Review

Insight into chemical mechanisms of sepal color development and variation in hydrangea

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Abstract: Hydrangea (Hydrangea macrophylla) is a unique flower because it is composed of sepals rather than true petals that have the ability to change color. In the early 20th century, it was known that soil acidity and Al³⁺ content could intensify the blue hue of the sepals. In the mid-20th century, the anthocyanin component 3-O-glucosyldelphinidin (1) and the copigment components 5-O-cafeoylquinic, 5-O-p-coumaroylquinic, and 3-O-cafeoylquinic acids (2–4) were reported. Interestingly, all hydrangea colors from red to purple to blue are produced by the same organic components. We were interested in this phenomenon and the chemical mechanisms underlying hydrangea color variation. In this review, we summarize our recent studies on the chemical mechanisms underlying hydrangea sepal color development, including the structure of the blue complex, transporters involved in accumulation of aluminum ion (Al³⁺), and distribution of the blue complex and aluminum ions in living sepal tissue.

Keywords: aluminum ion, anthocyanin, 5-O-acylquinic acid, Hydrangea macrophylla, sepal color, vacuolar pH

1. Introduction

The hydrangea plant (Hydrangea macrophylla) originates from Japan and East Asia. Hydrangea flowers are composed of sepals whose original color is blue, although various breeding efforts have resulted in a wide range of sepal colors (Fig. 1A). In Japan, hydrangea is loved as a flowering plant that blooms during the rainy season and is commonly portrayed in Flora Japonica by Siebold as Hydrangea ‘Otaksa’ Sieb. Et Zucc.1) Hydrangea sepal color can change depending on the cultivation conditions and/or transplantation. In general, flower coloration is thought to be genetically controlled by the expression of structural genes involved in anthocyanin biosynthesis.2)–4) However, in hydrangea, neighboring cells that should have similar gene expression profiles can exhibit different colors, indicating the importance of factors other than gene expression in sepal color determination.

Chemical studies on hydrangea sepal color development started in the 19th century.5) Early part of the 20th century, it was reported that in acidic soil hydrangea sepals tend to be blue, while in alkaline soil the sepals become red in color (Fig. 1A).6),7) This difference in coloration is because in acidic soils, Al³⁺ is highly water soluble and can be absorbed and transported to the sepals, where it chelates with anthocyanins.8)–11) Conversely, under alkaline conditions, Al³⁺ is water insoluble and is not absorbed by plant roots. This knowledge is effectively used by horticulturists for cultivating differently colored hydrangea. The major anthocyanin in hydrangea sepals is 3-O-glucosyldelphinidin (1),12) and blue and red hydrangeas express the same anthocyanin compounds (Fig. 2).13) Furthermore, copigments such as neochlorogenic acid (5-O-cafeoylquinic acid, 2), 5-O-p-coumaroylquinic acid (3), and chlorogenic acid (3-O-cafeoylquinic acid, 4) are present in all sepals regardless of color (Fig. 2).14) However, the chemical structure of the blue pigment

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in hydrangea is unknown, and the mechanisms that explain how the same components can develop different colors remain unclear.

Our group has been interested in studying the blue flower color development in a variety of plant species. Generally, any anthocyanin develops a blue color when its chromophore is in a quinoidal base anion form, although the mechanism that stabilizes this chemical structure is different in each flowering plant. We reported that blue coloration is caused by metalloanthocyanin, a self-assembled supramolecular metal complex pigment found in blue dayflower, cornflower, and several salvias; alkaline vacuolar pH (pHv) regulates blue coloration in morning glory; and nonstoichiometric metal complex anthocyanin is found in tulip and poppy. Our research on blue hydrangea started in the early 1990s to elucidate the attractive but curious properties of hydrangea coloration, and it has been carried out by combining various research areas, such as natural product chemistry, synthetic organic chemistry, plant physiology, and molecular biology. We have recently determined the chemical composition of the hydrangea blue-complex and have directly detected the blue pigment in vivo. Here, we summarize the last 30 years of our hydrangea studies.

2. Chemical analysis of colored sepal cells

Studying the mechanism of sepal color development required clarification of intravacuolar conditions such as vacuolar pH, the chemical structure of anthocyanins and copigment composition, and content of metal ion: aluminum ion in hydrangea at first, then reproduction of the sepal color using the obtained analytical data is essential. The
epidermal cell layer of hydrangea sepals is colorless, and the pigmented cells are located the second cell layer (Fig. 1B, 1C). Therefore, whole tissue samples are unsuitable for chemical analysis because colorless cells can affect sample and analysis quality (Table 1). Thus, we treated the tissue with pectinase and cellulase to obtain a protoplast mixture from which colored cells could be collected for analysis.

2.1. Vacuolar pH measurement. Anthocyanin equilibrium depends on pH (Fig. 3), and therefore, color development is largely affected by the pH of the vacuoles where the pigments are localized. There are many methodologies for measuring vacuolar pH (pHv), but the easiest one is to measure the pH of fluid isolates from pressed petal or sepal tissues. However, these isolates include cellular materials from both colored and colorless cell types. Therefore, we have found that using a pH-sensitive microelectrode method established by Felle is the best method to obtain accurate pHv measurements.

We previously measured the pHv of morning glory petals, *Ipomoea tricolor* ‘Heavenly Blue’ using a pH-sensitive microelectrode, because the pressed juice of the blue petals instantly changed from blue to purple during collection, the accuracy of the pHv measurements was compromised. The quick color change was due to a significant difference in the pHv values of colored epidermal cells and colorless parenchyma cells. Therefore, the pH of the pressed juice was a result of the mixture of both cell types. In morning glory petals, the colored cells are located in both epidermal layers: for measurement, the petal tissue was placed in the measurement vessel, and the electrode was directly inserted. However, in hydrangea sepals, epidermal cells were colorless, therefore, we measured pHv using protoplasts (Fig. 4). Before pHv measurement, the spectra of the selected colored cells were recorded using microspectrophotometry with approximately 10-µm beam flux under a microscope. Using a combination of microspectrophotometry and a proton-selective microelectrode technique, we correlated the color

**Table 1. Content of anthocyanin (1), copigments (2–4) and Al\(^{3+}\) in blue and red hydrangea sepals**

| Sepal color | 1 | 2 | 3 | 4 | \(\text{Al}^{3+}\) |
|-------------|---|---|---|---|-----------------|
| Blue        | 0.24 | 2.6 | 0.67 | 4.3 | 0.27 |
| Red         | 0.24 | 0.3 | 0.63 | 4.8 | 0.09 |

Blue cultivar: ‘Narumi blue’, Red cultivar: ‘Narumi red’.

