Chemical Studies on Different Color Development in Blue- and Red-Colored Sepal Cells of Hydrangea macrophylla

Daisuke Ito, Yosuke Shinkai, Yuki Kato, Tadao Kondo, and Kumi Yoshida

1Graduate School of Information Science, Nagoya University, Chikusa, Nagoya 464-8601, Japan
2Graduate School of Human Informatics, Nagoya University, Chikusa, Nagoya 464-8601, Japan

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To clarify the cause of the difference in blue and red color development of hydrangea sepals, Hydrangea macrophylla, we analyzed the organic and inorganic components in the colored cells. To obtain colored protoplasts, each blue and red sepal tissue was treated with a combination of cellulase and pectinase, and then from the suspension of the colored and colorless protoplast mixture colored single cells of the same hue were collected with a micro-pipette. The content of organic components (delphinidin 3-glucoside, chlorogenic acid, neochlorogenic acid and 5-O-p-coumaroylquinic acid) and Al³⁺ in each colored cell was quantified respectively by semimicro-HPLC and graphite furnace atomic absorption spectroscopy (GFAAS). In the blue cells 13 eq. of 5-O-acylquinic acids and 1.2 eq. of Al³⁺ to anthocyanin were contained. Contrary to this result, in the red cells, only 3.6 eq. of 5-O-acylquinic acids and 0.03 eq. of Al³⁺ were detected. A reproduction experiment of each blue and red sepal color by mixing those components concluded that, for blue coloration, both 5-O-acylquinic acids and Al³⁺ were essential.

Key words: analysis of aluminum ion; 5-O-acylquinic acid; colored-cell analysis; delphinidin 3-glucoside; sepal color difference, Hydrangea macrophylla

Flowers of Hydrangea macrophylla are just sepals, and their color is well known to change easily depending on the cultivation conditions and/or transplanting. Despite a long history of chemical and biological studies on hydrangea, the mechanism for this color variation is still not completely understood. A correlation between the blue sepal color and Al³⁺ has been investigated since the early 20th century. Hydrangeas cultivated in an acidic soil have blue sepals, and this phenomenon has been explained by the fact that in an acidic soil, Al³⁺ is soluble, and can thus be absorbed and transported to the sepals, where Al³⁺ forms complexes with anthocyanin resulting in a blue color. In contrast, Al³⁺ becomes insoluble under neutral and weakly alkaline solutions, therefore, the sepals become red (Fig. 1). However, all colored sepals, from red through purple to blue, contain the same anthocyanin, delphinidin 3-glucoside (1), and the same co-pigment components, chlorogenic acid (3-O-caffeoylquinic acid, 2), neochlorogenic acid (5-O-cafeoylquinic acid, 3) and 5-O-p-coumaroylquinic acid (4) (Scheme 1). Therefore, the reason why the same components give such a wide variety of colors remains to be clarified.

Takeda et al. have reported that blue sepals contained more 5-O-acylquinic acids (3 and 4) and Al³⁺ than red sepals. They confirmed that a stable blue solution could be obtained by mixing 1 and 3 or 4 and Al³⁺. Our group also obtained similar data on Al³⁺: the bluer the sepals became, the greater the Al³⁺ content in the sepals. Furthermore, we carried out a reproduction experiment of sepal colors by mixing various structures-diverse anthocyanins (Toyama and Yoshida, unpublished results) and co-pigments, including designed synthetic co-pigments. As a result, the blue color of the hydrangea may be developed by Al³⁺ complexation to 3',4'-dihydroxy residues of the B-ring in 1, and this complex should be solubilized and stabilized by intramolecular stacking of 5-O-acylquinic acid (3 and 4) as a co-pigment. Since sepal color development is a phenomenon occurring in only the colored cells, a chemical analysis of the components (1–4 and Al³⁺) that contribute to coloration must be carried out by using pure colored cells. However, until now, almost all the analyses have been carried out by using extracts of whole sepal tissues composed of colored and colorless cells, and the composition of 1–4 and Al³⁺ in the colored cells has not yet been quantified.

