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A Peculiar Yellow Flower Coloration of Camellia Using Aluminum-flavonoid Interaction

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The yellow coloration mechanism of *Camellia chrysantha* flowers has been a mystery because the pigments which produce their deep yellow color could not be found in the flower. We sought to solve the mystery regarding the characteristic accumulation of aluminum ions by camellia plants. Deep yellow *C. chrysantha* flowers contained aluminum ions at three times the concentration found in pale yellow flowers. Three quercetin derivatives, 3-rutinoside, 3-glucoside, and 7-glucoside, were identified as major flavonoids in both flowers. There were no significant differences in their flavonoid content or pH, which was 5.8. The deep yellow flowers of *C. chrysantha* contained flavonoids and aluminum ions in a ratio of 1 to 0.5. When quercetin 3-rutinoside solution, which was originally almost colorless and had an absorption maximum around 350 nm at pH 5.8, was prepared with an aluminum ion concentration similar to the endogenous ratio, the solution developed a deep yellow color. This mixture also had an absorption spectrum like that of *C. chrysantha* petals, which had an absorption maximum around 420 nm. After removing the cations using an ion exchange column, the yellow coloration of the *C. chrysantha* petal extract changed to a paler color; the coloration was restored by the addition of aluminum ions. We concluded that the deep yellow color of the *C. chrysantha* flower is generated by the chelation of aluminum ions by quercetin derivatives, which is a unique yellow 'dyeing' system of these flowers.

Key Words: aluminum, *Camellia chrysantha*, flavonoids, yellow flower.

Introduction

Humans recognize compounds absorbing 400–500 nm wavelength light as yellow pigments. In many plants, yellow flower pigments are carotenoids and flavonoids. Many carotenoids are deep yellow due to their heavy absorbance of light wavelengths with two or three absorption maxima around 440 nm, while many flavonoids are pale yellow due to their smaller absorbance of light wavelengths with an absorption maximum around 360 nm. Most deep yellow flower color is produced by carotenoid or a few kinds of flavonoid pigments, such as chalcones and aurones, which have a relatively high absorbance of 400–500 nm light wavelength.

An unexpected finding was that many kinds of plants cannot generate deep yellow coloration because they lack the ability to synthesize or accumulate large enough amounts of carotenoids, chalcones or aurones, and thus, these plants produce pale yellow flowers resulting from pale yellow flavonoids. Deep yellow varieties of these ornamental plants are often requested, thus, developing yellow flower pigmentation has been an important floricultural challenge similar to the blue pigmentation of roses, carnations, and some other species. Recently, yellow flower color was generated in *Torenia hybrida* using a transgenic technique (Ono et al., 2006), as were a 'blue' rose (Katsumoto and Tanaka, 2005) and carnation (Tanaka et al., 2005).

Camellia is an ornamental shrub used worldwide, and there is active interest in obtaining flowers with yellow coloration. Horticultural utilization of camellias has developed over the past 400 years in Japan, and many varieties have been produced during that time (Tuyama, 1968). Even fairly recently, flower coloration has been restricted to white, pink, and red; thus, it was an époque-making report in 1965 that introduced the deep yellow camellia flower, *Camellia chrysantha* (Hu, 1965).

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Although *C. chrysantha* has been used as a resource for potentially breeding yellow camellia varieties for the garden, progeny with comparable deep yellow flower color have not yet been obtained (Hwang et al., 1992; Nishimoto et al., 2004), suggesting complexity in the yellow coloration mechanism.

Three flavonoids, which are quercetin derivatives, and two carotenoids were reported to be yellow pigments in *C. chrysantha* flowers (Miyajima et al., 1985; Scogin, 1986). These flavonoids are pale yellow pigments, as opposed to aurones and chalcones, so it would seem difficult for these flavonoids to generate the deep yellow color of *C. chrysantha*. Nishimoto et al. (2004) discussed the contribution of carotenoids to the yellow coloration; however, in our opinion, the carotenoid content is too low to account for the deep yellow color. Yellow coloration is still a mystery, the resolution of which could contribute to breeding new yellow camellia varieties for the garden.