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Fig. 3. Reversible chemical reactions of anthocyanin and color development exemplified for 3-O-glucosyldelphinidin depending on pH in aqueous solution.
of a single hydrangea cell with its pHv (Table 2). The average pHv values of blue ($\lambda_{\text{vismax}}$: 589 nm) and red ($\lambda_{\text{vismax}}$: 539 nm) cells obtained from three blue (Narumi blue, Blue diamond, wild one from Mt. Chause) and two red cultivars (Kasterin and LK-49) were 4.1 and 3.3, respectively, indicating that the pHv of blue cells was significantly higher than that of red cells. We found that the pHv of colored hydrangea sepal cells was relatively low (e.g., acidic) compared to that of normal petals.

### 2.2. Composition of organic components in hydrangea sepal.

Organic components in colored cells of hydrangea sepal were quantified by collecting similarly colored cells. To clarify the cause of blue and red color development in hydrangea sepal, we prepared protoplasts from the blue and red cultivars ‘Narumi blue’ and ‘Kasterin’, respectively. Ideally, single-cell analysis is the best for this purpose; however, this was technically impossible at the time, so we manually collected the same colored protoplasts from a mixture using a micropipette under a microscope.

The number of cells necessary for quantitative analysis depended on the sensitivity of the analytical method used. For analysis of organic composition, we used a semimicro HPLC system equipped with an ODS column (1.5 mm i.d. 250 mm length) and a linear gradient elution with a flow rate of 0.1 mL/min. Anthocyanin was detected at 530 nm, and copigments were detected at 280 nm. Each compound was eluted with good separation and a high signal/noise ratio (S/N) of <10. For quantification of organic compounds, approximately 150 cells had to be collected, and for Al$^{3+}$, 200 cells were needed. The concentration of the organic and inorganic components in the colored cells was calculated using the average cell diameter and number of cells collected. As shown in Table 2, the average $\lambda_{\text{vismax}}$ was 586 nm in blue cells and 539 nm in red cells. In blue cells, 13 eq. of 5-0-acylquinic acids (2, 3) to anthocyanin (1) was recorded. Conversely, in red cells, only 3.6 eq. of 5-0-acylquinic acids was detected. Compared with our previous data of whole sepal tissue analysis, these results were remarkably different; the data from tissue samples varied and did not show any clear correlation between color and copigment composition. This

#### Table 2. The pHv, concentration of anthocyanin (1), and molar equivalents of the copigments (2–4) and Al$^{3+}$ to anthocyanin (1) in blue and red protoplasts prepared from hydrangea sepal tissue.

| Sepal color | $\lambda_{\text{vismax}}$ nm | pHv | $1$ mM | 2 (eq. to $1$) | 3 | 4 | Al$^{3+}$ eq. to $1$ |
|-------------|-----------------|-----|--------|--------------|---|---|------------------|
| Blue        | 586–589         | 3.6 | 12.9   | 8.3          | 4.5 | 10.4 | 1.2              |
| Red         | 537–539         | 3.3 | 15.4   | 2.4          | 1.2 | 18.1 | 0.03             |

Blue cultivar: ‘Narumi blue’, Red cultivar: ‘Kasterin’.

pHv data were from Ref. 35, and other analytical data were from Ref. 36.

![Fig. 4](image4.png) Micrographs of hydrangea protoplast mixtures. Protoplasts prepared from (A) blue sepal from the ‘Narumi blue’ cultivar and (B) blue sepal from the ‘Kasterin’ sepal. Scale: 50 µm.

![Fig. 5](image5.png) Sepal color change of chameleon hydrangea cultivar ‘Homigo’.

![Fig. 5](image5.png) Sepal color change of chameleon hydrangea cultivar ‘Homigo’.
strongly indicated that the distribution of anthocyanin in sepal is limited in colored cells, but copigments may also be present in colorless cells. Indeed, this was confirmed by cryo-TOF-SIMS analysis, as described in section 6.2 of this review.

2.3. Analysis of metals. Reducing the number of cells needed for the Al analysis has several challenges, the most significant is how to prevent environmental Al contamination. Hence, it was essential to wash all the plant materials and experimental equipment with aqueous HNO₃ and to use a clean chamber for cell collection.30 There are many methods for Al analysis, such as atomic absorption spectroscopy (AAS), graphite furnace AAS (GFAAS), induced coupled plasma atomic emission spectroscopy (ICP-AES), and ICP mass spectrometry (ICP-MS). We chose GFAAS because it has the lowest detection limit (0.04 µg/L) among these options.37

After the preparation of the protoplast mixture, we collected approximately 200 colored cells. The content of Al³⁺ in the colored cells was quantified using the cell number and diameter that were measured during the collecting procedure. The average concentration of Al³⁺ in the blue protoplast was high (14.8 ± 8.6 mM) while that in the red protoplast was low (0.4 ± 0.4 mM). The molar equivalent values of Al³⁺ to anthocyanin were 1.2 ± 1.1 eq. of Al³⁺ in blue cells and 0.03 ± 0.04 in red cells (Table 2).35 These results strongly indicated that Al³⁺ contributed to blue color development in sepal cells and that the difference in Al³⁺ content in blue and red cells could be caused by differences in the cultivar and/or soil pH.