In hydrangea sepals, the colored cells are located in the second layer, making it impossible to obtain colored cells by peeling the sepal epidermis. Therefore, we prepared protoplasts by treating with cellulase and pectinase. We have already established the color analysis of a single-cell by micro-electrophotometry followed by direct vacuum pH measurement of the cell by using a proton-selective microelectrode. Values for pH of the blue cells was significantly higher than that of the red cells. In this study we tried to resolve a correlation between cell color and chemical composition in blue and red sepals.
protoplasts obtained were washed twice by centrifugation and then washed with the buffer and centrifuged (reaction mixture was filtered through Miracloth (Calbiochem), washed of a buffered solution (0.6 M mannitol and 20 mM MES-Tris at pH 6.3). Protoplasts were prepared according to our previous report.18) To 10 ml Cellulase Y-C (Kikkoman), sepals cut to a 2-mm thickness (0.4–0.5 g) were added 3% aq. phosphoric acid (15 l), and then the mixture was centrifuged (35,000 g). The supernatant (5 l) was analyzed by micro-micro-spectrophotometry according to the previous report.18) The protoplast suspension was poured into a plastic dish (35 mm diameter). The dish was set on a microscope (IX70, Olympus), equipped with a micro-spectrophotometer (MCPD-7000, Photok). The absorption spectrum of the colored protoplasts was measured in the visible region (400–800 nm) with a 10-μm diameter optical light beam.

Measurement of the Vis absorption spectrum of the colored protoplasts. The Vis absorption spectrum of the colored protoplasts was measured by micro-micro-spectrophotometry according to the previous report.19) The protoplast suspension was poured into a plastic dish (35 mm diameter). The dish was set on a microscope (IX70, Olympus), equipped with a micro-spectrophotometer (MCPD-7000, Photok). The absorption spectrum of the colored protoplasts was measured in the visible region (400–800 nm) with a 10-μm diameter optical light beam.

Measurement of CD of the protoplast suspension. A piece of scotch tape was attached to both sides of the blue and red-colored sepal tissues, and then the tape stripped to give tissues from which the epidermis was removed. The peeled epidermis tissue was cut, and then the pieces were treated with cellulase and pectinase according to the procedure already described. The ratio of colored and colorless protoplasts in the suspension obtained was ca. 8:2. The protoplasts were washed and suspended again in the storage buffer already described. The protoplast suspension was poured into a quartz cell (0.05-mm path length), and CD was measured according to the previous report.19)

Collection of the colored protoplasts. A borosilicate glass capillary (IB 100 F-4, World Precision Instruments) was melted and pulled with a puller (PE-2, Narishige). The tip of the micropipette was broken and melted to adjust the diameter to ca. 60 μm. The micropipette was set into a micro-injector (IM-16, Narishige) filled with silicone oil, and then, the same colored protoplasts were collected from the protoplast mixture suspended in a plastic dish under microscopic observation. During cell collection, the diameter and absorption spectrum of ten cells that had been randomly selected were measured. After 10 to 30 protoplasts had been collected in the pipette, the cells were pushed out into 1% aq. TFA (100 μl), and this procedure was repeated until a sufficient number of cells had been collected (ca. 150 cells). The acidified mixture was then lyophilized and stored at −80°C before the HPLC analysis.

Quantification of the organic components (1–4). To the sample was added 3% aq. phosphoric acid (15 μl), and then the mixture was centrifuged (8,000 × g for 5 min). The supernatant (5 μl) was analyzed by the semimicro-HPLC system (PU-1580 pumps, UV-1570 detectors, HG-1580-32 mixer and DG-580-35 degasser; Jasco) according to our procedure20,21) with modifications. An ODS column (Develosil ODS-H5, 1.5 mm × 250 mm, Nomura Chemical) was eluted at 40°C with a linear gradient (0.1 ml/min) from 10% to 20% aq. of an CH3CN solution containing 0.1% TFA in 3 min. Two detectors were set in tandem, and 1 was detected at 530 nm and 2–4 at 280 nm. The amount of 1, 2, 3, and 4 was quantified directly by the calibration curve obtained by an HPLC analysis of the authentic solutions. The concentration of 1–4 in a protoplast was calculated by using the average cell volume and cell numbers in the sample solution.