The tea plant, *C. sinensis*, which belongs to the same subgenus *Thea* as *C. chrysantha* (Chang and Bartholomew, 1984), accumulates aluminum in its shoots (Chenery 1955; Matsumoto et al., 1976). It is a well-known *in vitro* phenomenon that some quercetin derivatives generate deep yellow coloration by the chelation of aluminum ions (Markham and Mabry, 1968). This spurred our hope to resolve the mystery, and prompted us to study the flavonoids and aluminum in the *C. chrysantha* flower. Here we report a new mechanism for generating deep yellow flower pigmentation, involving a flavonoid-metal ion complex.

Materials and Methods

Plant materials

Camellia plants have been growing in pot cultures at the National Institute of Floricultural Science (NIFS) for over 10 years. Some clones were made by grafts from one genetic resource, *C. chrysantha* (NIFS ID No. 566). Flowers from a plant with pale yellow blossoms, another with deep yellow ones, and flowers from *C. japonica* 'Chinshin' (NIFS ID No. 622), which were white, were sampled (Fig. 1) and analyzed using the following procedures, except carotenoid analysis, for which flowers of *C. chrysantha* accession, NIFS ID No. 565, were used.

Carotenoid analysis

Petals of *C. chrysantha* were extracted first with CH_3OH in order to avoid browning, followed by extraction three times with distilled water, twice with CH_3OH, and three times with acetone, successively using 5 mL per 1 g of fresh weight petals. The extracts were combined and evaporated to dryness. The residue was partitioned between water and diethyl ether. The diethyl ether fraction was evaporated to dryness and dissolved in *n*-hexane, and again evaporated to dryness. The residue was dissolved in CH_3OH and absorption at 440 nm was measured using a UV-VIS recording spectrophotometer (UV-2200, Shimadzu Co., Japan). Four flowers were measured and the average ± SE was obtained.

Measurements of Coloration and Absorption Spectra of fresh petals

Coloration values (C, 2°; L*, a*, b*) and absorption spectra of the abaxial side of fresh petals of each plant's flower were measured by a color sensor (C-1020, Hitachi, Ltd., Japan). Petal coloration is a little different among the outer, middle, and inner petals in a flower (Fig. 1), so measurements were performed at these three petal locations and the average was obtained as the value for the flower. Spectral reflectance factors (R) were measured using a C-1020 in the following 15 wavelength ranges; the absorption ratios were reported as values of 100-R at the mean wavelength of each range, which is in parentheses: 390–410 nm (400 nm), 420–430 nm (425 nm), 440–450 nm (445 nm), 460–470 nm (465 nm), 480–490 nm (485 nm), 500–510 nm (505 nm), 520–530 nm (525 nm), 540–550 nm (545 nm), 560–570 nm (565 nm), 580–590 nm (585 nm), 600–610 nm (605 nm), 620–630 nm (625 nm), 640–650 nm (645 nm), 660–670 nm (665 nm), and 680–730 nm (705 nm). Three flowers from each plant were measured and the averages were obtained.

Measurement of pH of fresh petals

One fresh petal from each plant's flower was homo-

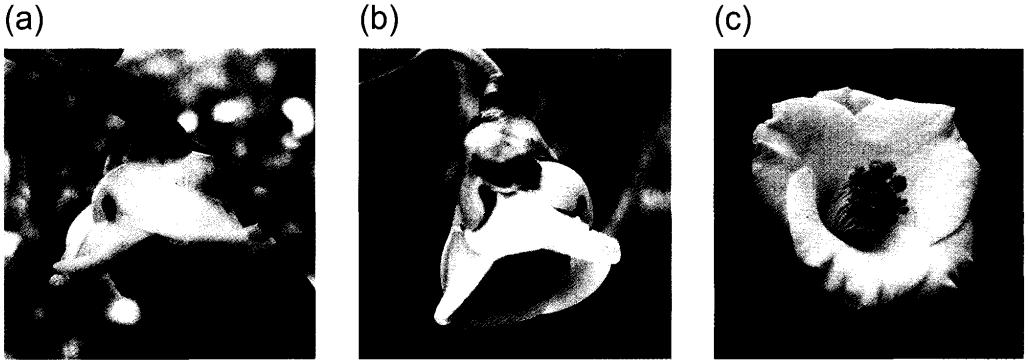

Fig. 1. Flowers of *C. chrysantha* (NIFS ID No. 566) and *C. japonica*. (a) Deep yellow *C. chrysantha*, (b) pale yellow *C. chrysantha*, and (c) *C. japonica* 'Chinshin'.

genized in a 1.5 mL test tube and the pH of the homogenate was measured using a compact pH meter (B-212, Horiba Ltd., Japan). As described above, three petals per flower were measured and the average was used as the value for the flower. Three flowers from each plant were measured and the averages ± SE were obtained.

