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Change of color and components in sepals of chameleon hydrangea during maturation and senescence

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ABSTRACT

The sepal color of a chameleon hydrangea, *Hydrangea macrophylla* cv. Hovaria™ 'Homigo' changes in four stages, from colorless to blue, then to green, and finally to red, during maturation and the senescence periods. To clarify the chemical mechanism of the color change, we analyzed the components of the sepals at each stage. Blue-colored sepals contained 3-*O*-sambubiosyl- and 3-*O*-glucosyldelphinidin along with three co-pigments, 5-*O*-*p*-coumaroyl-, 5-*O*-caffeoyl- and 3-*O*-caffeoylquinic acids. The contents of glycosyldelphinidins decreased toward the green-colored stage, with a coincident increase in the number of chloroplasts. During the last red colored stage, the two species of 3-*O*-glycosyldelphinidin almost disappeared, and another two anthocyanins, 3-*O*-sambubiosyl- and 3-*O*-glucosylcyanidin, increased in amounts. Mixing of 3-*O*-glycosylcyanidins, co-pigments, and Al^{3+} in a buffered solution at pH 3.0–3.5 gave not a blue, but a red, colored solution that was the same as that of the sepal color of the 4th stage. Sepals of hydrangea grown in an highland area also turned red in autumn, and contained the same cyanidin glycosides. The red coloration of the hydrangea during senescence was due to a change in anthocyanin biosynthesis.

1. Introduction

The sepal color of *Hydrangea macrophylla* is famous for its color changes under different cultivation conditions. In the maturation period, 3-*O*-glucosyldelphinidin is the only anthocyanin component in sepals of any color, including blue, purple and red (Hayashi and Abe, 1955; Asen et al., 1957b; Yoshida et al., 2003). Hydrangeas cultivated in an acidic soil have blue sepals, and those in an alkaline soil have red sepals (Allen, 1932, 1943; Chenery, 1937; Okada and Okawa, 1974). These phenomena are explained by the fact that in acidic soil, the aluminum ion is soluble, and can thus be absorbed and transported to the sepals, where Al^{3+} complexes with anthocyanin resulting in a blue color (Asen and Siegelman, 1957a; Takeda et al., 1990; Yoshida et al., 2003; Kondo et al., 2005; Toyama-Kato et al., 2007). In contrast, the aluminum ion becomes insoluble under alkaline conditions (Atkins, 1923; Okada and Okawa, 1974); therefore, the sepals become red (Allen, 1943; Toyama-Kato et al., 2003; Yoshida et al., 2003). Sepal color development is not determined by soil acidity alone; it is also affected by several other factors, such as the composition of the co-pigment, content of Al^{3+}, and vacuolar pH (Yoshida et al., 2003; Kondo et al., 2005; Toyama-Kato et al., 2007). However, there are still several unanswered questions about the causes of color variation and the change of hydrangea sepals.

H. macrophylla cv. Hovaria™ 'Homigo' is known as the chameleon hydrangea, because its sepals change color four times during maturation and senescence, from colorless to blue, then to green, and finally to cherry-red. To clarify the mechanism of color change of the chameleon hydrangea, we analyzed components at each color stage and determined the structure of sepal pigments and co-pigments. We also studied the sepal color in the blue and red stages by mixing the sepal components. Furthermore, the same color changes were found in hydrangea sepals grown in an highland area; therefore, the components in these sepals were also analyzed. Herein, the mechanism of the development of the red coloration of hydrangea during its maturation period is discussed.

2. Results and discussion

2.1. Change of sepal color of chameleon hydrangea

Sepals of the chameleon hydrangea, *H. macrophylla* cv. Hovaria™ 'Homigo', change in color during cultivation, as shown in Fig. 1. At first, the sepals were colorless (1st stage), then they became blue and maintained this color for about 1 month (2nd stage). In the next month, the blue color then faded and became green (3rd

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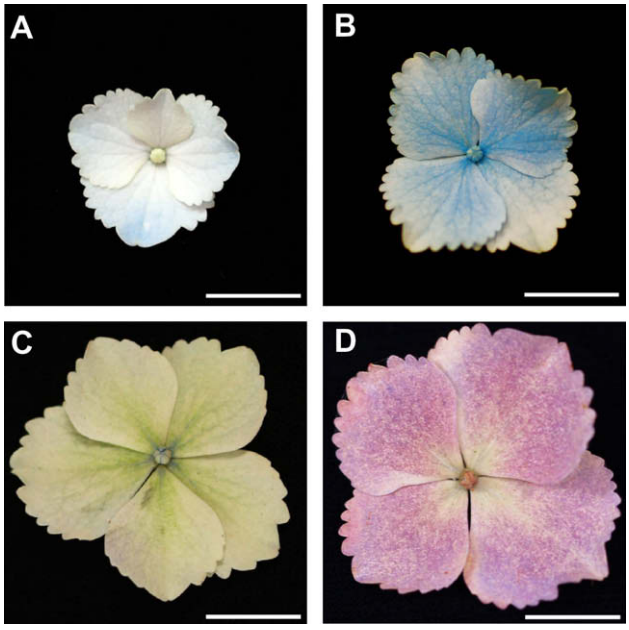