3. Color change mechanisms in chameleon hydrangea

It is well known that sepal color can change in hydrangea varieties. Among many hydrangea cultivars, the so-called chameleon hydrangea cultivar Hovaria™ ‘Homigo’ is unique in that its color changes from colorless to blue, then green, and finally red during its maturation and senescence (Fig. 5). To characterize the mechanisms underlying this color change, we observed the sepal tissue and analyzed the components according to maturation stage.38

3.1. Sepal color variations in chameleon hydrangea. For color measurement in hydrangea plant tissue, reflection spectra were usually recorded; however, the spectra tended to be affected by the cell surface shape. In comparison, when a tissue section was treated in vacuo with water, the cell air spaces are filled with water, and the noise from the surface structures can be reduced, which allows the measurement of the visible absorption spectra.39 Tissue from each developmental stage was pre-treated, and the visible absorption spectra were measured.

Except for the first colorless stage, cells in the second stage of chameleon hydrangea sepal development exhibited the same blue color with the same spectra measured in ordinary blue hydrangea cells. At the third green stage, the spectra showed a decrease in the peak at 579 nm, which appeared at 680 nm instead. At the fourth red stage, the spectra of the sepal showed a broad peak with λmax at 561 nm and a small peak at 680 nm. The transverse sections of the sepal at each stage indicated that at the first stage, almost all the cells were colorless, while at the second stage, the blue cells were mainly in the second layer of the adaxial epidermis. However, at the third stage, the blue color of the second layer of cells faded, and chloroplasts appeared in the cytoplasm of the cells in the second and inner cell layers. At the fourth stage, the second layer became red, while the number of chloroplasts decreased. These results suggested that the green stage was due to the appearance of chloroplast and the color of the second and the fourth stages could depend on the accumulation of anthocyanins.38

3.2. Chemical analysis of sepal components. The chemical components of hydrangea sepal were analyzed by the extraction of sepal tissue followed by 3D-detection ODS-HPLC. At the first stage, almost no pigment was detected, while at the second stage, 3-O-glucosyledelphinidin (1) and 3-O-sambubiosyledelphinidin (5) were detected as the major and minor pigments, respectively (Fig. 6). During the sepal color change to green near the third stage, the amounts of 3-O-glucosyledelphinidin (1) and 3-O-sambubiosyledelphinidin (5) decreased. At the fourth red stage, anthocyanins with a delphinidin chromophore almost disappeared, and two new anthocyna- ins, 3-O-glucosylcyanidin (6) and 3-O-sambubiosylcyanidin (7), appeared (Table 3). This result indicated that the blue and red coloration was due to different anthocyanins developing and accumulating in the tissues in a developmentally dependent manner. We concluded that the red sepal coloration in chameleon hydrangea was not caused by the same mechanism as that of other red cultivars during the maturation period but was instead due to de novo synthesis of pigments with a cyanidin chromophore, which was similar to autumn red
leaves and usually observed in hydrangea found in mountain areas.\(^38\)

4. Single-cell analysis for the clarification of mosaic purple color development

As we have described, chemical analysis of similarly colored cells (protoplasts) significantly advanced hydrangea color development studies. However, it is very often observed that when a potted hydrangea is planted in the garden, the sepal color changes, for example, from blue to purple or from red to purple. Microscopic observation of the purple tissue showed a surprising phenomenon: the single cells of the purple sepals each ranged in color from blue and purple to red, indicating that the purple color was due to a mosaic effect. To clarify the chemical mechanism of the color differences between neighboring hydrangea sepal cells, the establishment of a new method for single-cell analysis is required (Fig. 7).\(^40,41\)

4.1. Development of a single-cell analytical method for measuring organic components. To measure the chromatic compounds in hydrangea sepals, we needed more than 150 cells when an ODS column with an i.d. = 1.5 mm was used.\(^36\) In theory, the sensitivity of UV and visible light detection for HPLC analysis increases when the diameter of the column is reduced; therefore, a microcolumn with an i.d. = 0.3 mm could enable single-cell analysis. However, there are problems that need to be resolved for this method to be successful, including the optimization of the detection system, exclusion of impurities in the eluting solvent, manipulation of the narrow column, and reliability of the gradient elution system. The problem of the detection system was solved using a capillary flow cell system (i.d. = 75 µm, length = 20 mm, and cell volume = 35 nL). The elution solvent used was HPLC grade, and the acid was changed from trifluoroacetic acid to phosphoric acid. To reduce base-line fluctuation, a step-wise gradient elution was adopted to establish an HPLC method for quantifying organic components in single hydrangea cells (Fig. 7).\(^40,41\)

Interestingly, using this method, we reported that the molar equivalent of 5-O-acylquinic acids (\(2,3\)) to anthocyanins (\(1,5\)) was strongly correlated with blue sepal coloration; however, the content of anthocyanin and 3-O-cafeoylquinic acid (\(4\)) did not correlate with cell color. Nonetheless, these results indicated a clear contribution of 5-O-acylquinic acids to the blue sepal color in hydrangea.\(^40,41\)

The correlation between 5-O-acylquinic acids and blue sepal color was also confirmed from a single-cell analysis of blue and red cells prepared from blue and red sepals of the ‘Narumi blue’ and ‘Narumi red’ cultivars, respectively, which were grown in ordinal conditions. The molar equivalent of 5-O-acylquinic acids (\(2,3\)) to that of total anthocyanins (\(1,5\)) was 3.5 eq. in blue sepal cells while that in red cells was 0.9. These measurements were significantly different between samples, although the 3-O-cafeoylquinic acid (\(4\)) content did not show any significant difference between the blue and red cell samples. This strongly suggested that 5-O-acylquinic acids play a very important role in blue cell coloration in hydrangea sepals.\(^40,41\)

Table 3. Change in the organic components in chameleon hydrangea sepals during maturation and senescence\(^38\)

|    | 1st colorless | 2nd blue | 3rd green | 4th red |
|----|--------------|----------|-----------|---------|
| 1  | 0.044        | 0.227    | 0.110     | 0.080   |
| 2  | 0.868        | 0.959    | 0.934     | 0.581   |
| 3  | 6.90         | 5.43     | 3.51      | 2.56    |
| 4  | 4.84         | 5.67     | 6.22      | 5.20    |
| 5  | N.D.         | 0.077    | 0.009     | 0.007   |
| 6  | 0.060        | 0.063    | 0.090     | 0.426   |
| 7  | N.D.         | N.D.     | 0.040     | 0.231   |

The compound numbers are indicated in Figs. 2 and 6. n = 3, N.D.: not detected.
4.2. Development of a single-cell analytical method for analysis of aluminum ion content. To quantify the aluminum content in a single cell, GFAAS could not be used because of its detection limitation. Fluorescence detection has high sensitivity and when Al$^{3+}$ is complexed with lumogallion, it produces strong fluorescence and therefore, has been used as a tissue staining reagent for visualizing aluminum ion-localization. Moreover, fluorescence detection-HPLC can be used for the quantification of Al$^{3+}$ in aqueous solutions. Thus, we considered using fluorescence detection-HPLC to measure Al$^{3+}$ content in hydrangea cells. By reducing the reaction system and HPLC analytical condition, we established an Al$^{3+}$ analytical method for single cells.

