

## 黄花シクラメンの黄色発現に関与する主要色素の同定

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## Identification of the Main Agent Causing Yellow Color of Yellow-Flowered Cyclamen Mutant

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### Summary

The principal yellow pigment in petals of yellow-flowered cyclamen found in the inbred line of diploid cultivar of white-flowered cyclamen was isolated and identified chromatographically and spectrophotometrically as chalcononaringenin-2'-glucoside (isosalipurposide).

This mutant clone seems to lack the same recessive enzyme chalcone-flavanone isomerase as the mutant of *Callistephus chinensis*. The prospects on the breeding for deep yellow-colored cyclamen are discussed.

### Introduction

Flower colors of cyclamen have been restricted to red, pink, white, reddish-purple, and purple; therefore, a yellow- or blue-flowered cyclamen has been sought for a long time.

In 1982, the authors found a yellow-flowered individual in an inbred population of a diploid, white-flowered cyclamen 'Pure White Kage'. We have maintained this line by selfing and intrapopulational-crossing. Although the color of this flower is deep yellow at bud stage, it becomes paler after anthesis (Fig. 1). Anthocyanins and flavonols have been associated with flower pigments of cyclamen (5, 7, 8, 9, 10, 11, 12, 13), but a yellow pigment has never been reported. It seems,



Fig. 1. Yellow-flowered cyclamen in full bloom.

therefore, that our plant is a very valuable as a germplasm source for the breeding of new colored cyclamen cultivars.

In this study, the main agent of the yellow-flowered cyclamen was isolated and identified chromatographically and spectrophotometrically. Furthermore, the mechanism of occurrence of new yellow pigment was made clear and the prospects for breeding the deep yellow-flowered cyclamen are discussed.

### Materials and Methods

#### 1. Thin layer chromatography of crude extract

Fresh petals were homogenized in 80% ethanol and the homogenate filtered. The filtrate was evaporated to almost dryness under reduced pressure at 35°C, dissolved in small amounts of distilled water, and held on Sep-Pak cartridge (C<sub>18</sub>, Waters). The flavonoid pigments on the cartridge were washed sufficiently with distilled water to eliminate the water-soluble, hydrophilic contaminants and eluted with 2 ml of ethanol. The concentrated ethanol eluate was then applied to a 20 × 20 cm glass plate coated with micro-crystalline cellulose powder and developed two-dimensionally in the following solvent systems; *n*-butanol-acetic acid-water (4 : 1 : 5, v/v/v, upper phase) for the first dimension, and 30% acetic acid for the second. After development, color properties of each colored spot were recorded under visible and UV light

directly or after the treatment with ammonia, 5% ethanolic aluminum chloride, or 5% aqueous sodium carbonate.

## 2. Purification of the relevant pigment and its chromatographic and spectrophotometric characterization

To characterize the relevant pigment further, ethanol extract was purified successively by preparative paper chromatography using a 60 × 60 cm filter paper (No.526, Toyo) in two solvent systems: *n*-butanol-acetic acid-water (4:1:5, v/v/v, upper phase) and 30% acetic acid. After each development, the pigment band was marked under UV light, cut out, and then eluted with 80% ethanol. The eluate obtained was concentrated under reduced pressure and used for identification.

For the identification of the aglycone, a small portion of the purified pigment was hydrolyzed for 90 min at 100°C with 2 ml of 2N hydrochloric acid. The hydrolysate obtained was analyzed with some authentic specimens by HPLC, in which chromatograms were developed with a LC-6A pump (Shimadzu) equipped with a 4.6 mm I.D. × 250 mm Cosmosil 5C<sub>18</sub> column (Nakarai Tesque) and a SPD-6AV detector (Shimadzu) set at 290 nm. A flow rate of 1.0 ml/min was maintained and a mixture of acetonitrile-0.1 M acetic acid (30:70, v/v) was employed as the eluant.

For the identification of the sugar, the acid hydrolysate was neutralized with 1N sodium hydroxide, and the sodium chloride produced was eliminated through ion exchange resin. The residual sugar solution was evaporated to dryness, redissolved in ethanol, and co-chromatographed with authentic sugars using ethyl acetate-pyridine-acetic acid-water (5:5:1:2, v/v) as the solvent system. The color of the sugar spots on the chromatogram was developed by the method of Bryson and Mitchell (1).

Another portion of purified sample was used for the characterization of the nature of glycosides using HPLC. The analytical condition of HPLC system was the same as mentioned above, except that the wavelength was set at 369 nm and the column was eluted with acetonitrile-0.1 M acetic acid (25:75, v/v).

