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Co-pigmentation of Anthocyanin-flavonol in the Blotch Area of *Rhododendron simsii* Planch. Flowers

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***Rhododendron simsii* Planch. is distributed in eastern Asia, such as Ryukyu Islands of Japan, southern China, Taiwan, and Vietnam. It is a semideciduous shrub with five-lobed red corollas and reddish-purple blotches on the upper petals. From microscopic observation, reddish-purple colored cells were distributed only in the adaxial subepidermis of blotch areas in the upper petals, while red colored cells were observed in the whole epidermis of both upper and lower petals. Even though the anthocyanin constitutions of upper and lower petals were the same, the λ_{max} of absorbance of fresh upper petals was 514.9 nm, while it was 505.7 nm in the lower petals. In *R. simsii* flowers, cyanidin glycosides were major anthocyanins in whole petals. Quercetin glycosides were detected in reddish-purple blotch areas, but they were found only in trace amounts in lower petals. Anthocyanins and flavonols were extracted, and separated by column chromatography. Bathochromic shift (9.7 nm) was observed in mixed solution of anthocyanin and flavonol at a ratio of concentration of 1:7.5. These results were similar to the bathochromic shift obtained between upper and lower petals of intact flowers (9.2 nm). From these findings, co-pigmentation between anthocyanin and flavonol (quercetin glycosides) seems to be a key factor of reddish-purple color of the blotch area in the upper petals of *R. simsii* flowers.**

Key Words: bathochromic shift, blotches, co-pigments, flower color.

Introduction

The wild type of *Rhododendron simsii* is distributed in eastern Asia, such as Japan, southern China, Taiwan, and Vietnam (Galle, 1985). This species was introduced to Europe at the end of the 18th century, and its breeding has been started. Numerous cultivars with variation of colors, such as pink, reddish-purple, purple, or white, have been bred as Belgian azaleas. At present, these azalea varieties are one of the most important potted plants in Western Europe, the USA, and Japan.

The northern region of Vietnam is adjacent to China, which is considered as the center of the origin of wild azaleas. *Rhododendron simsii* are distributed in the mountainous areas of northern and central parts of Vietnam with above 800–1400 m altitude, and these accessions seem to be important genetic germplasms of azalea breeding in Vietnam. However, breeding programs of this species have not been conducted for domestic production.

Rhododendron simsii has five-lobed red corollas with reddish-purple blotches on the upper inside of the petals (Fig. 1A). As with all flowering plants, flower color is one of the most important features for breeding. In azalea, Mizuta et al. (2009) suggested that red-flowered azaleas, such as *R. indicum*, *R. kaempferi*, *R. oldhamii*, and *R. scabrum*, contain two major anthocyanins in their petals. Hang et al. (2011) reported that two major anthocyanins found in *R. simsii* accessions of Vietnam and Japan were identified as cyanidin 3-galactoside (Cy 3Ga) and cyanidin 3-arabinoside (Cy 3Ar). However, no report indicated the cause of the different color expressions between upper and lower petals of wild *R. simsii* flowers.

The purpose of this study was to clarify the pigmentation of reddish-purple blotches in upper petals of *R. simsii* flowers for future breeding using genetic germplasm in Vietnam.

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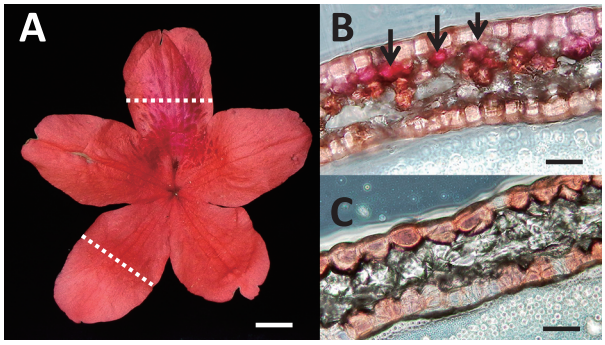

Fig. 1. Cross-sections of flower petals of *R. simsii*. A: Whole flower, B: Cross-section of upper petal, C: Cross-section of lower petal. Broken lines in photo A indicate sliced positions. Arrows in photo B indicate reddish-purple cells. Bars: photo A = 1 cm, photo B and C = 20 μm.