Preparation and collection of protoplasts for the Al analysis. All the equipment for Al-analysis was immersed in 5% aq. HNO3, washed with reverse-permeated water just before use, and set up in a clean chamber. The sepal suspensions were suspended in 5% aq. HNO3 for 5 s and washed with reverse-permeated water before the enzyme treatment. After the enzyme treatment, the protoplast mixture obtained was washed six times by using the procedure just mentioned. During protoplast collection, a plastic dish filled with the same storage buffer without any cells was put beside the cell suspension for a blank test. The other procedures were the same as those already described. The collected protoplasts (ca. 200 cells) were poured into 0.5% aq. HNO3 (100 μl), and then the sample was stored at −80°C before being analyzed.

Quantification of Aluminum. The sample mixture was unfrozen and centrifuged (8,000 × g for 5 min). To the supernatant, 0.5% aq. HNO3 was added to adjust the sample weight to 200 mg, before the prepared
Measurement of Aluminum by GFAAS

The concentration of Al$^{3+}$ in a protoplasm was calculated by using the average cell volume and cell numbers in the sample solution.

Reproduction of the sepal color. 1-4 and 100 mM AlNH$_4$(SO$_4$)$_2$ aq. were mixed in a buffer solution (100 mM AcOH–AcONa at pH 4.0, or 100 mM HCOOH–NaOH at pH 3.0) at a final concentration of 1 to give 10 mM. The UV/Vis spectrum and CD were measured in a quartz cell (0.05 mm path length) at 25°C.

Results and Discussion

Preparation and collection of the colored cells

Since hydrangea sepals show a wide variety of colors from the same organic (1-4) and inorganic (Al$^{3+}$) components (Scheme 1), we carried out the cell features according to cell color to clarify the mechanism for this color variation. As we have previously reported, the colored cells in hydrangea sepals are located in the second layer from the surface, and the epidermal cells are colorless. Thus, colored cells gathered by peeling the surface layer is impossible. We cut the sepals and treated them with cellulase and a mixture of colored and colorless protoplasts was obtained (Fig. 1). After searching for the best combination of cellulase and pectinase and reaction conditions, a combination of 0.2% Macerozyme R-200 and 2.0% Cellulase Y-C in 0.6 M mannitol at 30°C under pH 6.3 gave a relative good result; the ratio of colored and colorless cells in the protoplast mixture was ca. 50:50 (Fig. 1). We then tried to purify the protoplast mixture by density gradient centrifugation, but this no longer worked. We therefore planned to collect colored cells with the same hue one by one by using a micropipette connected to micro-injection apparatus under microscopic observation. Since the average cell diameter of the hydrangea protoplast was ca. 20 μm, a pipette with a tip diameter of ca. 60 μm was prepared. Ten to thirty cells were collected from the cell suspension in a plastic dish at once, and then the collected cells were pushed out into a tube filled with acidified water. This procedure was repeated several times until the total number of colored cells reached the requirement for analysis. During collection, ten colored protoplasts were randomly selected and their Vis absorption spectra and cell diameter were measured to record the cell data and to calculate the total cell volume.

Quantitative analysis of the organic components

Comparing the absorbance at λmax of a colored cell measured with micro-spectrophotometry and the absorbance of reproduction experiments by mixing 1-4 and Al$^{3+}$ at pH 4.0, the concentration of I in the colored cells was estimated to be ca. 5-10 mM. The results of the HPLC analysis of the extract of sepal tissue indicated that the concentration of 2-4 in the colored cells was 1 to 20 times higher than that of I. Meanwhile, the detection limit for the HPLC analysis of 1-4 defined the number of cells to be collected for the quantitative analysis. An ODS column (1.5 mmφ × 250 mm) with detection at 280 nm needed ca. 500 cells to be collected. To detect I with higher sensitivity, two detectors were set in tandem; the first detector was set at 530 nm for detecting I, and the second was set at 280 nm for detecting 2-4. This made the number of required cells less than 150.