The cell color obtained from purple sepals varied from 545 nm (red cell) to 588 nm (blue cell). The Al$^{3+}$ content in the reddest cells was only 0.3 eq. of anthocyanin, while that in bluest cells was approximately 3 eq. A clear correlation between blue cell color and Al$^{3+}$ levels was observed, similar to that of 5-O-acylquinic acids. Indeed, a similar result was obtained in the analysis of conventionally cultivated blue and red sepal cells. The Al$^{3+}$ to total anthocyanin ratio in blue cells was significantly higher (2.4 eq.) than that in red cells (0.3 eq.) ($P < 0.001$), indicating that this ratio is another critical condition for blue cell color in hydrangea sepals.

4.3. pHv and cell color in purple sepal cells. Vacuolar pH (pHv) measurement in differently colored cells from purple sepals resulted different data from our previous study, which reported that there was no significant correlation between pHv and cell color. From the reddest ($\lambda_{\text{vismax}} = 535$ nm) to the bluest ($\lambda_{\text{vismax}} = 580$ nm) cell, pHv values were randomly scattered from pH 2.8 to 3.9 with an average of 3.3 ± 0.3 and did not show any correlation with cell color. The average pHv of blue cells in blue sepals of cv. Narumi blue was approximately 3.3 ± 0.3, while that of red cells was approximately 2.8 ± 0.3.
3.6,35) The value is relatively low compared to the pHv of other cultivars’ blue cells, suggesting that pHv may be genetically controlled to some extent.

5. Structural study of the hydrangea blue-complex

The results of the colored cell analysis confirmed several factors essential for blue coloration, including the molar ratio of 5-O-acylquinic acids and Al³⁺ to anthocyanin, and pHv. We next wanted to clarify the mechanism of color variation and structural aspects of the blue pigment in hydrangea. For this purpose, we tried to reproduce the natural coloration by mixing vacuolar components under various pH conditions in vitro. Furthermore, to obtain structural information, artificial copigments were synthesized and used in in vitro reconstruction experiments. Finally, instrumental analysis revealed the composition and proposed structure of the hydrangea blue-complex.

5.1. Reproduction of the sepal color. The reproduction of the same blue and red cell colors was conducted by mixing the sepal components 1–4 and Al³⁺ to clarify the mechanisms of color development and how it changes in hydrangea. The similarity of the visible absorption spectrum and circular dichroism (CD) of the solution was evaluated with that of the visible absorption spectrum and circular dichroism of the sepal isolates and single cells. For this purpose, we used the natural coloration observed in hydrangea sepals. The similarity of the CD of the blue solution was similar to that of the blue cells. A lower concentration for anthocyanin [0.5 mM (using 1 mm path length), 1.0 mM (0.5 mm path length), 5 mM (0.1 mm path length), and 10 mM (0.05 mm path length)] than the actual intravacuolar concentration. Within this concentration range, all the blue colors through purple to red found in hydrangea sepal isolates and single cells could be reproduced by tuning the molar ratio of copigments, Al³⁺, and pH.

The simplest condition for reproducing the blue coloration was by mixing 3-O-glucosyldelphinidin (1), a copigment (2, 3, or 4), and Al³⁺ at 1, 3, and 1 mM, respectively, at pH 4.0. Without a copigment, the solution was purplish blue and gradually precipitated out of the solution. The CD was recorded just after mixing and showed an exciton-type negative Cotton effect, indicating the anticlockwise self-association of anthocyanidin chromophores.46,47) The addition of 5-O-caffeoylquinic acid (2) to this mixture resulted in a stable blue solution similar to sepal isolates and cells, and the CD indicating complex in the blue solution might have the same complex structure in blue cells (Fig. 9A, solid line).46,47) Conversely, the addition of 3-O-caffeoylquinic acid (4) produced a purple color (Fig. 9A, broken line) that was different from that of blue sepal isolates and cells, which also produced a precipitate.46 Using these simple experimental conditions, various synthetic copigments were tested to investigate the structure essential for the blue coloration observed in hydrangea sepal isolates.

The blue and red color development experiments were carried out using the analytical data (Table 2).36) By mixing 10 mM 3-O-glucosyldelphinidin (1), 100 mM 3-O-cafeoylquinic acid (4), 80 mM 5-O-cafeoylquinic acid (2), 45 mM 5-O-p-coumaroylquinic acid (3), and 10 mM Al³⁺ at pH 4.0, the same blue color (λmax = 585 nm) as that measured in blue protoplasts was reproduced (Fig. 9B).36) The CD of this blue solution was also similar to that of the colored protoplast suspension (Fig. 9B).36) This may indicate that the assembly of the components in the blue solution is similar to that of the blue cells. A mixture of 10 mM 3-O-glucosyldelphinidin (1), 180 mM 3-O-cafeoylquinic acid (4), 25 mM 5-O-cafeoylquinic acid (2), 10 mM 5-O-p-coumaroylquinic acid (3), and 0.3 mM Al³⁺ at pH 3.0 showed the same spectrum as that of the red protoplasts (Fig. 9C), although the CD data for the red solution did not show a clear slope, neither did that of the protoplast suspension (Fig. 9C).36)