Materials and Methods

Plant materials

Fresh flowers of wild *R. simsii* distributed in Than Uyen district, Lai Chau province, Vietnam were collected at anthesis. A part of fresh petals was used for microscopic observation and absorption spectra measurement. The remaining flowers were separated into upper and lower petals, and each part of petals was boiled at 100°C for 5 seconds, and immediately cooled in water. Then, the samples were dried in a forced convention oven overnight at 50°C. The dried samples were stored in a desiccator at 4°C until pigment analysis.

Microscopic observation of pigment distribution and absorption spectra of fresh petals

Upper and lower petals of fresh flowers of *R. simsii* were cut into small squares at the center positions, and fixed using 5% agar in the petri dish. Then they were sliced using a microslicer (DTK-1000; Dosaka EM, Kyoto, Japan) at a thickness of 150 μm. After slicing, cross-sections were observed under an optical microscope (Leica DM-2500; Leica Microsystems GmbH, Wetzlar, Germany).

Fully opened fresh petals were cut into squares in the center of the lobes of upper and lower petals. Then, the samples were attached to the glass cell using transparent tape, and immediately measured from 370 nm to 700 nm using a UV spectrophotometer (U-2910; Hitachi, Kyoto, Japan). Five samples were used as replication.

HPLC analysis

Dried petals (ca. 50 mg) of *R. simsii* were soaked overnight with 50% HOAc-H_2O. After filtration, analytical HPLC was conducted on a LC-20AD pump (Shimadzu, Kyoto, Japan), using a Cosmosil $5C_{18}$ MS-II column (4.6 Φ × 250 mm; Nakalai Tesque, Kyoto, Japan) at 40°C with a flow rate of 1 mL·min^{-1}, and monitoring at 520 nm and 360 nm for anthocyanins and flavonols, respectively. A linear gradient elution was applied for 40 min from 20% to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O).

Identification of pigments

Two-dimensional thin layer chromatography (TLC) was carried out on cellulose-coated glass plates (Merck, Darmstadt, Germany) using two mobile phases: BAW (*1*-BuOH/HOAc/H_2O, 4:1:2, v/v/v) and 10% HOAc. The plates were observed under UV light (365 nm), and the color of all spots was recorded. Subsequently, each spot was collected from the TLC plate, and dissolved using MeOH for HPLC analysis.

Isolation of major flavonols

Dried petals (ca. 0.7 g) of *R. simsii* were soaked overnight with 100% MeOH. After filtration, preparative HPLC was performed on an LC-6AD system (Shimadzu), using a Cosmosil $5C_{18}$ AR column (20 Φ × 250 mm; Nakalai Tesque) at 40°C with a flow rate of 9 mL·min^{-1}, and monitoring at 360 nm for isolation of major flavonol peaks. A linear gradient elution was applied for 40 min from 50 to 85% solvent B (10% formic acid, 40% MeCN in H_2O) in solvent A (10% formic acid in H_2O). Major peaks were obtained using a fraction collector.

Identification of flavonol aglycones

Each purified flavonol was acid hydrolyzed using 2N HCl at 100°C for 90 min. The flavonol aglycones were co-chromatographed with authentic standard flavonol aglycones, such as myricetin, quercetin and kaempferol, by HPLC with constant flow of 75% solvent A (0.1 M HOAc): 25% solvent B (MeCN). The HPLC system, column, and flow rate were the same as mentioned above (*HPLC analysis*). Wavelength was set at 360 nm.