The suspension of the 150 same-colored cells collected manually was lyophilized, and the residue was resolved in aq. phosphate (15 μl). Five μl of the sample solution was injected and analyzed by semi-micro HPLC. The HPLC chromatograms of the blue cells and red cells are shown in Fig. 2. Each chromatogram was obtained by injecting a content of ca. 50 cells. Each of the compounds, 1-4, were eluted with good separation and a high signal/noise ratio of S/N > 10. The

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**Table 1. Instrument Conditions and Furnace Program for the Measurement of Aluminum by GFAAS**

| Wavelength | 309.3 nm |
| Lamp current | 12.0 mA |
| Slit width | 1.3 nm |
| Inert gas | Argon |
| Gas flow-rate | 200 ml/min* |

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<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Duration time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>110</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>1,000</td>
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<tr>
<td>5</td>
<td>2,800</td>
<td>2,800</td>
</tr>
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</table>

*The gas-flow was stopped during the atomization step (Step 4).
Table 2. Difference in Concentration of Anthocyanin (1) and Molar Equivalents of the Co-pigments (2–4) and Al3+ to 1 in the Blue and Red Cells of Hydrangea

<table>
<thead>
<tr>
<th>Sepal color (cultivar)</th>
<th>blue (Narumi blue)</th>
<th>red (Kasterin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of experiments</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>( \lambda_{\text{vismax}} (\text{nm})^{a} )</td>
<td>586 ± 3.6(^{1})</td>
<td>539 ± 4.1(^{1} )</td>
</tr>
<tr>
<td>cell diameter (( \mu \text{m})^{b} )</td>
<td>24 ± 2.2(^{2})</td>
<td>27 ± 2.4(^{2})*</td>
</tr>
<tr>
<td>1 (mm)(^{b} )</td>
<td>12.9 ± 9.1</td>
<td>15.4 ± 16.2</td>
</tr>
<tr>
<td>2 (eq. to 1)</td>
<td>10.4 ± 12.0</td>
<td>18.1 ± 20.1</td>
</tr>
<tr>
<td>3 (eq. to 1)</td>
<td>8.3 ± 7.3(^{3})</td>
<td>2.4 ± 3.1(^{3})*</td>
</tr>
<tr>
<td>4 (eq. to 1)</td>
<td>4.5 ± 3.5(^{4})</td>
<td>1.2 ± 1.5(^{4})*</td>
</tr>
<tr>
<td>no. of experiments</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>( \lambda_{\text{vismax}} (\text{nm})^{a} )</td>
<td>586 ± 3.6(^{1})</td>
<td>539 ± 4.1(^{1} )</td>
</tr>
<tr>
<td>cell diameter (( \mu \text{m})^{b} )</td>
<td>23 ± 1.6(^{2})</td>
<td>26 ± 2.43(^{2})*</td>
</tr>
<tr>
<td>Al(^{3+} ) (mm)(^{b} )</td>
<td>14.8 ± 8.6(^{3})</td>
<td>0.4 ± 0.4(^{3})*</td>
</tr>
<tr>
<td>Al(^{3+} ) (eq. to 1)(^{4})</td>
<td>1.2 ± 1.1(^{4})</td>
<td>0.03 ± 0.04(^{4})*</td>
</tr>
</tbody>
</table>

\(^{a}\)Average wavelength of \( \lambda_{\text{max}} \) in the visible absorption spectra of protoplasts measured by micro-spectrophotometry.

\(^{b}\)Data are expressed as the mean ± SD.

\(^{1}\)The analysis of Al\(^{3+} \) was carried out independently of analysis of 1–4; therefore, the molar equivalent value of Al\(^{3+} \) to 1 was calculated by using the above-mentioned data.

Significant differences between \(^{1}\) and \(^{2}\) (\( P < 0.01 \)) and \(^{3}\) and \(^{4}\) (\( P < 0.001 \)) were observed.