Fig. 1. Color change in sepals of *H. Macrophylla* cv. Hovaria™ 'Homigo' during maturation and senescence periods. A: 1st stage, B: 2nd stage, C: 3rd stage, D: 4th stage. Bar: 1.0 cm.

stage); finally, in the next month, the sepal color changed to dark red (4th stage).

The VIS absorption spectra of each sepal were recorded using evacuated tissue (Mori et al., 2006; Yoshida et al., 2006) (Fig. 2). The blue sepal at the 2nd stage showed the same broad spectrum with a λvismax at 579 nm as for the other blue hydrangea sepals (Yoshida et al., 2003; Kondo et al., 2005; Toyama-Kato et al., 2007). The spectrum of the sepals at the 3rd green stage showed a decrease in the peak at 579 nm, and a sharp peak appeared at 680 nm (Fig. 2). At the 4th stage, the spectrum of the sepal showed a broad peak with a λvismax at 561 nm, with a small peak at 680 nm (Fig. 2).

The transverse sections of the sepals at each stage were observed by microscopy (Fig. 3). At the 1st stage, almost all of the cells were colorless. At the 2nd stage, blue colored cells were

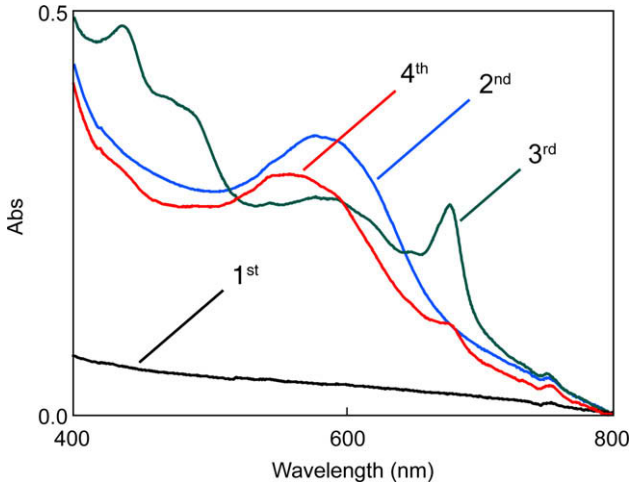


Fig. 2. Absorption spectra of evacuated sepals of *H. Macrophylla* cv. Hovaria™ 'Homigo' at each growing stage. Black line: 1st stage, blue line: 2nd stage, green line: 3rd stage, red line: 4th stage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed mainly in the second layer from the adaxial epidermis, and in the blue colored cells, pigments were found in the vacuoles, as previously reported (Yoshida et al., 2003). However, at the 3rd stage, the blue color of the cells became faint, and chloroplasts appeared in the cytoplasm of the cells in the second and inner layers. At the 4th stage, the vacuoles of the second layer became red, and while the number of chloroplasts decreased, some chloroplasts remained.

2.2. HPLC analysis of sepal components

The sepals at each stage were extracted with aq. CH_3CN containing 1% TFA, and the extracts were analyzed by 3D-detection ODS-HPLC (Fig. 4). The chromatograms, showing absorbance at 530 nm, detected changes in anthocyanin components in the sepals during the maturation and senescence periods. At the 1st stage, there was almost no pigment in the sepals, and at the 2nd stage, one major anthocyanin pigment (**2**) was observed with two minor pigments (**1** and **4**) (see Figs. 4 and 5). During the sepal color change to green near the 3rd stage, the amounts of **1** and **2** decreased and **4** appeared. At the 4th stage, **1** and **2** almost disappeared and instead, one new peak (**3**) appeared with **4** remaining as well.

The spectra of peaks **3** and **4,** obtained by 3D-detection, suggested that both had the same chromophore, which was different from that of **1** and **2**. The structures of **1** and **2** were identified as 3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranosyldelphinidin (Du and Francis, 1974; Toyama-Kato, 2006) and 3-*O*-β-D-glucopyranosyldelphinidin (Yoshida et al., 1996), respectively, by comparison with authentic samples (Fig. 5).

The HPLC chromatogram (detected at 280 nm) at cell growing periods resulted in detection of peaks **5**–**7**, which corresponded to the known co-pigments, neochlorogenic acid (**5**), *p*-coumaroylquinic acid (**6**), and chlorogenic acid (**7**) (Hayashi and Abe, 1955; Asen et al., 1957b; Takeda et al., 1985; Yoshida et al., 2003), respectively; however, their ratios changed during maturation and senescence periods.

