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Flower Color and Flavonoid Variation in *Clematis patens* (Ranunculaceae)

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Abstract Anthocyanins and other flavonoids in *Clematis patens* were surveyed in relation to their flower colors. Flower colors in the species were divided into three colors, white, pink and purple. Of their flower colors, the absorption maxima of the pink and purple were similar to each other, suggesting that the colors are due to intra-molecular copigment. Their anthocyanins were characterized as delphinidin and cyanidin glycosides in both flower colors. However, delphinidin is a major pigment in the purple flowers, together with minor cyanidin. On the other hand, small amounts of delphinidin and cyanidin were detected from the pink flowers. Thus, it was shown that their color differences are due to quantitative ones of anthocyanidins, especially delphinidin. Eight flavonols were detected from the pink flowers and characterized as kaempferol 3-*O*-rutinoside (F4), kaempferol 3-*O*-glucoside (F5), kaempferol 3-*O*-(caffeoylglucoside) (F1), quercetin 3-*O*-glycoside (F8), kaempferol 3-*O*-gentiobioside (F2), kaempferol 3-*O*-alloside (F6), and two kaempferol glycosides (F3 and F7). The white flowers also contained these flavonoids and were divided into two chemotypes by their flavonoid composition. However, the flavonoids could not be detected from the purple flowers. Thus, it was presumed that the purple flower color of *C. patens* is due to an intra-molecular copigment.

Key words : anthocyanins, *Clematis patens*, flavonols, flower colors.

Introduction

Clematis patens Morr. & Decne. (Ranunculaceae) is distributed in Honshu, Shikoku, Kyushu, Korea and China (Tamura and Shimizu, 1982). The species was listed in "Threatend Wildlife of Japan, Vascular Plants" (Environment Agency of Japan, 2000), since many plants have mainly been collected for ornamental in Japan. The flower colors are various from white, pink to purple.

The anthocyanins of some *Clematis* cultivars as flower pigments have been reported by Hosoki *et al.* (2003) and tentatively characterized as cyanidin and delphinidin glycosides. Cyanidin hexoside was detected from the flowers of *C. japonica* Thunb. (Ueno *et al.*, 1969). Cyanidin 3-*O*-glucoside was isolated from the fruits of *C. terniflora* DC. (Ishikura, 1975). Other flavonoids have been reported from some *Clematis* species, i.e., quercetin itself from the pollen of *C. apiifolia* DC., *C. japonica* and *C. stans* Sieb. & Zucc., and three quercetin glycosides, 3-*O*-glucoside, 3-*O*-glucuronide and 3-*O*-rutinoside from the leaves of *C. stans* (Kizu *et al.*, 1995). An acylated flavone glycoside, apigenin 7-*O*-(*p*-coumaroylglucoside) have been isolated from *C. terniflora* var. *robusta* Tamura (=*C. terniflora* var. *terniflora*) (Aritomi, 1963). The eight taxa of *Clematis* subsect. *Viornae* in USA, *C. addisonii* Britton, *C. texensis* Buckley, *C. glaucophylla* Small, *C. versicolor* Small ex Rydberg, *C. viorna* L., *C. reticulata* Walter, *C. pitcheri* Torrey & Gray var. *pitcheri* and *C. pitcheri* var. *dictyota* (Greene) Dennis, have chemotaxonomically been surveyed for foliar flavonoids, and seven flavones,

apigenin, apigenin 7-*O*-glucuronide, luteolin, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, luteolin 7-*O*-rhamnosylglucoside and chrysoeriol 7-*O*-glycoside; three flavone *C*-glycosides, isoorientin, isovitexin and vicenin-2; and five flavonols, quercetin 3-*O*-gluoside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnosylglucoside, quercetin 3-*O*-rhamnosylgalactoside and kaempferol 3-methyl ether, were isolated together with two unknown flavonoids (Dennis and Bierner, 1980). Quercetin and kaempferol glycosides were detected from the leaves of nine *Clematis* species in Europe, i.e., *C. cirrosa* L., *C. integrifolia* L., *C. flammula* L., *C. rehderiana* Craib., *C. serratifolia* Rehd., *C. viorna* L., *C. vitalba* L., *C. montana* Buch. and *C. alpina* Mill. (Lebreton, 1986). A triperpene, oleanolic acid, has been isolated from the roots of *C. patens* (Fujita *et al.*, 1974). However, the flower pigments and flavonoids are not reported from *C. patens*.