Flavonoid analysis

Fresh petals were frozen in liquid nitrogen and stored at −80°C. Petals were extracted overnight at room temperature using 50% CH_3CO_2H (2 mL per 1 g fresh weight sample). Extraction was performed two times. Aliquots of 10 μL of the combined extracts from each sample were analyzed using an HP1100 system with a photodiode array detector (Agilent Technologies-Yokogawa Analytical Systems, Inc., USA) and an Inertsil ODS-2 column (4.6 mm × 250 mm, GL Sciences Inc., Japan) at 40°C with a flow rate of 0.8 mL·min^{-1}. Absorption spectra were determined over the range 240–580 nm. A linear gradient of 10–50% of solvent B (1.5% H_3PO_4, 40% CH_3CN, 50% CH_3CO_2H) in solvent A (1.5% H_3PO_4) was run over a 40-min period. Flavonoids were identified based on the absorption spectra and retention times by comparison with authentic quercetin 3-rutinoside (Tokyo Chemical Industry Co., Ltd., Japan) and quercetin 3-glucoside (Extrasynthese, France). They were quantified by their absorbance at 360 nm as a quercetin 3-rutinoside equivalent. Four flowers from each plant were measured and the averages ± SE were obtained.

Aluminum analysis

Fresh petals were dried at 85°C over 4 h and stored in a desiccator. Ground plant material of ca. 150 mg was digested with 61% HNO_3 and 70% $HClO_4$ reagents. The following procedure was used. A 0.5 mL aliquot of HNO_3 was added to the sample and left overnight, then 1 mL of HNO_3 was added and it was heated for 2 h at 180°C. After this operation, 0.5 mL of $HClO_4$ was added and heated for 4 h at 180°C. Finally, 0.5 mL of HNO_3 was added and digested for 2 h at 180°C. After cooling, the digested solution was filtered and diluted with 1 M HNO_3 solution to a volume of 25 mL. Then, 10 mL of the solution was again diluted with 1 M HNO_3 to a volume of 25 mL. The aluminum concentration in the solution was measured with an inductively coupled plasma-atomic emission spectrometer (ICP-AES, P-4010, Hitachi). The accuracy of the elemental analysis was checked by using standard reference material, SRM 1515 Apple Leaves (National Institute of Standards and Technology, USA). Four flowers for *C. chrysantha* and three flowers for *C. japonica* were measured and the averages ± SE were obtained.

Measurement of the Absorption Spectra of flavonoid-aluminum solutions

Quercetin 3-rutinoside, which had been dissolved in a small amount of MeOH, was dissolved in a 0.1 M CH_3CO_2H buffer (pH 5.8) solution, and $AlCl_3$ (Wako Pure Chemical Industries, Ltd., Japan) solution (0.1 M CH_3CO_2H buffer, pH 5.8) was added. Thus, eleven solutions were made, which contained 50 μM quercetin 3-rutinoside with 0 μM to 50 μM of aluminum ions in 5 μM steps, in 0.1 M CH_3CO_2H at pH 5.8 (Fig. 4a). The absorption spectrum with a wavelength range of 300–600 nm was measured for each solution using a UV-2200.

Ion exchange chromatography

In order to avoid browning, petals of the deep yellow flower of *C. chrysantha* were heated with an iron and stored at −80°C. The yellow pigments were extracted twice with distilled water (each time using 5 mL per 1 g fresh weight petals) using a mortar and pestle. Part of the combined extracts was passed through an ion exchange column Dowex 50W × 8 (100–200 mesh, H-form; The Dow Chemical Company, USA). Both the extract and the column eluent were adjusted to 0.1 M CH_3CO_2H (pH 5.8) solution using 0.2 M CH_3CO_2H and 0.5 M NaOH. Furthermore $AlCl_3$ solution was added to part of the column eluent, and adjusted to the same pH. The absorption spectrum with a wavelength range of 300–600 nm was measured for each solution. The flavonoid content was analyzed using high performance liquid chromatography (HPLC).