2.3. Isolation and structural determination of anthocyanins

To determine the structures of **3** and **4**, both pigments were purified and analyzed. The frozen sepals at the 4th stage (400 g) were extracted with 3 L of 1% TFA-50% aq. CH_3CN, and the extract was repeatedly purified with Amberlite XAD-7 column chromatography. The column was eluted with aq. CH_3CN containing TFA to give a crude pigment fraction, which was purified by chromatography again with preparative ODS-HPLC (isocratic elution with 13%, or 15% aq. CH_3CN containing TFA) according to our general procedure (Yoshida et al., 1996; Mori et al., 2006; Yoshida et al., 2006) to give **3** (8.2 mg) and **4** (10.4 mg) as dark red TFA salts.

The structures of **3** and **4** were determined by ESI-TOF MS and various 1D and 2D NMR spectroscopic analyses. The ESI-TOF MS spectra of **3** and **4** indicate that both complexes had the same cyanidin nucleus with two or one sugar residues, respectively. By NMR (1D-TOCSY, COSY and ROESY) (Mori et al., 2006), the structure of **3** was determined to be 3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranosylcyanidin (cyanidin 3-sambubioside) (Reichel and Reichwald, 1977; Andersen et al., 1991). The structure of **4** was identified to be 3-*O*-β-D-glucopyranosylcyanidin (Yoshida et al., 1996). This is the first time that both of these anthocyanins were found in hydrangea.

2.4. Quantitative analysis of sepal components during senescence

The contents of **1**–**7** in sepals at each stage were quantified by HPLC using authentic samples. The major pigment at the 2nd stage

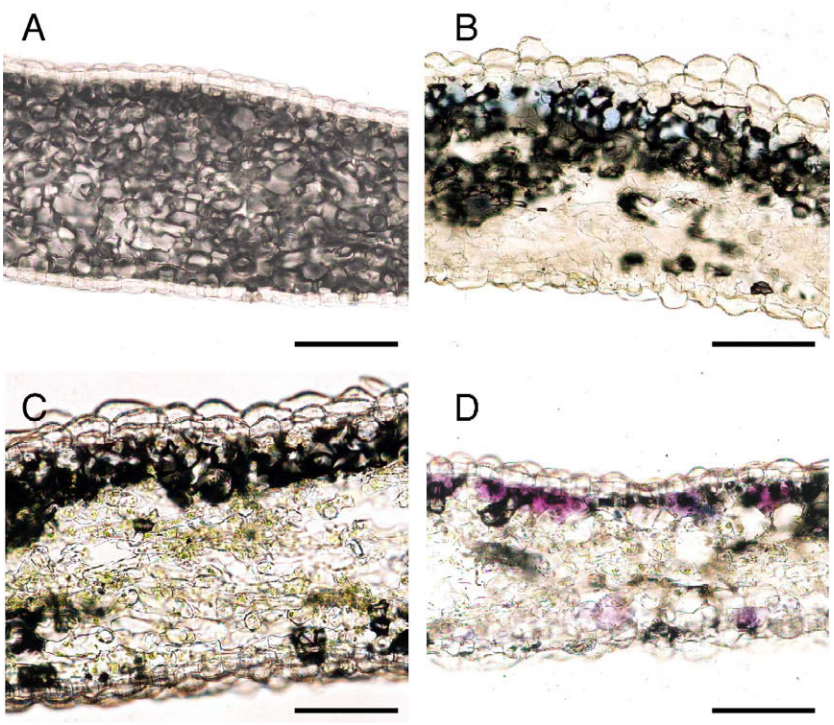


Fig. 3. Transverse section of the sepals of *H. Macrophylla* cv. Hovaria™ 'Homigo' at each growing stage. A: 1st stage, B: 2nd stage, C: 3rd stage, D: 4th stage. Bar: 100 μm.