In this paper, we describe anthocyanins and other flavonoids from the flowers of *Clematis patens* in relation to its various flower colors.

Materials and Methods

Plant materials

Eighteen individuals of *Clematis patens* from eighteen populations in Japan were used as plant materials. Their flower colors, collection sites and acc. No. were as follows.

White flowers: Miyagi Pref. (TBG142575), Nikota, Uchihara, Ibaraki-gun, Ibaraki Pref. (TBG132792), Koibuchi, Uchihara, Ibaraki-gun, Ibaraki Pref. (TBG132786), Muramatsu, Tokai, Naka-gun, Ibaraki Pref. (TBG143503), Kasumigawa, Ohme-shi, Tokyo (TBG142581), Funabashi-shi, Chiba Pref. (TBG142577), Ashigakubo, Yokose, Chichibu-gun, Saitama Pref. (TBG123485), Nagano Pref. (TBG141573).

Pink flower: Nikko-shi, Tochigi Pref. (TBG 134741).

Purple flowers: Oshibe, Iwama, Nishi-ibaraki-gun, Ibaraki Pref. (TBG133548), Nikko-shi, Tochigi Pref. (TBG134739), Mamada, Oyama-shi, Tochigi Pref. (TBG134738), Ohme-shi, Tokyo (TBG142576), Inba-mura, Susui, Inba-gun, Chiba Pref. (TBG142580), Kawarayu, Naganohara, Azuma-gun, Gunma Pref. (TBG 137173), Osaka Pref. (TBG134742), Sanda-shi, Hyogo Pref. (TBG142574), Hiroshima Pref. (TBG123436).

Plant materials are growing in Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan.

Extraction and isolation of anthocyanin and other flavonoids

Fresh sepals of each individual were extracted with HCOOH/MeOH (8 : 92). In cases of anthocyanin isolation, the extracts were concentrated *in vacuo*, applied to Sephadex LH-20 column chromatography (solvent system: 5% HOAc), and applied to preparative paper chromatography (prep. PC) using solvent systems: BAW (*n*-BuOH/HOAc/H_2O = 4 : 1 : 5, upper phase) and then 15% HOAc. Isolated anthocyanin was furthermore purified by preparative HPLC (see below).

In cases of other flavonoids, the concentrated extracts were applied to prep. PC using solvent systems: BAW, 15% HOAc and then BEW (*n*-BuOH/EtOH/H_2O = 4 : 1 : 2.2). The obtained flavonoids were purified by Sephadex LH-20 column chromatography using solvent system: 70% MeOH.

High performance liquid chromatography (HPLC)

Qualitative and quantitative HPLC was performed with Shimadzu HPLC systems using Shim-Pak CLC-ODS column (I.D. 6.0×150 mm: Shimadzu), at a flow-rate of 1.0 ml min^{-1}; detection was 190 – 700 nm and eluents were H_3PO_4/HOAc/MeCN/H_2O (3 : 8 : 6 : 83) for anthocyanins, and H_3PO_4/MeCN/H_2O (0.2 : 22 : 78) for other flavonoids. Fresh sepals (0.2 g) were extracted with FAH (HCOOH/MeCN : H_2O = 8 : 20 : 72) for quantitative HPLC survey. Preparative HPLC was performed with Tosoh HPLC systems using Senshu Pak PEGASIL ODS column (I.D. 10×250 mm: Senshu Scientific Co. Ltd.), at a

flow-rate of 1.5 ml min^{-1}; detection was 530 nm and eluent was HCOOH/MeCN/H_2O (5 : 13 : 82).

Direct visible absorption spectra of the sepals

Direct visible absorption spectra of the sepals from *C. patens* were measured between 400 and 700 nm using Shimadzu multipurpose recording spectrophotometer MPS-2000.

Characterization of anthocyanins

After concentration, crude extracts were directly hydrolyzed with 12% HCl, 100°C for 3 min. After cooling in water, the solution was shaken with 3-methyl-1-butanol (isoamyl alcohol). Anthocyandins were identified by direct TLC and HPLC comparisons with authentic pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin.