Results

Carotenoid content

We analyzed deep yellow flowers of *C. chrysantha* (NIFS ID No. 565) to discuss the contribution of carotenoids to the yellow coloration of general *C. chrysantha*. The extracts were purified by solvent partitioning and total carotenoid content was calculated as 3.72 ± 0.44 nmol lutein equivalent·g^{-1} FW.

Coloration and Absorption Spectra of Fresh Petals

The yellow color intensity of the flowers was reflected by the b* values, which indicate yellowness; significant differences were not found among the a* values, which indicate redness (Table 1). The absorption maximum of deep yellow *C. chrysantha* fresh petals was detected at around 420 nm (Fig. 2). The absorption maximum of pale yellow flower of *C. chrysantha* was also detected around 420 nm; however, the absorption attenuated more quickly at higher wavelengths than in the deep yellow flower type. Using the white flower of *C. japonica* 'Chinshin', the absorption maximum could not be detected in the 400–500 nm wavelength region, and the absorption ratio in the region was much less than those of both *C. chrysantha* flowers (Fig. 2).

pH values, flavonoids, and aluminum content

Homogenates of petals of both deep and pale yellow flowers of *C. chrysantha* had a pH of 5.8, while that of

Table 1. Chromatic components, pH, the content of the major flavonoids, and of aluminum ions of camellia flowers. The peaks detected in *C. japonica* were too weak to use to identify the flavonoids.

	C. chrysantha (deep yellow)	*C. chrysantha* (pale yellow)	*C. japonica* (white)
L*	81.8	84.6	93.5
a*	−3.9	−5.7	−1.6
b*	67.8	56.8	9.2
pH	5.8 ± 0.1	5.8 ± 0.1	4.2 ± 0.1
Component contents (μmol·g^{-1} FW)			
Quercetin 7-glucoside	1.75 ± 0.09	1.64 ± 0.04	—
Quercetin 3-rutinoside	2.42 ± 0.14	2.04 ± 0.07	—
Quercetin 3-glucoside	0.67 ± 0.03	0.55 ± 0.02	—
Total flavonol	4.84 ± 0.25	4.23 ± 0.11	—
Aluminum	2.33 ± 0.18	0.79 ± 0.08	0.48 ± 0.05

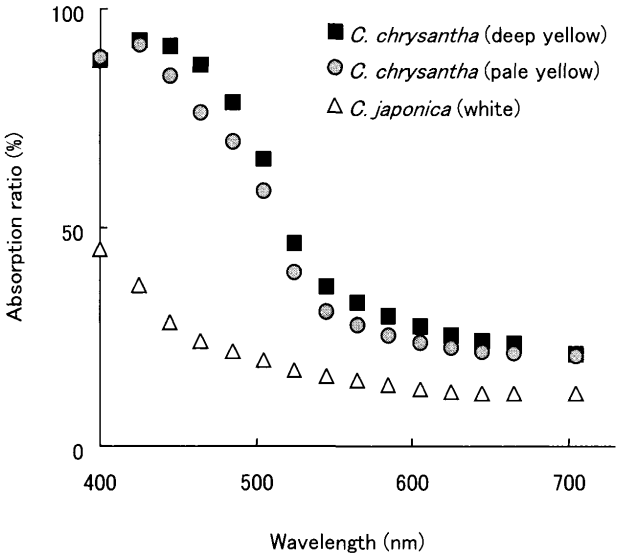

Fig. 2. Absorption spectra of fresh petals of *C. chrysantha* and *C. japonica*. Spectral reflectance factors (R) were measured using a C-1020 in 15 wavelength ranges; absorption ratios were reported as values of 100-R at the mean wavelength of each range.