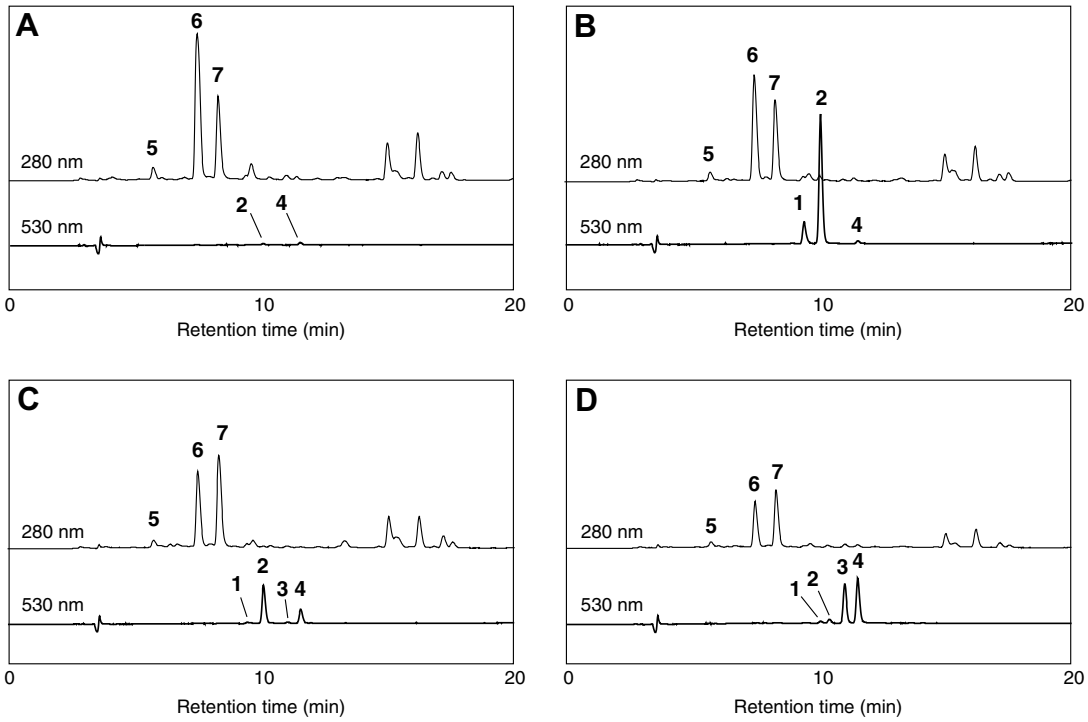
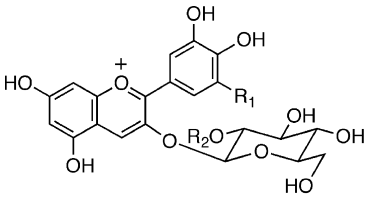
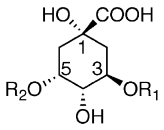


Fig. 4. HPLC chromatograms of the sepal extracts at each stage of *H. Macrophylla* cv. Hovaria™ 'Homigo'. A: 1st stage, B: 2nd stage, C: 3rd stage, D: 4th stage.

was **2**, and the content of anthocyanins with a delphinidin nucleus was ca. 0.3 μmol/g FW. However, the amounts of **1** and **2** decreased near senescence, and at the 4th stage, there was less than 0.1 μmol/g FW of each. In contrast to **1** and **2**, the amounts of **3** and **4** during the 1st and the 2nd stages were lower than 0.07 μmol/g FW, but increased toward the 4th stage: the contents of those pigments with the cyanidin chromophore were ca. 0.13 μmol/g FW at the 3rd stage, and more than 0.65 μmol/g FW at the 4th stage. During all stages, the amounts of **5** and **7** were steady, around 0.8 and 5 μmol/g FW, respectively; however, the amount of **6**, 5-*O*-*p*-coumaroylquinic acid, decreased during the growing period from 6.9 μmol/g FW at the 1st stage to 2.6 μmol/g FW at the 4th stage. Therefore, the major co-pigment component at the 1st stage was **6**, and later changed to 7 (chlorogenic acid) during senescence.



1 : R_1= OH , R_2= β-xylopyranosyl
2 : R_1= OH , R_2= H
3 : R_1= H , R_2= β-xylopyranosyl
4 : R_1= H , R_2= H



5 : R_1= H , R_2= caffeoyl
6 : R_1= H , R_2= *p*-coumaroyl
7 : R_1= caffeoyl , R_2= H

Fig. 5. Structures of components in hydrangea sepals, of *H. Macrophylla* cv. Hovaria™ 'Homigo'.

2.5. Reproduction of blue and red colors by mixing components

We studied the difference in the mechanisms of the blue and red colorations in the sepals at the 2nd and 4th stages by mixing the sepal components in vitro. Since the different sugar residues, 3-glucoside and 3-sambubioside, in the anthocyanin did not affect the blue coloration by Al^{3+}-complexation (Toyama-Kato, 2006), compounds **2** and **4** were used for reconstitution experiments. The concentration of components was determined by HPLC analysis of the sepals as outlined in our previous reports (Yoshida et al., 2003; Kondo et al., 2005; Toyama-Kato et al., 2007). The concentration of anthocyanin components (**2** or **4**) were 1 mM, and that of the co-pigment components, **5** and **7**, were determined to be 1 mM and 5 mM, whereas, that of **6** and Al^{3+} ranged from 1–3 mM and 0–5 mM, respectively. Since the vacuolar pH of colored hydrangea cells ranged from 3 to 4 (Yoshida et al., 2003), we carried out the reconstitution experiments in buffered solution at pH 3.0, 3.5 or 4.0. All the components were mixed, and the UV–VIS absorption spectrum was recorded in a cuvette (path length: 0.5 mm).