In vitro co-pigmentation test

Approximately 20 g dried upper petals of *R. simsii* was immersed with 1 L of 10% formic acid in H_2O for 24 hours under 5°C. After filtration, the filtrate was diluted with the same amount of water (1 L), and pass through Diaion HP-20 resin (350 mm × 60 mm i.d.; Mitsubishi Chemical, Tokyo, Japan). The column was thoroughly washed with 5 L water, and pigments were eluted using 5% formic acid in MeOH. After concentration of the eluent, it was again purified, and fractionated using Sephadex LH-20 (200 mm × 40 mm i.d.; Sigma-Aldrich, St. Louis, MO, USA) with 5% formic acid in 50% EtOH solution. The absorption spectra of the obtained fractions were measured using a UV spectrophotometer (U-2910; Hitachi) at wavelengths from 300 nm to 700 nm to avoid contamination between anthocyanins and flavonols. Then separated anthocyanin and flavonol fractions were concentrated below 40°C

in vacuo to almost dryness using an evaporator for powdering.

To examine anthocyanin-flavonol co-pigmentation, powder of anthocyanins and flavonols were dissolved in 0.1 M Glycine-HCl buffer (pH = 2.5), and mixed together at various ratios. Since the pH value of squeezed juice of fresh petals was determined to be 2.52 using a compact pH meter (TWIN pH waterproof B-212; Horiba Ltd., Kyoto, Japan), and that value was similar in both upper and lower petals of *R. simsii*, we decided to prepare the buffer of co-pigmentation test with the same pH value. The absorbance of each mixed solution was measured using a UV spectrophotometer (U-2910; Hitachi).

Results and Discussion

Pigments accumulate in the epidermis of petals of many flower species. However, the cross-sections of blotches from fresh upper petals of *R. simsii* showed that reddish-purple pigment cells accumulated in the adaxial subepidermis of upper petals, while red colored cells were numerously distributed in the epidermis of both upper and lower petals (Fig. 1B, C). The upper epidermis of blotch areas was flat, and the shape of reddish-purple pigmented cells was not different from that of red pigmented cells. Generally, in blotches of *Rhododendron* species flowers, the upper and lower epidermal cells have weaker color intensity compared with adaxial subepidermal cells, which are strongly colored by pigments (Pecherer, 1992). The same results were observed in the transverse section of red blotches in *R. schlippenbachii* flowers. In this species, red pigments were distributed in subepidermal cells while light pink colored cells accumulated in the upper epidermis (Yamagishi and Akagi, 2013). Our results indicated that reddish-purple blotches arise from pigment accumulation in adaxial subepidermal cells of upper petals.

The absorption spectra of fresh petal samples showed the λ_{max} in the visible region at 514.9 nm and 505.7 nm for upper and lower petals, respectively (Fig. 2). The wavelength of upper petals shifted 9.2 nm longer than that of lower petals.

Both upper and lower petals contained two major anthocyanins (Fig. 3A), which have been reported as cyanidin 3-galactoside (Cy 3Ga) and cyanidin 3-arabinoside (Cy 3Ar) (Hang et al., 2011). In the HPLC analysis at 360 nm, two major peaks were detected in upper petals, while no major peak was detected in lower petals (Fig. 3B). Thus, these two flavonoids seem to be present only in upper petals of *R. simsii* flowers.

To identify these two major flavonoids in upper petals, two-dimensional TLC was carried out. Eight discrete spots appeared in the TLC plates (Fig. 4) and color properties were recorded under visible and UV light (Table 1). Spot numbers 1 and 2 expressed red-lilac color under visible light and violet under UV light. These two spots were considered to be two major anthocyanins (Cy 3Ga and Cy 3Ar). In addition, spots 3 and 4 appeared pale brown under visible light, and showed yellow florescence under UV light. Spethmann (1980) investigated flavonoids of *Rhododendron* flowers, and reported that some flavonol glycosides, such as

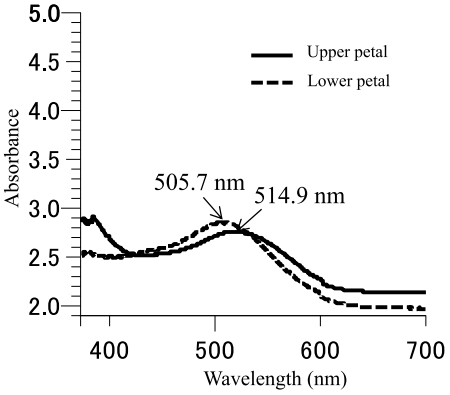

Fig. 2. Absorption spectra of intact petals of *R. simsii*.