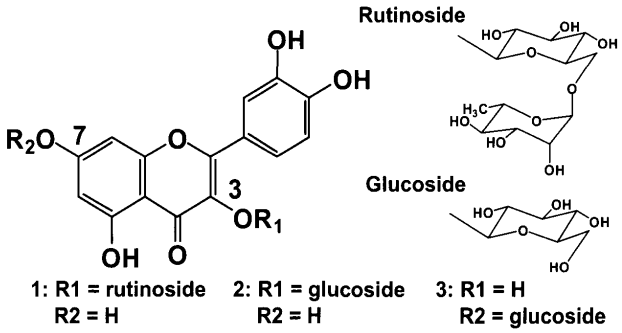

Fig. 3. Structures of the major flavonoids in *C. chrysantha* petals. 1. Quercetin 3-rutinoside, 2. quercetin 3-glucoside, and 3. quercetin 7-glucoside.

the white flower of *C. japonica* 'Chinshin' had a pH of 4.2 (Table 1). Three major flavonoids were detected in *C. chrysantha* using HPLC. Among them, quercetin 3-rutinoside and quercetin 3-glucoside were identified based on the coincidence of retention times and absorption spectra with those of standard samples. The other major flavonoid was determined to be quercetin 7-glucoside by referring to previous work (Miyajima et al., 1985; Scogin, 1986). The concentration of each of these compounds was similar in these flowers (Table 1 and Fig. 3). No significant flavonoid signal could be detected by HPLC in the 'Chinshin' extract, suggesting that the flavonoid content of the white flower petals was much lower than that of *C. chrysantha* flowers. The aluminum content in the petals of pale yellow *C. chrysantha* and white *C. japonica* was 34% and 21%, respectively, of that of deep yellow *C. chrysantha*.

Effect of aluminum ions on flavonoid solution

The changes in color and absorption spectra were investigated after the addition of aluminum ions to quercetin 3-rutinoside (50 μM) dissolved in acetic acid buffer at pH 5.8, which is the pH of homogenates of *C. chrysantha* petals. In the absence of aluminum ions, the flavonoid solution was almost colorless (Fig. 4a), the absorption maximum was detected around 350 nm, and there was little absorbance in the 400–500 nm region (Fig. 4b). With increasing aluminum ion concentration, the yellow color of the solution became deeper (Fig. 4a), the width of the wavelength band where absorption took place broadened, and the absorption maximum progressively changed, with a new maximum appearing at 420 nm (Fig. 4b).

Effect of aluminum ions on ion exchange eluent

After the *C. chrysantha* deep yellow petal distilled water extract, ion exchange column eluent, and the eluent with aluminum ions were adjusted to pH 5.8, a comparison was made of their coloration and absorption spectra. The yellow color of the distilled water extract (Fig. 5a-1) was reduced to pale yellow by passing it through an ion exchange column (Fig. 5a-2), and there was a decrease in absorbance around 420 nm (Fig. 5b-2). Adding aluminum ions to this column eluent raised the