Identification of other flavonoids

The isolated flavonoids were identified by UV spectral survey according to Mabry *et al.* (1970), and direct TLC and HPLC comparisons of original glycosides and their hydrolysates with authentic samples. TLC, HPLC and UV spectral properties of the isolated flavonoids are as follows.

Kaempferol 3-*O*-(caffeoylglucoside) (F1). TLC: Rf 0.63 (BAW), 0.66 (BEW), 0.67 (15%HOAc); UV – dark purple, UV/NH_3 – yellow fluorescence. UV: λmax (nm) MeOH 267, 333; +NaOMe 274, 325sh, 386 (inc.); +$AlCl_3$ 268, 305, 357; +$AlCl_3$/HCl 276, 303, 336, 390sh; +NaOAc 274, 312, 373; +NaOAc/H_3BO_3 267, 305sh, 353.

Kaempferol 3-*O*-rutinoside (nicotiflorin, F4). TLC: Rf 0.51 (BAW), 0.57 (BEW), 0.49 (15% HOAc); UV – dark purple, UV/NH_3 – dark yellow. HPLC: *t*R 5.88 min. UV: λmax (nm) MeOH 266, 352; +NaOMe 275, 326, 400 (inc.); +$AlCl_3$ 274, 304, 354, 396; +$AlCl_3$/HCl 275, 303, 350, 392; +NaOAc 274, 306, 375; +NaOAc/H_3BO_3 267, 351.

Kaempferol 3-*O*-glucoside (astragalin, F5). TLC: Rf 0.68 (BAW), 0.73 (BEW), 0.32 (15% HOAc); UV – dark purple, UV/NH_3 – dark yellow. HPLC: *t*R 6.57 min. UV: λmax (nm) MeOH 266, 350; +NaOMe 275, 325, 400 (inc.); +$AlCl_3$ 274, 304, 352, 395; +$AlCl_3$/HCl 275, 302, 346, 395; +NaOAc 274, 303, 372; +NaOAc/H_3BO_3 267, 351.

Quercetin 3-*O*-glycoside (F8). UV: λmax (nm) MeOH 257, 265sh, 359; +NaOMe 274, 329, 407 (inc.); +$AlCl_3$ 274, 429; +$AlCl_3$/HCl 266, 302, 362, 400sh; +NaOAc 272, 310sh, 399; +NaOAc/H_3BO_3 262, 376.

Results and Discussion

Direct visible absorption spectra of the sepals

Eighteen individuals which were used as the materials in this experiment, were divided into three flower colors, i.e., white (8 individuals), pink (1 individual) and purple (9 individuals) (Figs. 1 – 3). Direct visible spectra of the purple and pink flower colors are shown in Figs. 4 and 5. In the purple flowers, its absorption maxima are present in 571 nm, together with 534 and 617 nm. Though the absorbance intensity of the pink flower is lower than that of the purple flowers, their absorption property is similar to each other. Saito (1967) classified the absorption spectra into four groups according to number and position of maxima in the visible region. According to him, the purple and pink flowers of *C. patens* belong to GROUP C, of which the flower colors are due to inter-molecular or intra-molecular copigment (Honda and Saito, 2002; Saito, 2002).

Characterization of anthocyanidins

Two anthocyanidins were liberated by acid hydrolysis of the crude exracts from the purple and pink flowers. They were identified as delphinidin and cyanidin (Fig. 6) by direct TLC comparisons with authentic samples. Their anthocyanidins were contained in both flower colors. However, delphinidin is major pigment in the purple flowers, together with minor cyanidin. On the other hand, small amount of delphinidin and cyanidin were detected from the pink flower. Thus, it was shown that the color differences between the purple and pink flowers are due to quantitative ones of the anthocyanidin, especially delphinidin. A

Fig. 1. White flowers of *Clematis patens* (TBG143503).

Fig. 2. Pink flowers of *Clematis patens* (TBG134741).

Fig. 3. Purple flower of *Clematis patens* (TBG142576).