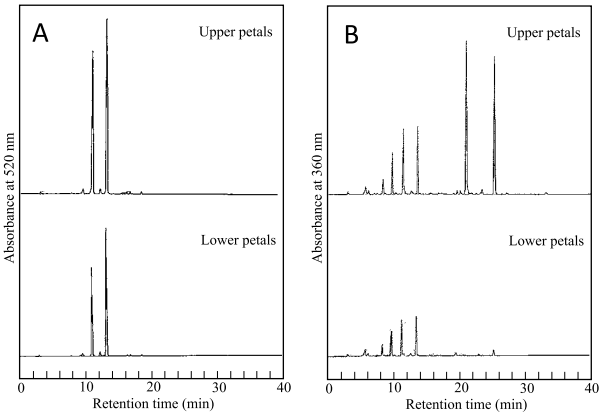

Fig. 3. HPLC analysis of extracts from upper and lower petals of *R. simsii* under 520 nm (A) and 360 nm (B).

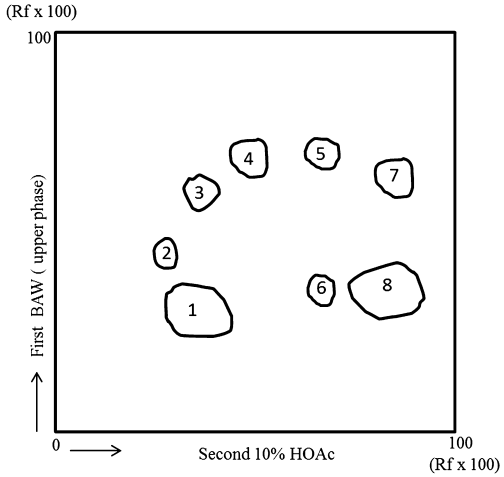

Fig. 4. Two-dimensional thin layer chromatogram of MeOH extract from upper petals of *R. simsii* flowers. BAW (*1*-BuOH/HOAc/H_2O, 4:1:2, v/v/v). See Table 1 for color properties of spots.

quercetin 3-arabinoside, quercetin 3-rhamnoside, or kaempferol 5-methylether, appeared brown or pale brown under visible light and colored greenish-yellow to yellow florescence under UV light on TLC plate. These findings suggested that spots 3 and 4 are flavonols. The HPLC analysis of spot 3 and 4 was carried out after collection and extraction from TLC plate. From HPLC analysis, spots 3 and 4 coincided with two major peaks in upper petals (Fig. 3B).

Next, these two major peaks in upper petals were isolated by preparative HPLC and acid hydrolyzed using 2N HCl. In comparison to authentic standard samples, such as myricetin, quercetin and kaempferol, the aglycones of two major peaks were identified as quercetin (Table 2).

A co-pigmentation test was carried out to observe the effect of flavonols on anthocyanins in the bluing phenomenon of reddish-purple blotches in upper petals. After purifying and isolating the dried petal extraction by column chromatography, 11 fractions of pigment were obtained (Fig. 5). By the measurement of absorption spectra of each fraction, absolute purified anthocyanin fractions (Fr. 1 to Fr. 4) and flavonol fractions (Fr. 8 to Fr. 11) were obtained, and used as materials for the co-pigmentation test. Since fractions 5, 6, and 7 were mixtures of anthocyanins and flavonols, they were discarded. The result of the *in vitro* co-pigmentation test showed that as the density of flavonol increased in the mixed solution, the λ_{max} increased gradually (Fig. 6). The λ_{max} of mixed solution was 510 nm when it contained only anthocyanins (1.0 mg·mL^{-1}), and a 15.2 nm bathochromic shift was obtained from the mixed solution at the ratio of 1:10 equivalent of anthocyanins to flavonols (Table 3). In comparison, the *in vitro* co-pigmentation test showed that a 9.7 nm bathochromic shift was obtained from mixed solution of anthocyanin and flavonol at the ratio of concentration of 1:7.5. These results were similar to the bathochromic shift obtained between upper and lower petals of intact flowers (9.2 nm) (Fig. 2).