At first, the reproduction of the blue color at the 2nd stage was investigated. The VIS spectra of the mixture of **2**, **5**, and **7** together with addition of 3 mM of **6** and various concentrations of Al^{3+} are shown in Fig. 6. At pH 3.0, the solution with or without Al^{3+} did not reproduce the blue color, but reddish (λvismax at 521 nm) and reddish-purple (λvismax at 571 nm) colors were obtained, respectively. However, at pH 3.5 with 3 mM of Al^{3+}, the solution became the same blue color (λvismax: 579 nm); furthermore, at pH 4.0 the addition of only 1 mM of Al^{3+} gave the same blue color (λvismax: 580 nm).

Next, reproduction of the red color at the 4th stage was investigated. The VIS spectra of the mixture of **4**, **5** and **7** with addition of 3 mM or 1 mM of **6** with Al^{3+} are shown (Fig. 7). In contrast to the results of **2** (Fig. 6), anthocyanin **4** with the cyanidin chromophore gave a different color as a redder colored solution was obtained under the same conditions. At pH 3.0, the mixture of **4**, **5** and **7** with **6** (3 mM) without Al^{3+} was red (λvismax: 517 nm), but after the addition of Al^{3+} (5 mM), the mixture became bluer (λvismax at 559 nm) i.e., the same color as that of the sepals at the 4th stage (Fig. 7A). When the components were mixed in the same ratios at pH 3.5, the λvismax of the solution shifted toward blue at 565 nm. However, with 1 mM of Al^{3+} at pH 3.5, the same red solution (λvismax at 560 nm) was obtained (Fig. 7A). As observed in the quantitative analysis of sepal components (Table 1), the content of **6** decreased during senescence; therefore, the reconstitution experiments were carried out with 1 mM of **6** (Fig. 7B). At pH

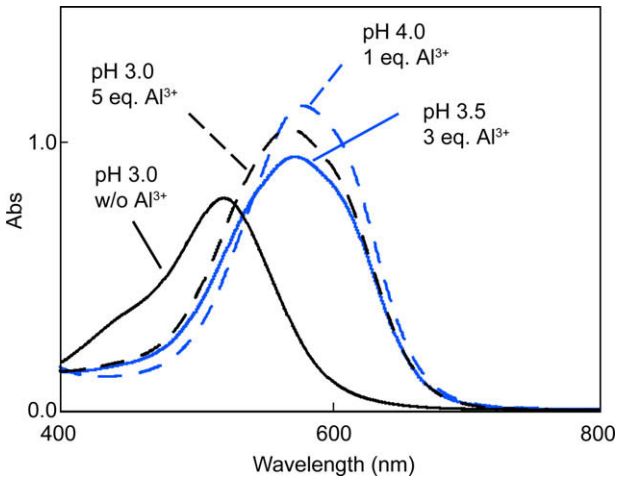


Fig. 6. VIS spectra of reproduction solutions mixed with **2** (1 mM), **5** (1 mM), **6** (3 mM), **7** (5 mM), with/without Al^{3+} (0–5 mM) at various pH buffered solutions (path length: 0.5 mm). Black line: without (w/o) Al^{3+} at pH 3.0, dotted black line: with Al^{3+} (5 mM) at pH 3.0, blue line: with Al^{3+} (3 mM) at pH 3.5, dotted blue line: with Al^{3+} (1 mM) at pH 4.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