To confirm the glycoside further, the purified sample was co-chromatographed with authentic chalcone glycoside isolated from petals of yellow

carnation (14) on the cellulose TLC-plate in four solvent systems: *n*-butanol-acetic acid-water (4:1:5, v/v/v, upper phase), 30% acetic acid, water, and phenol saturated with water. The absorption spectra in 95% ethanol and the bathochromic shift in an alkaline 95% ethanolic solution of a purified sample and those of authentic specimens were measured spectrophotometrically (Beckman model DU-65) and compared.

## 3. Comparison of the constitutions of flavonol aglycone in petals of yellow-flowered and white-flowered cyclamen

Ten mg of each lyophilized petals of yellow- and white-flowered cyclamen were soaked in 2 ml of 2 N hydrochloric acid and hydrolyzed for 90 min at 100°C. The acid hydrolysates were passed through the membrane filter and applied to Sep-Pak C<sub>18</sub> cartridge. The aglycones trapped on the cartridge were washed with 10 ml of water to eliminate any water soluble contaminants and then eluted with 2 ml of ethanol. Five μl of each sample were injected to HPLC. The conditions of HPLC were the same as described in the method of identification of aglycone, except that the wavelength was set at 360 nm.

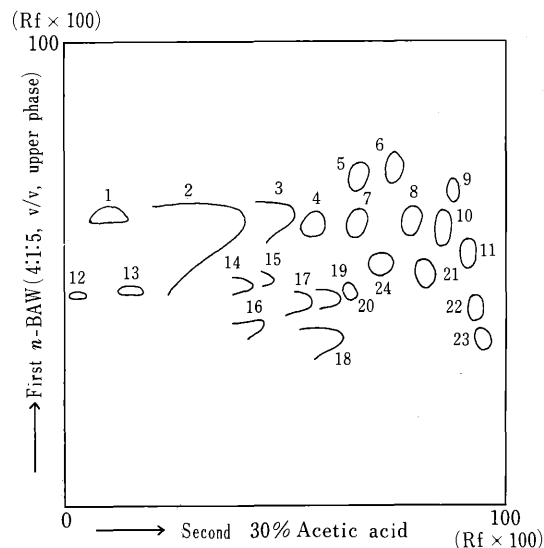


Fig. 2. Two dimensional thin-layer chromatogram of an ethanol extract from petals of yellow-flowered cyclamen. *n*-BAW: *n*-butanol-acetic acid-water. See Table 1 for the pigment properties of the numbered spots.

**Table 1.** Color properties of pigments extracted from the petals of yellow-flowered cyclamen and separated by two-dimensional thin-layer chromatography.

Spot no.	None		+ NH <sub>3</sub>		+ AlCl <sub>3</sub> <sup>z</sup>		+ Na <sub>2</sub> CO <sub>3</sub> <sup>y</sup>	
	VIS.	UV.	VIS.	UV.	VIS.	UV.	VIS.	UV.
1	—	F <sup>x</sup> .G <sup>w</sup> .blue	—	Yellow	—	F.G.blue	—	Yellow
2	Yellow	Black	B <sup>v</sup> .yellow	F.orange	B.yellow	Orange	Yellow	F.orange
3	P <sup>u</sup> .yellow	Brown	P.yellow	Yellow	P.yellow	G.yellow	P.yellow	Yellow
4	—	P.purple	P.yellow	Green	—	F.yellow	—	Yellow
5	—	P.purple	—	G.yellow	—	F.yellow	—	G.yellow
6	—	P.purple	—	Yellow	—	F.yellow	—	G.yellow
7	—	P.purple	—	—	—	F.yellow	—	P.yellow
8	—	Purple	—	Black	—	F.yellow	—	Purple
9	—	Brown	P.yellow	Black	—	F.G.yellow	—	F.G.blue
10	Yellow	Brown	B.yellow	Orange	B.yellow	Orange	Yellow	F.orange
11	—	P.purple	—	Black	—	F.yellow	—	Purple
12	—	F.orange	—	Yellow	—	F.orange	—	Yellow
13	—	F.G.yellow	—	Orange	—	F.G.yellow	—	F.orange
14	—	Purple	P.yellow	Orange	—	Purple	—	F.orange
15	—	Purple	—	Yellow	—	F.yellow	—	F.orange
16	—	Purple	—	Black	—	Purple	—	P.orange
17	P.yellow	Purple	P.yellow	Orange	Yellow	Blue	Yellow	F.yellow
18	—	Purple	—	Blue	—	Blue	—	P.purple
19	—	F.blue	—	Yellow	—	F.blue	—	G.yellow
20	—	F.blue	—	—	—	F.blue	—	P.purple
21	—	F.blue	—	F.blue	—	F.blue	—	P.blue
22	—	F.G.yellow	Yellow	Black	—	F.yellow	Yellow	F.orange
23	—	F.G.yellow	—	—	—	F.yellow	Yellow	F.orange
24	—	Purple	Yellow	Yellow	Yellow	F.yellow	Yellow	F.yellow

<sup>z</sup> 5% ethanolic aluminum chloride.<sup>y</sup> 5% aqueous sodium carbonate.<sup>x</sup> F, fluorescent.<sup>w</sup> G, greenish.<sup>v</sup> B, bright.<sup>u</sup> P, pale.