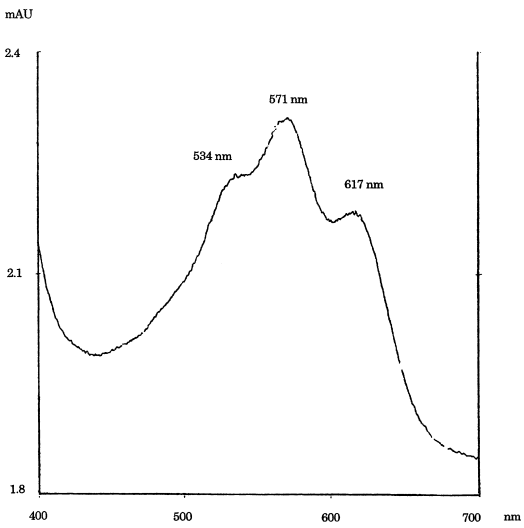


Fig. 4. Absorption spectral curve of *Clematis patens* purple flower.

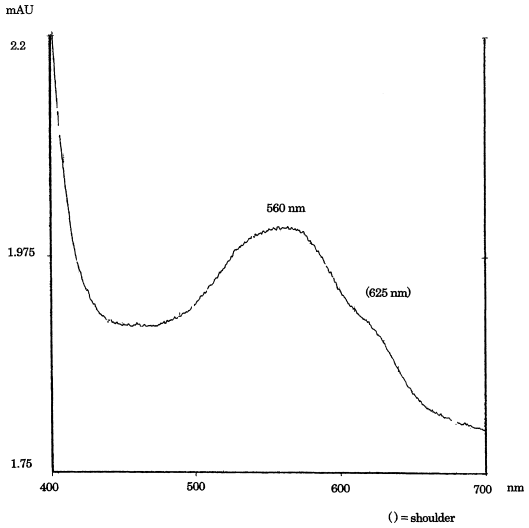


Fig. 5. Absorption spectral curve of *Clematis patens* pink flower.

delphinidin glycoside was isolated from the purple flowers (TBG133548), and glucose and caffeic acid were liberated by acid hydrolysis, together with delphinidin. The complete identification of the anthocyanins are now in progress.

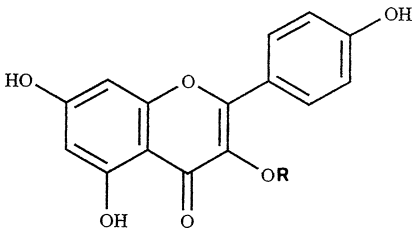
Identification of other flavonoids

Four flavonoids were isolated from the pink flowers (TBG134741). Of their compounds, flavonoids F4 and F5 were identified as kaempferol 3-*O*-rutinoside and 3-*O*-glucoside

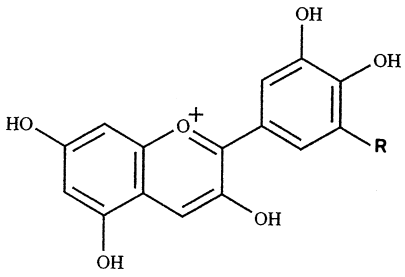
(Fig. 7) by UV and TLC properties, and direct TLC and HPLC comparisons with authentic nicotiflorin from the aerial parts of *Osyris alba* L. (Santalaceae) (Iwashina *et al.*, 2008) and astragalin from the fronds of *Cyrtomium falcatum* (L. f.) C. Presl (Dryopteridaceae) (Iwashina *et al.*, 2006).

Major flavonoid F1 liberated kaempferol, glucose and caffeic acid by acid hydrolysis. The presence of free 5, 7 and 4′-hydroxyl and a substituted 3-hydroxyl groups was shown by UV spectral mesurement in addition to various shift reagents, suggesting the attachment of both glucose and caffeic acid to 3-position of kaempferol. Thus, flavonoid F1 was characterized as kaempferol 3-*O*-(caffeoylglucoside) (Fig. 7). The glycoside has only been reported from the aerial parts of the fern, *Pteridium aquilinum* (L.) Kuhn as kaempferol 3-*O*-(6″-caffeoylglucoside) (Imperato and Minutiello, 1997).