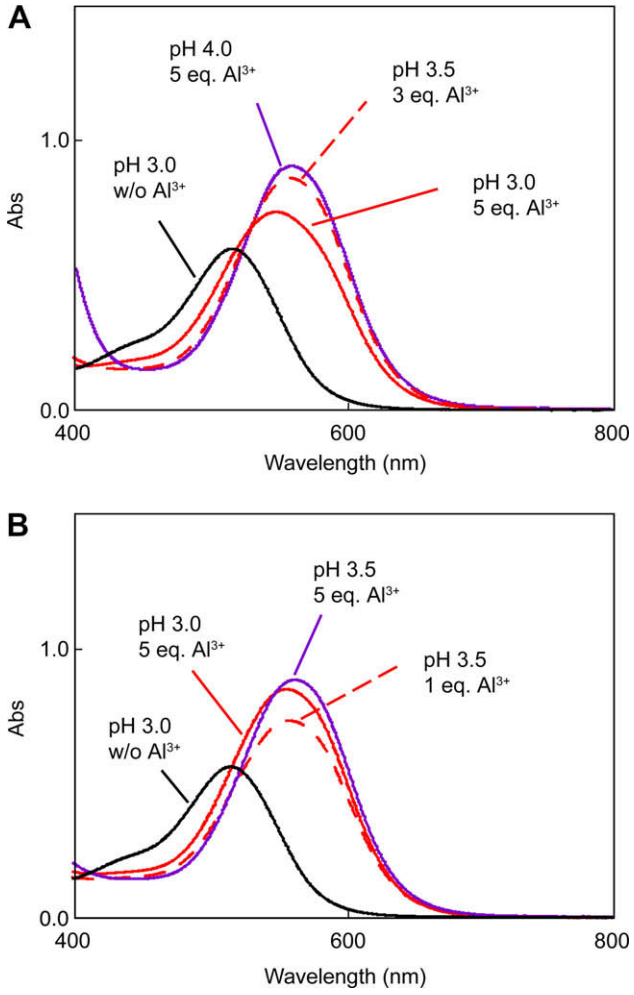


Fig. 7. VIS spectra of reproduction solutions mixed with **4** (1 mM), **5** (1 mM), **7** (5 mM), with 3 mM of **6** (A) and 1 mM of **6** (B) with/without Al^{3+} (0–5 mM) at various pH buffered solutions (cell length: 0.5 mm). (A) Black line: without (w/o) Al^{3+} at pH 3.0, red line: with Al^{3+} (5 mM) at pH 3.0, dotted red line: with Al^{3+} (1 mM) at pH 3.5, purple line: with Al^{3+} (5 mM) at pH 3.5. (B) Black line: without (w/o) Al^{3+} at pH 3.0, red line: with Al^{3+} (5 mM) at pH 3.0, dotted red line: with Al^{3+} (3 mM) at pH 3.5, purple line: with Al^{3+} (5 mM) at pH 4.0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Change of anthocyanins (**1**–**4**) and co-pigments (**5**–**7**) in sepals during maturation and senescence

	Content (μmol/g FW)[a]			
	1st	2nd	3rd	4th
1	N.D.[b]	0.077 ± 0.013	0.009 ± 0.002	0.007 ± 0.012
2	0.044 ± 0.006	0.227 ± 0.021	0.110 ± 0.018	0.080 ± 0.034
3	N.D.[b]	N.D.[b]	0.040 ± 0.027	0.231 ± 0.047
4	0.060 ± 0.021	0.063 ± 0.011	0.090 ± 0.008	0.426 ± 0.211
5	0.868 ± 0.171	0.959 ± 0.055	0.934 ± 0.135	0.581 ± 0.164
6	6.90 ± 0.261	5.43 ± 0.195	3.51 ± 0.372	2.56 ± 0.305
7	4.84 ± 1.25	5.67 ± 0.353	6.22 ± 0.733	5.20 ± 1.89

[a] Mean ± S.D. (n = 3).
[b] Not detected.

3.0, the same red color was not obtained even when Al^{3+} was increased to 5 mM (λvismax: 550 nm). However, when 3 mM of Al^{3+} was added to the mixture at pH 3.5, the red color of the sepals was obtained (λvismax: 561 nm). Furthermore, at pH 4.0, only 1 mM of Al^{3+} was needed to produce the same red color, and with the addition of 5 mM Al^{3+}, almost the same red color (λvismax at 562 nm) was obtained. Therefore, both co-pigments (**5**–**7**) and Al^{3+} were essential for red color development by anthocyanins with a cyanidin chromophore in the sepals of chameleon hydrangea.

2.6. Red coloration of usual hydrangea sepals

Sepals of usual cultivars of *H. macrophylla* become brown during the senescence period, and then die. However, some wild blue hydrangeas that are grown in highland areas can survive over the summer and turn red in autumn (Fig. 8A). To clarify the correlation between red coloration of the wild hydrangeas and chameleon hydrangea, we collected sepals at the Chausuyama Highland (ca. 1000 m above sea level) and analyzed the components. The blue sepals collected in early summer only contained **2** (Fig. 8B), and the red sepals (λvismax: 550 nm) collected in autumn contained **3** and **4** as the major pigments (Fig. 8C). Those results indicate that usual hydrangea also exhibit the same red-coloration if the sepals are able to survive until autumn.

2.7. Considerations on sepal color change of chameleon hydrangea

The above-mentioned observations indicate that the blue color at the 2nd stage develops via a mechanism similar to that of other blue hydrangea sepals: an unstable supramolecular metal-complex pigment composed of anthocyanins with a delphinidin chromophore (**1** and **2**), 5-*O*-acylquinic acid (**5**, **6**) and Al^{3+} (Takeda et al., 1990; Yoshida et al., 2003; Kondo et al., 2005; Toyama-Kato, 2006; Toyama-Kato et al., 2007). During maturation, the blue sepal color faded because of degradation of **1** and **2**, then, the green color by chloroplasts was developed. This may be similar to the phenomenon that occurs in juvenile leaves, which are frequently red due to accumulation of anthocyanin to protect against UV radiation, before turning green by synthesis of chlorophyll to start photosynthesis (Chalker-Scott, 1999; Shirley, 1996; Gould et al., 2000; Oughham et al., 2005).