Previously, co-pigmentation tests were done in some flower species, such as Japanese garden iris (Yabuya et al., 1997), torenia (Aida et al., 2000), or blue

Table 1. Color properties of pigments extracted from the petals of *R. simsii* flowers and separated by two-dimensional thin layer chromatography.

Spot no.	Color in	
	Visible light	UV-light
1	Red-lilac	Violet
2	Red-lilac	Violet
3	Pale brown	F.[y] yellow
4	Pale brown	F. yellow
5	—[z]	F. blue
6	—	F. blue
7	—	F. blue
8	—	F. blue

[z] Without color.
[y] Fluorescent.

Table 2. Retention time of standard flavonols and flavonol aglycones of *R. simsii* flowers.

Aglycones	Retention time (min)
Myricetin	8.0
Quercetin	15.2
Kaempferol	28.8
Peak 1	15.3
Peak 2	15.3

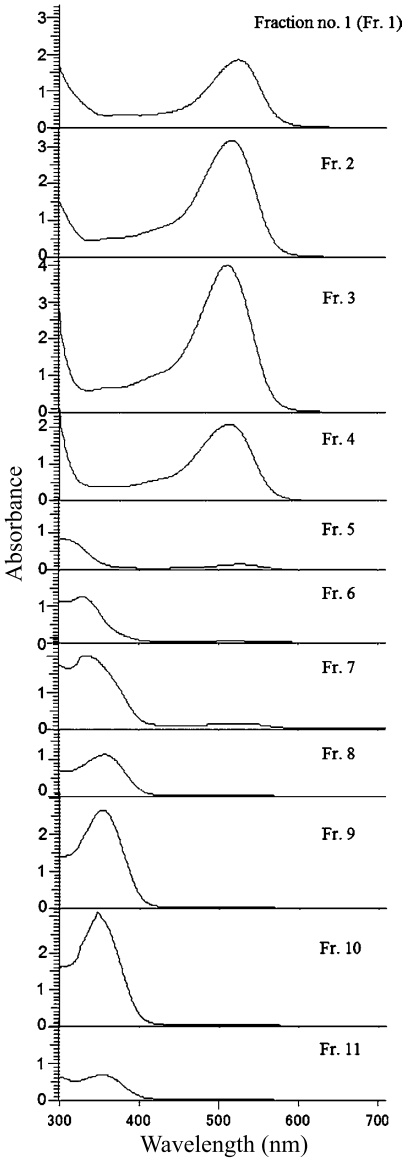

Fig. 5. Absorption spectra of fractions from *R. simsii* petal extraction purified and isolated using a Sephadex LH-20 column. Fractions (Fr.) were numbered from 1 to 11. Fr. 1–Fr. 4: anthocyanin fractions; Fr. 5–Fr. 7: mixture of anthocyanin and flavonol fractions; Fr. 8– Fr. 11: flavonol fractions.