To verify the mechanisms of various color development (blue, purple, and red) in hydrangea,
further reproduction experiments with varying several factors were conducted. The reproduction conditions referred to the analytical results of the single-cell analysis of purple sepals. The mixture comprised 5 mM 3-O-glucosyldelphinidin (1), 25 mM 3-O-cafeoylquinic acid (4), 0–15 mM 5-O-cafeoylquinic acid (2), and 0–15 mM Al3+ at pH 4.0, 3.5, and 3.0, respectively. Hence, a total of 48 solutions were tested and depending on these conditions, the color gradually changed, and all the colors from blue (\(\lambda_{\text{vis}} = 585\text{ nm}\)), purplish blue (\(\lambda_{\text{vis}} = 570\text{ nm}\)), purple (\(\lambda_{\text{vis}} = 560\text{ nm}\)), reddish purple (\(\lambda_{\text{vis}} = 542\text{ nm}\)), and red (\(\lambda_{\text{vis}} = 525\text{ nm}\)) were obtained.\(^{40,41}\) The blue solutions had more than 3 eq. of 5-O-cafeoylquinic acid and more than 1 eq. of Al3+, with a pH of >3.5. For red coloration, the amount of Al3+ had to be less than 1 eq., with less than 1.0 molar equivalent of 5-O-cafeoylquinic acid (2) at pH 3.0. Under other conditions, a purple solution was obtained, with the hue depending on the combination of these factors.\(^{40,41}\)

**5.2. Synthesis of copigments.** Synthetic studies are a powerful tool for characterizing natural product chemistry. In the structural study of the hydrangea blue-complex, no analyzable NMR spectra were obtained, and MS analysis did not produce molecular ions in our previous studies. Therefore, we designed and synthesized various copigments to investigate the essential functional structure in copigments for a stable blue complex: (1) is affected by the position of the acyl moiety when it is 3-O-equatorial or 5-O-axial, (2) is sensitive to the number of phenolic hydroxyl groups in the cinnamate moiety, (3) is affected by the size of the 5-O-aromatic moiety, (4) requires the 1-OH of quinate, (5) requires a 1-COOH, and (6) requires a 5-O-ester structure.\(^{46,47}\)

In the synthetic study of 5-O-acetylquinic acid, there were two problems that affected the synthetic route and yield. The first was the choice of the protecting group at 1-COOH and 3,4-OHs, and the second was the acylation reaction of the axial 5-OH. In the first-generation synthesis,\(^{46,47}\) we chose the protocol described by Montchamp et al.,\(^{48}\) in which 1-COOH was protected by a methyl ester and 3,4-OHs were protected by butane 2,3-bisacetel.\(^{48}\) The 5-O-acylation was carried out using acid chloride with pyridine, while the deprotection reaction was conducted with 2N HCl.\(^{49}\) We obtained all the proposed copigments and were able to complete the reproduction experiments; however, in this method, the preparation of acid chloride, low yield of 5-O-acylation, and competitive reaction of 5-O-deacetylation during the deprotection reaction limited the success of the experiment.

We continued the synthetic studies and established a second-generation synthesis protocol (Fig. 10).\(^{50}\) We designed \(p\)-methoxybenzyl quinate as a key intermediate, which could be deprotected using trifluoroacetic acid (TFA)- or BCl3/C6HMe5 in good yield. Esterification of the sterically hindered 5-OH group was improved by applying a modified Tanabe’s method, in which the esterification reaction is performed with free carboxylic acid and a combination of TsCl/NMI and i-Pr2NEt to provide...
a good yield of the proposed ester.\textsuperscript{50} This second-generation synthesis enabled us to obtain various 5-O-acylquinic acids in seven steps with a 45–60\% overall yield.\textsuperscript{50}

5.3. Sepal color reproduction using synthetic copigments. Reproduction using various synthetic copigments with 3-O-glucosyldelphinidin and Al\textsuperscript{3+} at pH 4.0 revealed which partial structure was essential for blue hydrangea sepal coloration (Fig. 11).\textsuperscript{46,47,51} The essential parts of the copigments were determined to be 1-OH, 1-COOH, and 5-ester. The 1-dehydroxy and 1-OMe derivatives 1-COOMe compound, 5-cinnamyl ether, and 3-O-cafeoylquinic acid did not produce a blue solution but did make a blue precipitate. Thus, these oxygen atoms might chelate to AI\textsuperscript{3+} in the blue complex.\textsuperscript{46,47,51} When the 5-O-cafeoyl residue was replaced with a cinnamoyl moiety, a blue solution was produced, indicating that the phenolic hydroxyl group(s) on the 5-O-acyl residue was not essential for a soluble blue complex. The size of the aromatic component of the 5-O-acyl moiety increased the stability of the complex. Therefore, 5-O-naphthoylequinic acid gave the most stable blue solution, suggesting that the aromatic component of the 5-O-acyl residue may stack with the anthocyanidin chromophore through hydrophobic interactions and has a copigmentation effect on the blue complex.\textsuperscript{46,47,51}

To obtain further information on the properties of the blue complex, the blue precipitate was isolated from the mixture of 3-O-glucosyldelphinidin (1), 3-O-cafeoylquinic acid (4), and Al\textsuperscript{3+} at pH 4.0, and the findings clarified that the blue precipitate was composed of only 1 and Al\textsuperscript{3+}.\textsuperscript{46} This strongly indicates that Al\textsuperscript{3+} complex with the OHs of the B-ring of 1 shows a blue color, however, the water solubility of the complex may be very low. When 5-O-acylquinic acid coexists with 1 and Al\textsuperscript{3+}, the critical oxygen atoms, namely, 1-OH, 1-COOH, and 5-0-C=O, in copigments may chelate to Al\textsuperscript{3+}, and the aromatic acyl moiety shall stack to the anthocyanidin chromophore, then, the complex becomes water soluble to show a stable blue color.