After the 3rd stage, the senescence programs of the sepals may begin; therefore, degradation of chlorophyll and salvaging of nitrogen might occur and simultaneous synthesis of anthocyanins in the sepals starts, producing an autumn like color. Autumnal red leaves contain very simple anthocyanins, such as mono- or di-glycosylcyanidins; without regard to plant species (Hayashi and Abe, 1955; Ishikura, 1972). This suggests that the red color change of leaves by *de novo* synthesis of anthocyanins may be controlled by a general metabolic program, although the full mechanism is still unknown. This gives a reasonable explanation for the red coloration of hydrangea sepals with anthocyanins containing a cyanidin chromophore (**3** and **4**) at the 4th stage. During maturation, the Al^{3+} content in sepals may increase (Toyama-Kato et al., 2003), and in chameleon hydrangea, the content of **6** decreases (Table 1, Fig. 4). In the pH range of 3.0–4.0, the same red color as that of the 4th stage was reproduced.

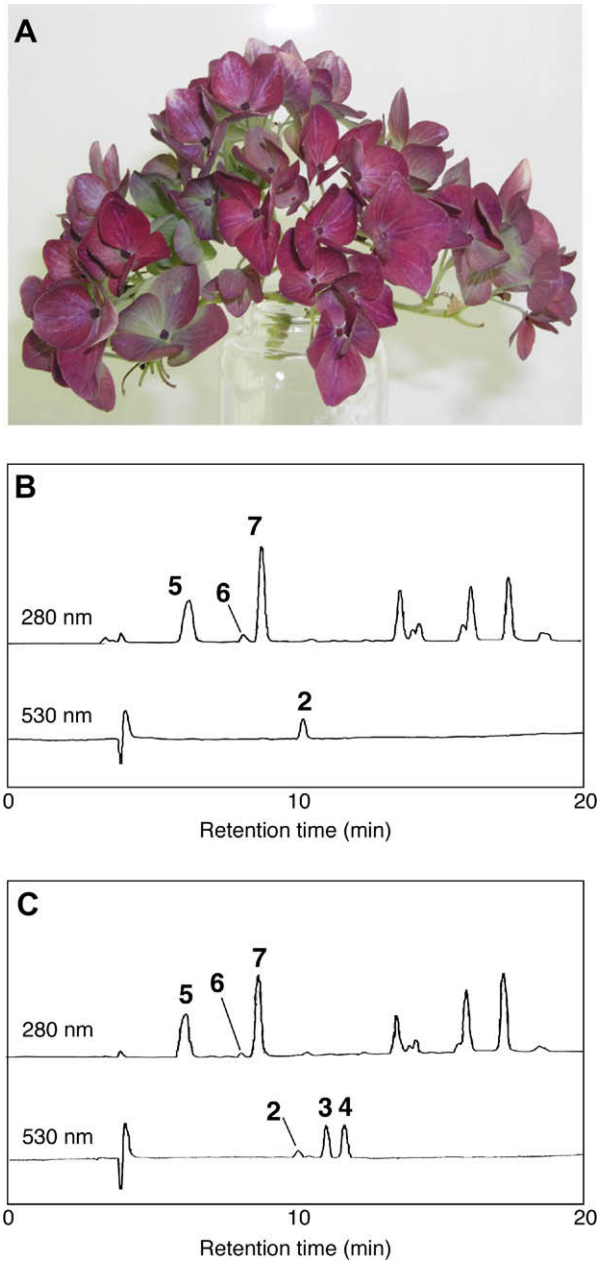


Fig. 8. Red coloration of wild hydrangea with the change of sepal components. (A) Red sepals of wild hydrangea collected at Chausuyama Highland in autumn. (B) HPLC chromatogram of extract of blue sepals collected in the early summer. (C) HPLC chromatogram of extract of red sepals collected in autumn.

3. Conclusion

The red coloration of sepals of chameleon hydrangea cv. Hovaria™ 'Homigo' was not caused by the same mechanism as that of other red cultivars at the maturation period, but was instead due

to *de novo* synthesis of pigments with the cyanidin chromophore. A similar red-color change was observed in sepals of wild *H. macropylla* in a highland area; therefore, these phenomena might be common. The mechanism and biological purpose of this red coloration may a topic of future studies.