The concentration of 1–4 in the colored cells was calculated by using the average cell diameter and number of cells collected and is shown in Table 2. The average \( \lambda_{\text{vismax}} \) of the cells was 586 nm in blue cells and 539 nm in red cells. The concentration of anthocyanin (1) in the blue cells was 12.9 ± 9.1 nm and that in the red cell was 15.4 ± 16.2 nm. There is no significant difference between these two figures. The concentration of the three co-pigments was found to be the molar equivalent to anthocyanin (1) (Table 2). The data obtained show a remarkable difference between the blue and red cells.

The molar equivalent of neochlorogenic acid (3) and 5-O-p-coumaroylquinic acid (4) to 1 in the blue cells was high, 8.3 ± 7.3 eq. and 4.5 ± 3.5 eq., respectively, but that in red cells was very low, 2.4 ± 3.1 eq. and 1.2 ± 1.5 eq. The molar ratios of 3 and 4 to 1 in blue cells were significantly higher than those in the red cells. In contrast to 5-O-acylquinic acid, the ratio of 3-O-acylquinic acid (chlorogenic acid, 2) in the blue cells was lower (10.4 ± 12.0 eq.) than that in the red cells (18.1 ± 20.1 eq.).

We have previously analyzed the content of 1–4 in sepal tissue by extracting the sepal with acidic CH\(_3\)CN aq. (Toyama-Kato, thesis, Nagoya University). The data varied and were very much different from the results in this study. The analytical data of the tissue extracts did not show any clear correlation between the colors. The ratio of 2:3:4 in the blue sepal tissue was 4.3:2.6:0.7 (mg/g of fresh sepal) and that in the red sepal tissue was 4.8:2.3:0.6; no difference in the ratio of the co-pigments was apparent between the blue and red sepals. Furthermore, the molar ratio of 5-O-acylquinic acids (3 and 4) to anthocyanin (1) in the extracts was very high; in the blue sepal extracts, the molar ratio of 3 to 1 was 22 eq., and that of 4 to 1 was 14 eq. This higher molar ratio of 3 and 4 to 1 would have been caused by contamination by the colorless cells that contained 3 and 4, but not 1.

Therefore, the data obtained by the analysis of the sepal-tissue extracts may be meaningless for clarifying the in vivo mechanism for the sepal color differences. Our data shown in Table 2 are the first results of an analysis of the pigment and co-pigment components (1–4) in only colored cells of hydrangea.

**Quantitative analysis of Al in the colored cells**

Downsizing the number of collected cells for the Al analysis, has several difficulties. One of the most serious problems is contamination by trace Al from the environment. Al can come from everywhere from soil dust, use of aluminum foil in the laboratory, and even from plastic tubes, because an Al catalyst was used for polymerization. To avoid Al contamination, we washed all the plant materials and experimental equipment with aq. HNO\(_3\), and the cell collecting operation was carried out in a clean chamber. Among the various methods for Al analysis, such as atomic absorption spectrometry (AAS), graphite furnace atomic absorption spectrometry (GFAAS), induced coupled plasma atomic emission spectrosopy (ICP-AES), and induced coupled-plasma mass spectrometry (ICP-MS), we chose GFAAS for its low detection limit (0.04 µg/l).22)

We collected ca. 200 colored cells by using the same procedure, and the cells were destroyed in aq. HNO\(_3\), before the sample solution was analyzed. All the obtained raw data were higher than the detection limit of this instrumentation (Fig. 3). The content of Al\(^{3+} \) in the colored cells was quantified by using the cell numbers and cell diameters measured during the collecting procedure (Table 2). The average concentration of Al\(^{3+} \) in the blue protoplast was high, 14.8 ± 8.6 nm and that in the red protoplast was very low, only 0.4 ± 0.4 nm. The molar equivalent of Al\(^{3+} \) to 1 in each blue and red cell was estimated by dividing the Al\(^{3+} \) concentration with the concentration of 1 obtained from the above-mentioned experiments. In the blue cells, 1.2 ± 1.1 eq. of Al\(^{3+} \) was detected, and in the red cells, the value was only 0.03 ± 0.04. These results strongly indicate the contribution of Al\(^{3+} \) to the blue color.
development in sepal cells. The difference of Al\(^{3+}\) content in the blue cells and that in the red cells may have been caused by the difference of cultivar and/or soil pH.