5.4. Structural analysis of hydrangea blue-complex. Reproduction experiments clarified that the blue hydrangea sepal color is due to a metal complex called the “hydrangea blue-complex” that is composed of 3-O-glucosyldelphinidin (1), 5-O-cafeoylquinic acid (2) and/or 5-O-p-coumaroylquinic acid (3) as copigments, and Al\textsuperscript{3+} in an aqueous solution at approximately pH 4.0. However, the ratio of each component is not stoichiometric; it fluctuates within a certain range. The complex could exist only in aqueous solution, and attempts to crystallize the complex were unsuccessful. Therefore, to clarify the chemical structure of the complex, we used NMR\textsuperscript{51} and MS analyses.\textsuperscript{52}

The 1H-NMR spectrum of the hydrangea blue-complex in deuterated acetate buffer (100 mM, pH 4.0) gave very broad signals that we could not analyze; however, in a highly concentrated sodium acetate buffer (6 M, pH 4.0), we could measure the 1H-NMR spectrum of the hydrangea blue-complex.\textsuperscript{51} We also recorded the spectra of simple Al\textsuperscript{3+} complexes of 3-O-glucosyldelphinidin (1), copigments, and those composed with structurally different anthocyanins and copigments to obtain their structural information.
Therefore, as a pre-experiment, UV–vis, CD, and 1H-NMR measurements of the copigment and Al3+ without anthocyanin were conducted, and the results showed that the 5-O-acylquinic acids have a copigmentation effect as a complex with Al3+, while 3-O-acylquinic acid did not.52) The addition of 0.5–2.0 eq. of Al3+ at pH 4.0 to 5-O-caffeoylquinic acid (2) showed a bathochromic shift in $\lambda_{\text{vismax}}$, suggesting an exciton-type positive Cotton effect. In contrast, no such spectral change was observed when Al3+ at pH 4.0 was added to 5-O-p-coumaroylquinic acid (3) or 3-O-caffeoylquinic acid (4).52) In NMR experiments, the signal for 0.5 eq. of Al3+ to 2 became more complex, and with 1.0 eq. of Al3+, the spectra converged. When all the signals were assigned, we deduced that 2 may form a 1 : 1 complex with Al3+ and that a half amount of Al3+ gave a 1 : 1 mixture of free copigment and that complexed with Al3+.52) The position of Al3+ in the complex should be the catechol moiety of the caffeoyl residue; however, 1-OH, 1-COOH, and 5-O=C=O oxygen atoms might be essential for complex production, and this molecular characteristic may play a role in the development of the blue color in hydrangea.

The NMR measurement condition that included 6M acetate buffer was found coincidentally, and the spectra gave helpful information for the analysis of the hydrangea blue-complex (Fig. 12).51) The spectrum of 1 eq. of Al3+ and 3-O-glucosyldelphinidin (1) gave another set of signals that was deduced to be a flavylvium ion structure (Fig. 12B). A mixture of 1 and 5-O-caffeoylquinic acid (2) produced a simple combination of the signals attributed to both compounds with a small high-field shift of signals that were attributed to the delphinidin chromophore, which indicated copigmentation between anthocyanin and quinic acid (Fig. 12C). However, the spectrum of the hydrangea blue-complex was very different from the above spectra in that the anthocyanin signal became very broad and shifted toward higher field, and while $\alpha$ and $\beta$ protons of the copigment also shifted toward higher field, these shifts were sharp (Fig. 12D).51) The NMR analysis of the hydrangea blue-complex solution indicated that its structure is not fixed but under an equilibrium.

Many trials to determine the structure of the hydrangea blue-complex failed, and the ratio of the components seemed to fluctuate rather than have stoichiometric characteristics. To determine the composition of the complex, we tried direct observation of the molecular ion of the complex using electrospray-ionization mass spectrometry (ESI-TOF-MS).27) We had previously reproduced the hydrangea blue-complex in approximately 100–200 mM buffered solutions; however, such a high-concentrated salt was known to suppress ionization, therefore, we tested whether the same blue color could be realized in a very low-concentration buffer solution. After several trials, the same hydrangea blue-complex was obtained by mixing 3-O-glucosydellphinidin (1), 5-O-caffeoylquinic acid (2) and Al3+ in ratios of 1 : 1 : 1, 1 : 2 : 1, and 1 : 3 : 1 in a diluted buffer solution at pH 4.0. From the comparison of the three UV–vis spectra, the ratio of 1, 2 and Al3+ was thought to be 1 : 2 : 1 because this solution was the bluest in color and its absorbance at $\lambda_{\text{vismax}}$ reached its maximum.27) The ESI-TOF-MS measurements indicated that all the blue solutions tested (the ratios of 1, 2 and Al3+ are 1 : 1 : 1, 1 : 2 : 1 and 1 : 3 : 1) gave the same molecular ion peak at $m/z = 843$, which was attributed to the 1 : 1 : 1 ratio for the complex.
This composition was confirmed by high-resolution MS analysis. Furthermore, the observed mass number for 5-O-p-coumaroylquinic acid (3) was \( m/z = 827 \), which was attributable to the ratio of 1, 3 and Al\(^{3+} \) to be 1 : 1 : 1, although the blue solution was obtained by mixing ratio of 1, 3 and Al\(^{3+} \) of 1 : 2 : 1 (Fig. 13B). Replacement of the copigment with 3-O-caffeoylquinic acid (4) did not produce a blue solution, rather, the solution was purple and did not produce a molecular ion peak at \( m/z = 843 \). These results strongly suggested that the hydrangea blue-complex was composed of 1, Al\(^{3+} \) and 2 or 3 with a 1 : 1 : 1 ratio.\(^{27} \)

Combining these results, we proposed a quasi-stable chemical structure for the hydrangea blue-complex (Fig. 14).\(^{27,28} \) This model showed that Al\(^{3+} \) chelates to the catechol at the B-ring of anthocyanin to produce a quinoidal base anion in weak acidic media. In addition, Al\(^{3+} \) chelates to the 1-COOH, 1-OH, and 5-O–C=O residues in 5-O-acylquinic acids. This complex with five coordination points should give a relatively stable Al\(^{3+} \) structure and stabilize the anthocyanidin chromophore via hydrophobic interactions between the aromatic acyl group of quinic acid. However, the coordination might not have high stability, and thus, the hydrangea blue-complex may exist in equilibrium with the coordinated and dissociated states of the copigments. The equilibrium point may vary depending on the concentration of the components and pH of the solution. Hence, the NMR spectrum of the blue solution may give both broad and insufficient signals for components in complexes with free copigments, which are visualized by sharp signals in the spectra.\(^{27} \)

6. **In vivo analysis of hydrangea sepals coloration**

Recent innovations in MS spectrometry have promoted improved MS imaging to map intracellular organic and inorganic components. However, compared with animal tissues, mapping plant tissues has its unique difficulties as high water content and high osmotic pressure can compromise the analyte when cells break. Among imaging MS techniques, the most promising method for *in planta* visualization of water-soluble chemicals localized in vacuoles is cryo-time-of-flight secondary ion mass spectrometry (cryo-TOF-SIMS) of freeze-fixed samples.\(^{28,53–55} \) This preparation and analysis method have high spatial resolution, and therefore, we applied cryo-TOF-SIMS for mapping Al\(^{3+} \) and hydrangea blue pigments.