4. Experimental

4.1. General

VIS spectra were recorded on a JASCO V-560. NMR spectra were obtained with a JEOL ECA-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) in a 5-mm ϕ tube at variable temperature 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 standard. ESI-TOF MS data were recorded on a PE Biosystems QSTAR and analyzed with PE SCIEX Analyst QA software. Analytical HPLC was conducted using two Jasco PU-1585 pumps, a Jasco MD-1515 photodiode-array detector, Jasco CO-1565 oven, with a Develosil ODS-HG-5 column (2.0 ϕ × 250 mm, Nomura Chemical). The column was eluted with 30-minute linear gradient elution from 10% to 30% aq. MeCN solution containing 0.5% TFA at 40 °C.

4.2. Plant materials

A chameleon hydrangea, *H. macrophylla* cv. Hovaria™ 'Homigo', was purchased from Chigusa Engei Co. Ltd., Otsu, Japan, in 2004 and 2007. The plants were cultivated in an incubator (under light and dark conditions: 12 h light (15,000 lx) at 23 °C and 12 h dark at 18 °C until the 2nd stage. During the 3rd and 4th stages, the temperature was controlled at 26 °C in light (15,000 lx) and at 21 °C in dark conditions. For isolation of pigments, red colored sepals at the 4th stage were stored at −30 °C until use. Sepals of wild hydrangea, *H. macrophylla,* were collected at Chausuyama Highland in Toyone village, Aichi prefecture, Japan in June through September of 2003.

4.3. VIS absorption spectrum of sepal

VIS absorption spectra of sepals were recorded with a pre-evacuated tissue according to our previously reported procedure (Mori et al., 2006).

4.4. Microscopic observation of transverse section of sepal

Fresh sepals were cut into ca. 10 × 10 mm squares, and a piece was placed between the two halves of a split elder stem and cut using a Nippon Ikakikai MT-3 plantmicrotome. The obtained transverse section was observed under an Olympus BX50WI microscope.

4.5. Extraction and HPLC analysis of anthocyanins from sepals

Small pieces of cut sepals were frozen with liq. N_2, and then crushed. To each crushed mass, a solution of CH_3CN:H_2O containing 1% TFA (1:1, v/v) was added, with the mixture allowed to stand at rt for 3 h. The extract was then analyzed by HPLC using the condition described in 4.1.

4.6. Isolation of anthocyanins (**3**, **4**) from sepals at the 4th stage

Frozen red colored sepals at the 4th stage (400 g) were extracted with CH_3CN:H_2O containing 1% TFA (3 L, 1:1, v/v). The extract was evaporated under reduced pressure to ∼300 mL, then, the concentrated extract was purified with an Amberlite XAD-7 column. The column was eluted with a stepwise gradient elution from 0.3% TFA-H_2O to CH_3CN:H_2O (50:50, v/v) containing 0.3% TFA. The 15% CH_3CN aq. fraction containing 0.3% TFA was evaporated to give a crude pigment (0.9 g), which was applied to an Amberlite XAD-7 column, eluted with a stepwise gradient of 0.3% TFA-5% aq. CH_3CN to 50% aq. CH_3CN solution containing 0.3% TFA. Crude compounds **3** (200 mg) and crude **4** (120 mg) were obtained from 13% and 15% CH_3CN aq. containing 0.3% TFA elutes, respectively. By further purification with a preparative ODS-HPLC (Develosil ODS-HG-5 20 mm ϕ × 250 mm, Nomura Chemical) eluted with 10% aq. CH_3CN solution containing 0.5% TFA, **3** (8.2 mg) was obtained as a red amorphous TFA salt. Purification by preparative HPLC with 0.5% TFA-14% aq. CH_3CN as solvent gave pure **4** (10.4 mg) as a red amorphous TFA salt.

4.6.1. 3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranosylcyanidin (**3**)

UV–VIS (0.1% HCl–MeOH) λ_{max} nm (ε): 524 (19,100), 284 nm (13,500); MALDI-TOF MS: *m/z* = 743 $[M]^+$. ^{1}H 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), 3.70 (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 gl-1, H-6 and g2-1, H-8 and g2-1, g1-2 and xy-1, g1-3 and xy-1. HMBC correlation; gl-2 and xy-1.

4.6.2. 3-*O*-β-D-glucopyranosylcyanidin (**4**)

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

4.7. Reproduction of sepal color by mixing components

An aq. solution of compounds **2** or **4**, co-pigments (**5**, **6**, and **7**), and Al^{3+} at the final concentrations of 1 mM, 1 mM, 1 or 3 mM, 5 mM and 0–5 mM, respectively, were added to a buffer solution (100 mM HCOOH–HCOONa, pH 3.0, 3.5 or 4.0), and mixed for 2 s, then, VIS absorption spectra were immediately recorded in a quartz cuvette (path length: 0.5 mm) at 25 °C.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.02.024.

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