## Results and Discussion

A two-dimensional TLC-plate of the crude extract disclosed 24 distinct spots (Fig. 2); their color properties are shown in Table 1. Spots 2, 3, 10 and 17 were visible under white light without treatment with any color developing reagents. Spot 2, the major pigment, had color properties when sprayed with three reagents, which suggested that it might be a chalcone (2). Therefore, further characterization of this spot was done after its purification by successive preparative paper chromatographies.

It was reported that chalcone was isomerized readily to a flavanone by acid treatment (3). Therefore, in this study, three kinds of authentic flavanone, naringenin, hesperetin and dihydro-quercetin were used for co-chromatography. Upon acid

hydrolysis the resulting aglycone was identified as naringenin (Fig. 3). Accordingly, spot 2 was deter-

**Table 2.** Thin layer (cellulose) chromatographic separations of sugar moiety obtained by the acid hydrolysis of spot 2 isolated from the petals of yellow-flowered cyclamen.

Compounds	R <sub>f</sub> values (×100) in EPAW <sup>z</sup>	Color <sup>y</sup>
Spot 2 sugar	29.2	Brown
Authentic sugars		
Galactose	28.5	Brown
Glucose	29.2	Brown
Arabinose	38.0	Dark-brown
Xylose	45.3	Dark-brown
Rhamnose	56.2	Dark-green

<sup>z</sup> Ethyl acetate-pyridine-acetic acid-water (5 : 5 : 1 : 2, v/v).<sup>y</sup> Reaction with aniline-phosphoric acid (1).

mined to be a glycoside of chalcononaringenin. Moreover, the sugar residue attached was identified as glucose (Table 2), indicating that this compound was a chalcononaringenin glucoside.

R<sub>f</sub> values from co-chromatographies, using four solvent systems, and spectral properties of spot 2 were compared with those of chalcononaringenin-2'-glucoside, the presumptive yellow pigment of carnation (3, 14). As summarized in Table 3, the R<sub>f</sub> values, the maximum absorption wavelength and the bathochromic shift in alkaline solution of spot 2 were basically the same as those of chalcononaringenin-2'-glucoside. Furthermore, the data on HPLC coincided completely with

chalcononaringenin-2'-glucoside (Fig. 4).

Fig. 5 shows the constitution of flavonol aglycones in petals of yellow- and white-flowered cyclamen. Van Bragt (12) reported the eight flavonol glycosides were isolated from cyclamen petals; two of their aglycones were identified as quercetin and kaempferol. In the present experiment, quercetin and/or kaempferol were also detected. Petals of white-flowered cyclamen contained a large amount of flavonols, especially kaempferol which made up over 80% of total flavonols. On the other hand, in petals of yellow-flowered cyclamen, only a trace

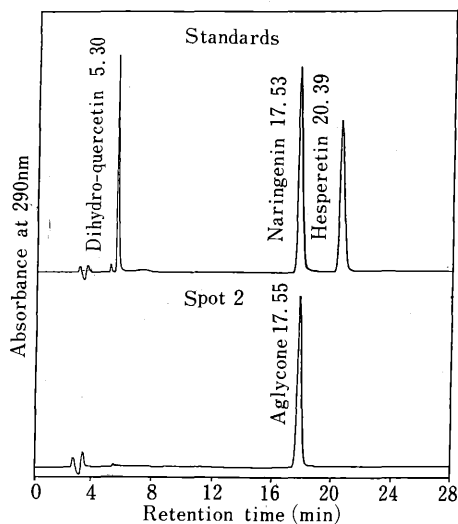


Fig. 3. HPLC tracing of authentic flavanones and the spot 2 aglycone of yellow-flowered cyclamen. Chromatograms were run on a LC-6A HPLC system using a Cosmosil 5C<sub>18</sub> column (4.6 mm I.D. × 250 mm) and SPD-6AV detector set at 290 nm. A flow rate 1.0 ml/min was maintained employing acetonitrile-0.1M acetic acid (30 : 70, v/v) as an eluant.