UV spectral properties showed that minor flavonoid F8 is quercetin 3-*O*-glycoside. However, its identification could not perform for small amount of original glycoside. HPLC properties of this compound did not agree with those of common quercetin 3-*O*-glycosides, 3-*O*-glucoside and 3-*O*-sophoroside.

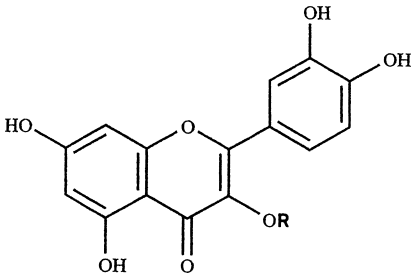


R=caffeoylglucosyl: kaempferol 3-*O*-(caffeoylglucoside) (F1)
R=gentiobiosyl: kaempferol 3-*O*-gentiobioside (F2)
R=rutinosyl: kaempferol 3-*O*-rutinoside (nicotiflorin, F4)
R=glucosyl: kaempferol 3-*O*-glucoside (astragalin, F5)
R=allosyl: kaempferol 3-*O*-alloside (asiaticalin, F6)



R=H: cyanidin
R=OH: delphinidin

Fig. 6. Chemical structures of anthocyanidins from the purple and pink flowers of *Clematis patens*.



R=glycosyl: quercetin 3-*O*-glycoside (F8)

Fig. 7. Chemical structures of flavonol glycosides from the white and pink flowers of *Clematis patens*.

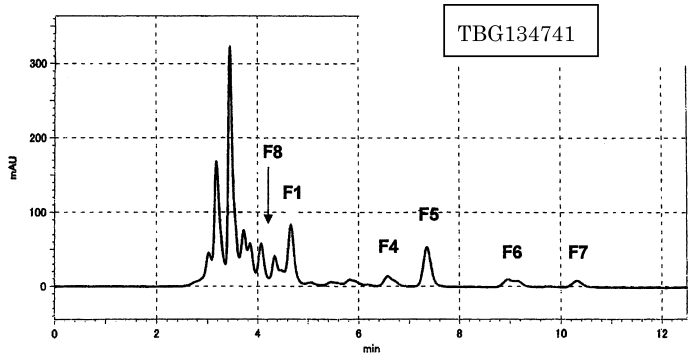


Fig. 8. HPLC chromatogram of the extracts from the pink flowers of *Clematis patens*.

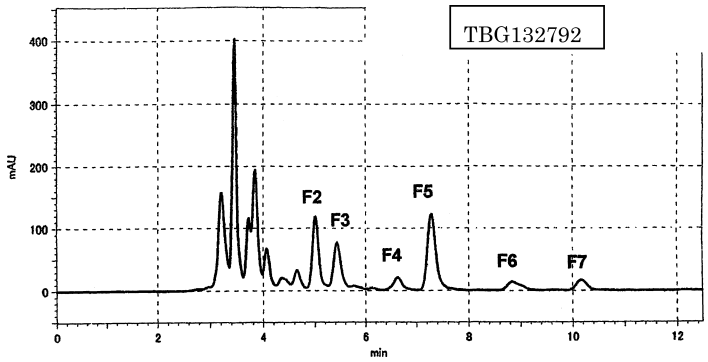


Fig. 9. HPLC chromatogram of the extracts from the white flowers (B) of *Clematis patens*.

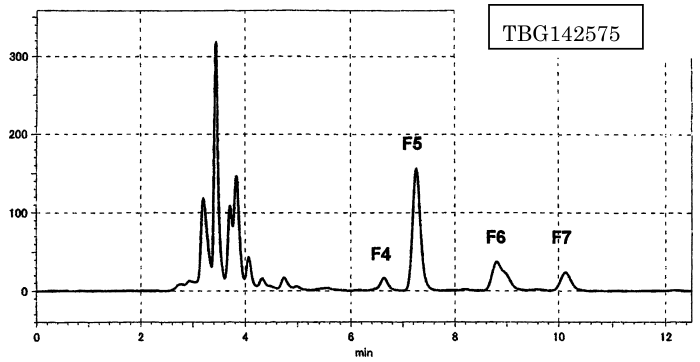


Fig. 10. HPLC chromatogram of the extracts from the white flowers (A) of *Clematis patens*.