**Reproduction of sepal color by mixing the sepal components**

A great difference was apparent between the content of organic (1–4) and inorganic (Al\(^{3+}\)) components in the blue- and red-colored cells. We therefore tried to reproduce the same blue or red color as that of the cells by mixing the components (1–4 and Al\(^{3+}\)) at the ratio clarified by the analysis already mentioned. The concentration of Al was determined to be 10 mM and the pH condition was fixed at 3.0 and 4.0 according to our previous report.\(^{16}\)

In a buffered solution, 1–4 and Al\(^{3+}\) were mixed, and the visible absorption spectrum and CD of the solution was measured in a cuvette with a path-length of 0.05 mm (Fig. 4). By mixing 1 (10 mM) at a molar ratio with 2 (10 eq.), 3 (8.0 eq.), 4 (4.5 eq.) and Al\(^{3+}\) (1.0 eq.) at pH 4.0, being equal to that of the blue cells, the same blue color as that of the blue protoplasts was reproduced. The mixture of 1 (10 mM) at a molar ratio with 2 (18 eq.), 3 (2.5 eq.), 4 (1.0 eq.) and Al\(^{3+}\) (0.03 eq.) at pH 3.0 showed the same spectrum as that of the red protoplast. The CD value of the reproduced blue solution was similar to that of the colored protoplast suspension, in which more than 80% was colored cells. Neither CD showed any exition-type of Cotton effect, but a small positive slope at around pH 3 without any clear slope, and neither did that of the protoplast.

Our previous results of reproduction experiments indicated that the color of 1 was affected by the composition of the co-pigments and Al\(^{3+}\) as well as by pH.\(^{16–18}\) A solution mixing 1 (1.0 mM), 4 (1.0 mM) and Al\(^{3+}\) (1.0 mM) at pH 3.0 showed a red color, but the same solution at pH 4.0 did not give a stable blue solution instead giving a blue-black precipitate.\(^{16,17}\) To obtain a stable blue solution with 1 eq. of Al\(^{3+}\) to 1, more than 3 eq. of 3 or 4 was essential.\(^{16–18}\) In the blue cells analyzed here, the average molar ratio of 3 and 4 to 1 was 8 eq. and 4.5 eq., respectively, suggesting that the intravacuolar composition of the co-pigments was sufficient for blue color development. In the red cells, the average molar ratio of 3 and 4 to 1 was 2.4 eq. and 1.2 eq.; therefore, if the amount of Al\(^{3+}\) to 1 was higher than 1 eq., the resulting color should not be red, but purple at pH 3.0. However, in these red cells of cv. Kasterin, the Al\(^{3+}\) content was very low, less than 0.1 eq. to 1. Thus, the cells could develop the red color.

In conclusion, we obtained the same blue and red colored cells of the hydrangea for the first time. We observed a clear difference in the contents of 5-O-acetylquinic acid (3 and 4) and Al\(^{3+}\) between the blue and red cells. In the blue cells a very high amount of 3, 4, and Al\(^{3+}\) was contained compared to that in red cells. The difference in composition of the co-pigments (2–4) might have been caused by some unknown biosynthetic control and/or difference of genomic background of these two cultivars. The difference of Al\(^{3+}\) content may have been caused by the acidity of the soil for cultivation and Al-fertilizer application.\(^{29}\) Using these data, we could reproduce the same blue and red color as that of sepal cells in vitro. It was clarified that the blue sepal color in the hydrangea might develop by the coexistence of two species of 5-O-acetylquinic acid, neo-chorogenic acid (3) and 5-O-p-coumaroyl quinic acid (4), and Al\(^{3+}\) with anthocyanin (1) at around pH 4.0, and that the red sepal color might develop under more acidic conditions of around pH 3 without 3, 4, or Al\(^{3+}\).

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