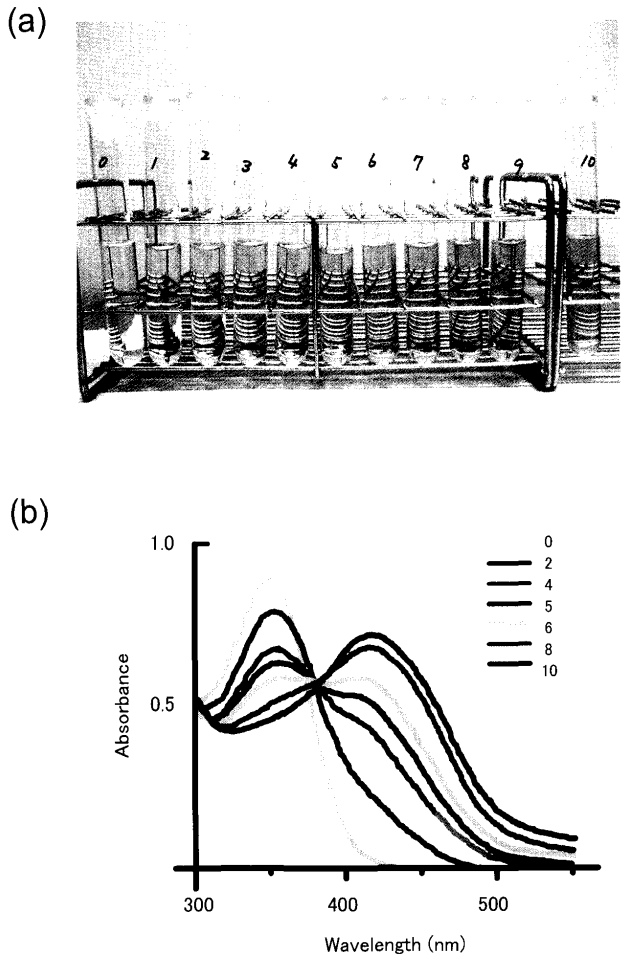

Fig. 4. Coloration and absorption spectra of quercetin 3-rutinoside/aluminum solutions. (a) Picture of the solutions. Solutions with ratios of aluminum ions to flavonoids ranging from 0 : 10 (left) to 10 : 10 (right) were prepared with a 0.1 M CH_3CO_2H, pH 5.8 buffer. Aluminum ions were added to 50 μM quercetin 3-rutinoside solutions in 5 μM steps from 0 μM to 50 μM. (b) Absorption spectra of the solutions. Numbers assigned to the spectra coincide with those of the solutions in (a). The ratio of 5 : 10, represented by a red line, was nearly the same as the composition ratio in deep yellow *C. chrysantha*.

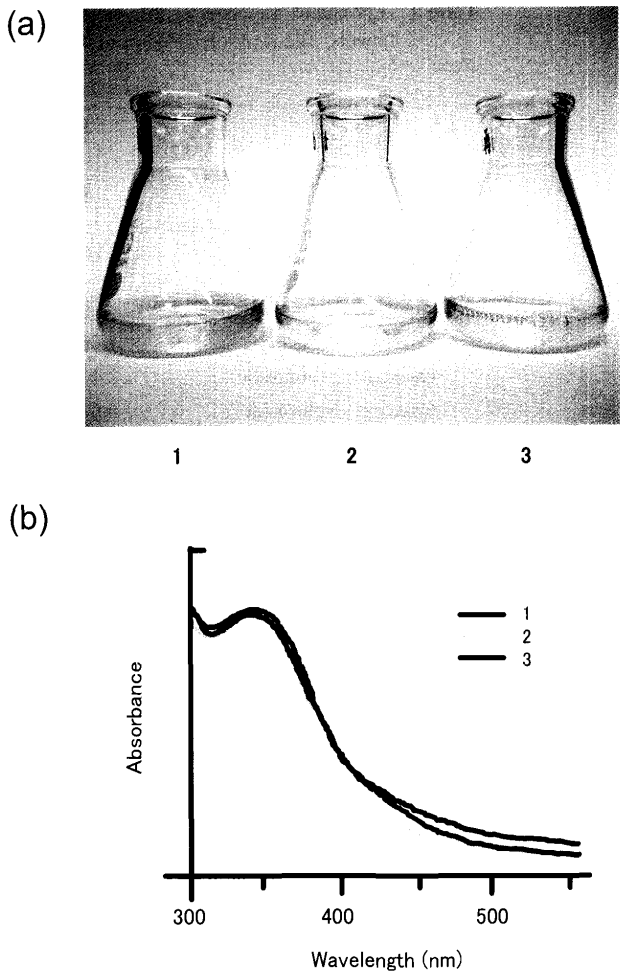

Fig. 5. Coloration and absorption spectra of extracts from deep yellow flowers of *C. chrysantha*. (a) Picture of the solutions. 1. extract of the petals, 2. eluent of an ion exchange column, and 3. the eluent with added 15 μM of aluminum ions. Solutions were prepared with a 0.1 M CH_3CO_2H buffer at pH 5.8. (b) Absorption spectra of the solutions. Absorbance of spectrum 1 was 1.5 and those of 2 and 3 were 1.0. Flavonoid content in solution 1 (30 μM) was reduced to 20 μM in solutions 2 and 3 by means of column elution. The absorbance of each spectrum was adjusted based on the flavonoid concentration.

absorbance around 420 nm (Fig. 5b-3) and deepened the yellow color (Fig. 5a-3); this solution contained 21 μM of flavonoids and 15 μM of aluminum ions were added.

Discussion

The yellow color intensity of camellia petals represented by the b* values corresponded well with their visual color tones (Table 1 and Fig. 1). The characteristics of the absorption spectra of fresh *C. chrysantha* petals, with absorption maxima occurring at 420 nm (Fig. 2), are different from those of carotenoids. The carotenoid content in deep yellow flowers of *C. chrysantha* was 3.72 ± 0.44 nmol lutein equivalent·g^{-1} FW, which was less than the 16 nmol lutein equivalent·g^{-1} FW in pale yellow flowers of *Eustoma* (Nakayama et al., 2006). We concluded that carotenoids are not the major responsible pigments for the deep yellow coloration of *C. chrysantha*.