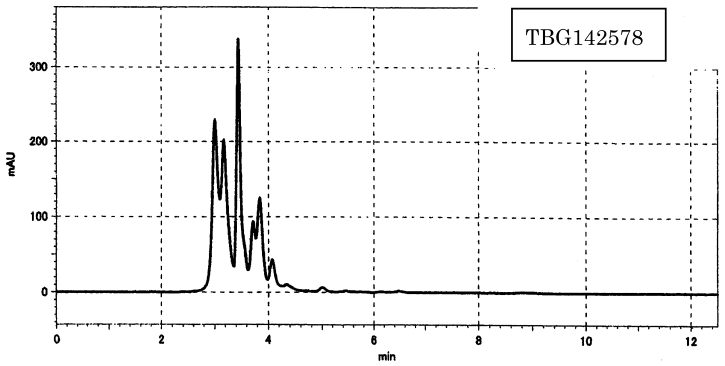


Fig. 11. HPLC chromatogram of the extracts from the purple flowers of *Clematis patens*.

Four flavonoid peaks (F2, F3, F6 and F7), which were not isolated, appeared on HPLC chromatograms of crude extracts from the white flowers (Fig. 9). Two of them were identified as kaempferol 3-*O*-gentiobioside (F2) and kaempfrol 3-*O*-alloside (F6) (Fig. 7) by direct HPLC comparisons with authentic samples from the flowers of *Glycine max* (L.) Merr. (Leguminosae) (Iwashina *et al.*, 2007) and the leaves of *Glaucidium palmatum* Sieb. & Zucc. (Glaucidiaceae) (Iwashina and Ootani, 1990), respectively. Remained two glycosides F3 and F7 were partially characterized as kaempferol glycosides.

Table 1. Distribution of anthocyanidins and flavonols in the purple, pink and white flowers of *Clematis patens*.

Flower colors	Anthocyanins		Flavonols							
	Dp	Cy	D1	D2	D3	D4	D5	D6	D7	D8
purple[1]	+	t	−	−	−	−	−	−	−	−
pink[2]	t	t	+	−	−	t	+	t	t	+
white A[3]	−	−	−	−	−	+	+	+	+	−
white B[4]	−	−	−	+	+	t	+	t	t	−

[1] TBG133548, TBG134739, TBG134738, TBG142576, TBG142580, TBG137173, TBG134742, TBG142574 and TBG123436.
[2] TBG134741.
[3] TBG142575, TBG142581, TBG142577 and TBG123485.
[4] TBG132792, TBG132786, TBG143503 and TBG141573.
Dp=delphinidin, Cy=cyanidin, D1=kaempferol 3-*O*-(caffeoylglucoside), D2=kaempferol 3-*O*-gentiobioside, D3=kaempferol glycoside, D4=kaempferol 3-*O*-rutinoside, D5=kaempferol 3-*O*-glucoside, D6=kaempferol 3-*O*-alloside, D7=kaempferol glycoside and D8=quercetin 3-*O*-glycoside. +=presence, t=trace and −=absence.

Other flavonoid composition of white, pink and purple flowers

Of eighteen individuals examined in this survey, pink flower one (TBG134741) synthesized six flavonol glycosides (F1 and F4 – F8) (Table 1 and Fig. 8). Of their flavonoids, an acylated flavonol, kaempferol 3-*O*-(caffeoylglucoside) was only found in the pink flowers. Eight white flower individuals could be divided into two chemotypes by the presence (B) (TBG132792, 132786, 143503 and 141573) or absence (A) (TBG142575, 142581, 142577 and 123485) of flavonoids F2 and F3 (Table 1, and Figs. 9 and 10). In the purple flowers, flavonol glycosides were apparently not recognized (Fig. 11). However, as described above, the purple flowers of *C. patens* are colored by copigment effects. Though delphinidin glycosides of the species are not enough characterized the chemical structures, they may be polyacylated anthocyanins and pigmented by intra-molecular copigments.

Flower color variations are reported from various anthocyanin-containing plants, and they are due to the polymorphism (Bohm, 1987). In this case of *Clematis patens*, it was also presumed that their flower color variations are due to polymorphism.

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