6.1. **TOF-SIMS analysis of hydrangea stem.**

As we have described, the major cause of toxicity in acidic soil is Al\(^{3+} \), but hydrangea is tolerant to Al\(^{3+} \) and can adsorb, transport, and accumulate Al\(^{3+} \) in its leaves and sepals. To observe Al\(^{3+} \) and other inorganic ion distribution, both freeze-dried and freeze-fixed stems were analyzed by TOF-SIMS.\(^{56} \) Simultaneously, the inorganic metal content was quantified by ICP-AES.\(^{56} \)

The actual amounts of inorganic elements in the stem were quantified by ICP-AES, which showed that Al soil treatment increased the amount of Na,
Mg, Al, and Ca and decreased the amount of K in the stem. The results of TOF-SIMS analysis of freeze-dried and freeze-fixed stems were similar. After Al soil treatment, the Al in stems was more widely distributed, and mainly located in the inner part of the cortex and in the xylem. This Al distribution was different from that of the other inorganic elements in both sample types. The results of both TOF-SIMS and ICP-AES were generally consistent with each other, which showed the possibility of semiquantitative visualization of the in planta distribution of inorganic elements within a nearly living state.

6.2. Cryo-TOF-SIMS analysis of hydrangea sepal.

We have already discussed the molecular weight of the hydrangea blue-complex. To determine whether the complex exists in sepal tissues, and to visualize the distribution of Al$^{3+}$ as well as other inorganic elements and organic components, we conducted cryo-TOF-SIMS analysis of hydrangea sepal tissue. Before conducting these measurements, cryo-TOF-SIMS measurements of synthetically produced hydrangea blue-complex solution and other standard solutions were carried out. In ESI-TOF-MS measurements of the blue solutions, both positive and negative detections gave a molecular ion peak at $m/z = 843$ and $m/z = 841$, respectively. However, in cryo-TOF-SIMS analysis, only the negative mode of detection gave a molecular ion peak. This might be because relatively high concentration of KCl ($100 \mu$M) to imitate the vacuolar condition might suppress ionization under positive mode.

Next, we performed cryo-TOF-SIMS analysis of blue sepal tissues. The total positive and negative ion images were consistent with the SEM imaging, indicating that the resolution of the MS imaging was high (Fig. 15A). In blue sepal tissues, the blue cells were located in the second cell layer, and the mass spectrometry imaging of the ions that attributed to the hydrangea blue-complex overlapped with the blue cell layer (Fig. 15B). The distribution of Al$^{3+}$ also overlapped with the distribution of the complex (Fig. 15C), while in the colorless epidermal cells, the atomic Al$^{3+}$ ions were hardly detected, and the potassium adduct ions of 5-O-caffeoyl and/or 3-O-acetylquinic acid were observed. Compared to the blue sepal tissue, red sepal tissue showed different images; red sepal tissue did not contain the hydrangea blue-complex or Al$^{3+}$ at detectable levels, but the potassium adduct ions of 5-O-caffeoyl and/or 3-O-acetylquinic acid were present. This was therefore the first report of the in vivo detection of the hydrangea blue-complex and Al$^{3+}$ in blue sepal cells that also showed that these factors are involved in the blue coloration of hydrangea sepal.

7. Aluminum transporters in hydrangea

The source of sepal and leaf Al$^{3+}$ is acidic soils. Most plants are sensitive to acidic soils and this is because aluminum ion in soils becomes water-soluble in acidic environment below pH 5.0. A very low amount of Al$^{3+}$ less than few ppm is toxic to plant roots. One-third of the global arable land is acidic soil, which can be a very serious problem in crop production; therefore, much research has been conducted to identify plants that are tolerant to acidic soil conditions. Several Al$^{3+}$-tolerant plants secrete organic acids from their roots into the soil to prevent the incorporation of Al$^{3+}$. Very few species, such as tea plants, buckwheat and hydrangea, are naturally tolerant to Al$^{3+}$ as Al-hyperaccumulators; the vacuolar concentrations is approximately 15 mM Al$^{3+}$ in blue hydrangea cells. However, the molecular mechanisms underlying Al transport and storage are poorly understood. Therefore, we attempted to identify Al transporters in hydrangea.

7.1. Search of aluminum transporter candidates from sepal.

Hydrangea is not a model plant and its complete genome has not yet been determined. Furthermore, no Al transporter gene has been found in yeast or other microorganisms. Thus, to identify candidate Al transporter genes in hydrangea, we used the following approach: (1) preparation and sequencing of a full-length cDNA library from blue hydrangea sepal tissue, (2) generation of a custom microarray based on these cDNAs, (3) selection of candidate genes by hybridization on the microarray, and (4) implementation of a complementation assay using an Al-sensitive yeast strain (Fig. 16).

