration of anthocyanins and carotenoids (Lightborn et al., 2008). Although we extracted carotenoids and chrollophylls from the black flowers of A. rosea 'Nigra' using 80% acetone, but these pigments were hardly contained in the flowers (data not shown).

**Table 2. Chromaticity of *in vitro* expression from the black petals of Alcea rosea 'Nigra'.**

<table>
<thead>
<tr>
<th></th>
<th>(L^*)</th>
<th>(a^*)</th>
<th>(b^*)</th>
<th>(h)</th>
<th>(C^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg FW/3 ml</td>
<td>6.55 ± 0.18</td>
<td>1.79 ± 0.44</td>
<td>0.71 ± 0.13</td>
<td>21.7</td>
<td>1.9</td>
</tr>
<tr>
<td>4 ml</td>
<td>12.95 ± 1.30</td>
<td>11.64 ± 2.65</td>
<td>3.46 ± 0.63</td>
<td>16.5</td>
<td>12.1</td>
</tr>
<tr>
<td>5 ml</td>
<td>24.46 ± 2.07</td>
<td>25.36 ± 2.74</td>
<td>5.58 ± 1.07</td>
<td>12.4</td>
<td>26.0</td>
</tr>
<tr>
<td>6 ml</td>
<td>38.26 ± 3.11</td>
<td>31.75 ± 2.60</td>
<td>3.68 ± 0.79</td>
<td>6.6</td>
<td>32.0</td>
</tr>
</tbody>
</table>

\(L^*\) = lightness; \(a^*\) and \(b^*\) = chromatic components; \(h\) = hue angle (°); \(C^*\) = chroma.

Mean values ± SE (n = 3). FW = fresh weight.

**Fig. 4.** Visible spectrum of *in vitro* color expression of the crude extract of Alcea rosea 'Nigra' petals in pH 4.5 buffer.
References