The deep yellow and pale yellow flowers of *C. chrysantha*, which are from clones generated from the same plant, have the same flavonoid components, including quercetin 7-glucoside, which has been considered the main contributor of the yellow pigmentation (Miyajima et al., 1985). No significant difference in the flavonoid content of these flowers has been found, and both flowers had the same pH, 5.8. A commercial reagent solution of quercetin 3-rutinoside, which was used as a representative of the quercetin derivative contained in *C. chrysantha*, was almost colorless and had an absorption maximum at 350 nm at pH 5.8 (Fig. 4). Therefore, it is difficult to ascribe the deep yellow coloration of *C. chrysantha* simply to flavonoids.

A significant difference was found in the aluminum ion content of the deep yellow and pale yellow flowers of *C. chrysantha* (Table 1). Among the major flavonoids in the petals, only quercetin 7-glucoside has a hydroxyl

group at the 3-position (Fig. 3); this hydroxyl group is one of the chelation positions of aluminum (Markham and Mabry, 1968). As a result, quercetin 7-glucoside seems to be a better ligand of aluminum ions than either quercetin 3-rutinoside or quercetin 3-glucoside. In this investigation, we used quercetin 3-rutinoside to study the effect of aluminum ions on the coloration of flavonoids because it was readily available. We added aluminum ions to quercetin 3-rutinoside solution at a concentration 0.5 times that of the flavonoid content, which is the same content ratio as is found in deep yellow flowers of *C. chrysantha*. Even quercetin 3-rutinoside solution under these conditions shifted its absorption maximum from 350 nm to 420 nm, increased light absorbance in the 400–500 nm wavelength region, and generated a deeper yellow coloration (Fig. 4). These characteristics are similar to those of fresh petals of *C. chrysantha*. After using an ion exchange column to remove the cations, including aluminum ions, the yellow coloration of the deep yellow *C. chrysantha* flower extract changed to a paler color, the 400–500 nm wavelength light region absorbance decreased, and furthermore, these properties were restored by the addition of aluminum ions (Fig. 5). We concluded that the deep yellow coloration of *C. chrysantha* flowers is generated by the chelation of aluminum ions by quercetin derivatives.

The *C. chrysantha* petal extract did not have a clear absorption maximum around 420 nm even when aluminum ions were added to the ion charge column eluent at a higher concentration than the original petal content (Fig. 5b). It is known that citric acid and other organic acids form complexes with aluminum ions (Hue et al., 1986); therefore, inter- or intra-cellular contamination by these molecules may interfere with the effect of aluminum ions in the extract.

Yellow color intensity mainly depended on the aluminum ion level rather than the flavonoid level in *C. chrysantha* flowers (Table 1). White coloration of *C. japonica* 'Chinshin' flower is probably due to low levels of flavonoids and aluminum ions, and lower pH. Introduction of deep yellow property into *C. japonica* flowers has not been successful by crossing with *C. chrysantha* yet. As some progeny of *C. japonica* and *C. chrysantha* seem to have sufficient flavonoids (Nishimoto et al., 2004), they may lack the conditions for yellow coloration in aluminum ions and/or pH. By selecting plants of *C. chrysantha* and other yellow *Camellia* species, and especially *C. japonica* possessing a relatively higher level of aluminum ions and higher pH value, we would obtain deeper yellow flower progeny.

Aluminum and other metal ion complexes have been found to be involved in flower coloration in *Hydrangea macrophylla*, *Commelina communis*, *Centaurea cyanus*, *Salvia patens* (Takeda, 2006), *Meconopsis grandis* (Yoshida et al., 2006), and *Tulipa gesneriana* (Shoji et al., 2007), where they are chelated to anthocyanins and other compounds, and generate a blue color. In this study, we found the involvement of metal ions in yellow flower coloration as well. As suggested by a poem, No. 3101, recorded in Vol. 12 of the *Man'yoshu*, which is the earliest extant anthology of Japanese verse, compiled in ca. 8th century, aluminum ions accumulated in camellia shoots were used for dyeing cloth. Camellia plants also use accumulated aluminum ions for 'dyeing' their flowers, probably an earlier use than that by humans.

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