According to the abovementioned strategy, followed by analysis of the subcellular localization to the membrane by SOSUI application, we obtained six candidate genes. To functionally assay these candidate genes, we used the $\Delta hsp150$ yeast mutant (Fig. 16). We found one gene that conferred Al-tolerance to the transformed yeast. The intracellular Al content of the transformants had significantly increased. These results strongly suggested that we had identified a vacuolar Al transporter gene ($Hm\text{VALT}$). Using the same strategy, we searched for plasma membrane-localized genes and found the candidate genes $Hm\text{PALT1}$ and $Hm\text{PALT2}$.61,63
**HmVALT** encodes a 252-amino acid (aa) polypeptide that belongs to the tonoplast-localized aquaporin family (TIP family).\(^6^1\) **HmPALT1** encodes a 304-aa protein that was similar to other nodulin-26-like intrinsic proteins (NIP).\(^6^1\) **HmPALT2** encodes 206-aa polypeptide and is predicted to be a member of the anion permease ArsB/NhaD family.\(^6^3\)

### 7.2. Functional studies of aluminum transporter candidates.

The subcellular localization of these three proteins indicated that **HmVALT** localized to the vacuolar membrane, while **HmPALT1** and **HmPALT2** were observed on the plasma membrane.\(^6^1\),\(^6^3\) The **HmVALT** transformants had Al-tolerance, while **HmPALT1** and **HmPALT2** transformants were Al-sensitive.\(^6^1\),\(^6^3\) The mutation of **HmVALT** with L162T (162nd leucine to threonine) caused a loss of Al-tolerance. All other TIPs have threonine at this site, therefore, the change is presumed to facilitate substrate selectivity, then the L162T mutation may cause a conformational change that decreases aluminum-selectivity and transport activity.\(^6^1\)

Lastly, we examined the effects of **HmVALT**, **HmPALT1**, and **HmPALT2** expression in planta.\(^6^1\),\(^6^3\) We assessed Al-tolerance by quantifying root growth in transgenic *Arabidopsis thaliana* seedlings expressing the hydrangea gene candidates. **HmVALT** significantly conferred Al-tolerance, while the introduction of **HmPALT1** and **HmPALT2** significantly conferred Al-hypersensitivity. Moreover, both double mutants of **HmVALT** and **HmPALT1** as well as **HmVALT** and **HmPALT2** recovered Al-tolerance.\(^6^1\),\(^6^3\) These results suggested a mechanism by which **HmPALT1** and **HmPALT2** transport aluminum into the cytosol, from where **HmVALT** transports aluminum into the vacuoles (Fig. 17).\(^6^1\),\(^6^3\) The **HmVALT**-overexpressing plants were more tolerant to aluminum than wild-type *Arabidopsis*, indicating that this gene might be a promising candidate for breeding crops with acidic soil tolerance.

### 8. Conclusion

Blue color development in hydrangea and hue variation are some of the oldest research subjects in flower coloration, and it has been more than a hundred years since the first publication on the subject. During this time, many Japanese researchers have contributed to the field; however, the mystery of the ephemeral blue coloration in hydrangea has remained uncharacterized. We have shown that the blue pigment can exist only in aqueous solution and still remains to be crystallized for further structural analysis. By developing and advancing various analytical methods, the chemistry of blue color development in hydrangea has been described. The hydrangea blue-complex is a metal complex composed of anthocyanin, 3-O-glucosyldelphinidin (1), 5-O-acylquinic acids (2, 3), and Al\(^{3+}\), which are assembled through weak bonds that support the metal complex and hydrophobic interactions. This complex consists of an anthocyanin-AI\(^{3+}\)-5-O-acylquinic acid in a 1:1:1 ratio that changes its formation in vacuoles due to various factors, such as the ratio of co-pigments, Al\(^{3+}\) content, and vacuolar pH. These alterations cause the color...
change, which is characteristic in hydrangea. However, why neighboring cells with similar genomic backgrounds and similar environmental conditions exhibit different colors is still an open question. In the near future, this question should be solved by a combination of single-cell analysis of chemical components and gene expressions.

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Profile

Kin-ichi Oyama was born in Kanazawa (Ishikawa Prefecture) in 1970 and was raised in Komaki (Aichi Prefecture) and Iwanuma (Miyagi Prefecture), Japan. He received his B.E. (1994) and M.E. (1996) degrees from the Graduate School of Engineering, Nagoya University. In 1997, he joined the Chemical Instrument Center at Nagoya University as a technical staff and started a study on the coloration of blue flowers using a combination of synthetic chemistry and instrumental analysis. He received his Ph.D. (2004) degree from the Graduate School of Bioagricultural Sciences, Nagoya University, under the supervision of Prof. Tadao Kondo. In 2004, he moved to the Chemical Instrumentation Facility, Research Center for Materials Science, Nagoya University, as a technical staff. At the facility, he contributed to the management, operation, and development of various instruments for the structural characterization of organic compounds. In particular, he has dedicated himself to the development and accumulation of mass spectrometry techniques. He received the Taisho Pharmaceutical Co., Ltd., Award in Synthetic Organic Chemistry Japan in 2007 and a SANBORE GRANT from the Suntory Institute from 2008 to 2010 for his research on supramolecular pigments of blue flowers. From 2016 to 2019, he has been an adjunct lecturer on organic chemistry at Chubu University. His current research interests include the synthesis, design, and study of biologically and materially important molecules such as oligosaccharides, polyphenols, and supramolecules.

Profile

Tadao Kondo was born in Nagoya (Japan) in 1941. He received his B.Sc. degree from Gifu Pharmaceutical University in 1965 and MS degree from the Department of Agricultural Chemistry, Nagoya University, in 1967. After joining the department as a research staff on the synthesis of nucleoside chemistry, he obtained his Ph.D. degree from Nagoya University in 1973 under the direction of Professor Toshio Goto. He then joined Professor R.U. Lemieux group as a post-doc in the Department of Chemistry at the University of Alberta, Canada (1973–1975). In 1979, he moved to the Chemical Instrument Center at Nagoya University as a research associate, and then in 1993, he became an Associate Professor. In 1998, he received the Chemical Society of Japan Award for Creative Work, and in 2014, he was awarded the Groupe Polyphenols Medal for his contribution on polyphenol research. In 2000, he moved to the Graduate School of Bioagricultural Sciences, Nagoya University, as a Professor of Polymer and Organic Chemistry. After his retirement in 2005, he has been working as a research fellow in the Graduate School of Informatics (Dr. Kumi Yoshida) at Nagoya University and serving as a Buddhist monk. His research field is natural product chemistry, including the synthesis of nucleosides, carbohydrates, and flavonoids; mechanism of flower color development; and structural analysis of natural products.