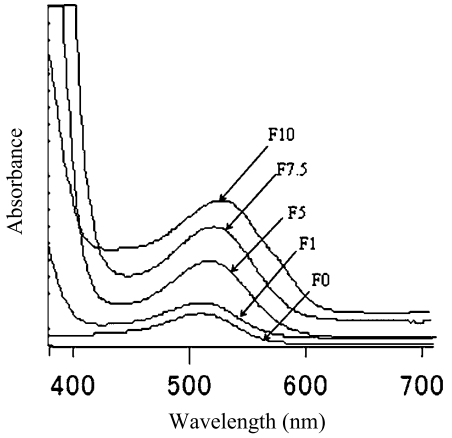

Fig. 6. *In vitro* co-pigmentation test with anthocyanins and flavonols. The anthocyanin and flavonol fractions extracted from upper petals of *R. simsii* flowers were dissolved in 0.1 M Glycine-HCl buffer (pH = 2.5), and mixed together at different ratios. All samples include 1.0 mg·mL^{-1} anthocyanin. F0; flavonol 0 mg·mL^{-1}, F1; flavonol 1.0 mg·mL^{-1}, F5; flavonol 5.0 mg·mL^{-1}, F7.5; flavonol 7.5 mg·mL^{-1}, F10; flavonol 10.0 mg·mL^{-1}.

Table 3. Effect of flavonols on the wavelength of maximum absorbance of anthocyanins *in vitro* co-pigmentation test.

Density (mg·mL^{-1})		λ_{max} in 0.1 M glycine-HCl buffer[z] (nm)	$\Delta\lambda_{max}$
Anthocyanin	Flavonol		
1.0	0.0	510.0	—
1.0	0.1	511.3	1.3
1.0	0.5	511.5	1.5
1.0	1.0	513.3	3.3
1.0	2.0	514.7	4.7
1.0	5.0	516.3	6.3
1.0	7.5	519.7	9.7
1.0	10.0	525.2	15.2

[z] 0.1 M Glycine:0.1 M HCl:H_2O (50:2.5:47.5, v/v/v), pH = 2.5.

Veronica persica flowers (Ono et al., 2010). In these flowers, blue flower color is expressed by co-pigmentation between anthocyanin and flavone. Asen et al. (1971) determined the cause of the difference in color expression between 'Red Wing' azalea and an orange sport of this cultivar. The orange color of the mutant was due to cyanidin glycosides, whereas the color of 'Red Wing' azalea was due to the same cyanidin glycosides co-pigmented with quercetin glycosides. These results suggested that the co-pigmentation of anthocyanin-flavonol occurs in the blotches in upper petals of *R. simsii* flowers.

In nature, plants and pollinators have co-evolved physical characteristics that make them more likely to interact successfully. The plants benefit from attracting a particular type of pollinator to their flower by color signal, ensuring that pollen will be carried to another flower of the same species, and resulting in successful reproduction. A study of flower morphology of *Clarkia gracilis* species has demonstrated that insect pollinators have the ability to distinguish between spotted and non-spotted flowers, and seed number of plants having spots in flower petals was 32% higher than that in non-spotted flower plants (Jones, 1996), suggesting that color spots on flower petals may significantly influence pollinator-mediated pollen transfer and seed production. In some *Rhododendron* species, swallowtail butterflies and lasioglossums frequency visited reddish flower, while bees and flower flies preferred to visit light-purplish flowers (Tagane, 2008). Thus, the reddish-purple color of blotches in *R. simsii* flowers, which is caused by the co-pigmentation between anthocyanin and flavonol, might attract pollinators due to the wide color range perception.

When breeding program using *R. simsii* will be done, not only anthocyanin inheritance, but also flavonol inheritance should be considered because the co-pigmentation effect is one of the most important factors of flower color expression in azaleas. Heursel and Horn (1977) identified the gene Q that is responsible for the presence of quercetin glycoside in *R. simsii* flowers. De Loose (1969) noted that scarlet and salmon flowers of the Belgian hybrids of *R. simsii* have a low flavonol content, while the petals of deep red, magenta and blue red flowers contain a high flavonol content. Red flowered *R. simsii* is believed to have a small prospect for gaining bluish flower color due to the low flavonol content and epidermal pH (Heursel, 1975). However, interspecific crossing with blue tone flowered species may be a promising mean to produce bluer shade flowers by increasing the amount of flavonols sufficient to form full co-pigmentation in the hybrids. Interspecific crossing should be carried out in further studies to confirm the color inheritance, and produce wide color variation in *R. simsii* in Vietnam.

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