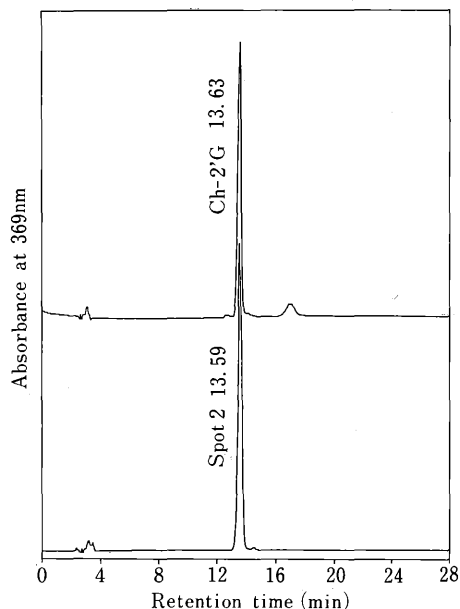


Fig. 4. HPLC profiles of spot 2 isolated from petals of yellow-flowered cyclamen and chalcononaringenin-2'-glucoside (Ch-2'G) isolated from petals of yellow-flowered carnation. See Fig. 3 for HPLC system and column condition. Detector was set at 369 nm. A flow rate 1.0 ml/min was maintained employing acetonitrile-0.1M acetic acid (25 : 75, v/v) as an eluant.

Table 3. Chromatographic and spectral properties of the spot 2 isolated from the petals of yellow-flowered cyclamen.

	R <sub>f</sub> values (× 100) in				λ <sub>max</sub> in 95%EtOH(nm)	Δλ <sub>alk</sub> <sup>x</sup> (nm)
	<i>n</i> -BAW <sup>z</sup>	30%HOAc	H <sub>2</sub> O	PhOH <sup>y</sup>		
Spot 2	67	39	07	55	370	70
Ch-2'G <sup>w</sup>	68	39	05	56	370	70

<sup>z</sup> *n*-Butanol-acetic acid-water (4 : 1 : 5, v/v/v, upper phase).

<sup>y</sup> Phenol saturated with water.

<sup>x</sup> 95% Ethanol + 2 drops of 1N sodium hydroxide.

<sup>w</sup> Chalcononaringenin-2'-glucoside.

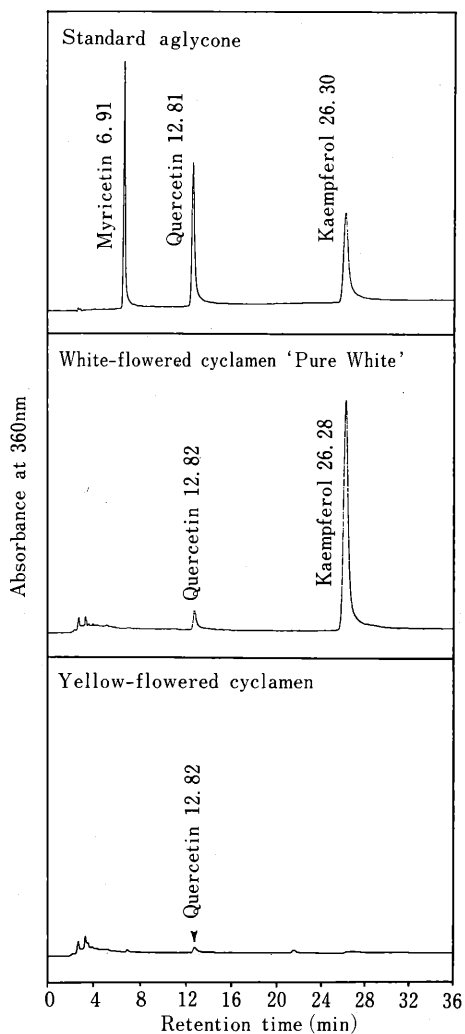


Fig. 5. HPLC tracings of flavonol aglycones found in the hydrolysates of petals of yellow- and white-flowered cyclamen. The numbers are retention time. The detector was set at 360 nm. See Fig. 3 for the other analytical conditions.

amount of quercetin could be detected. This finding suggests that the biosynthetic pathway from chalcone to flavanone is blocked in yellow-flowered cyclamen; i.e., this is a recessive mutant which lacks the chalcone-flavanone isomerase.

In *Callistephus chinensis*, Khun et al. (6) found that the recessive mutant 'chch' genotype with yellow flower accumulated a large amount of tetrahydroxychalcone. This genotype is deficient in chalcone-flavanone isomerase, whereas the

dominant wild type allele with this enzyme could synthesize the higher oxidized flavonols and anthocyanins. This finding also indicates that 1) in the flavonoid biosynthesis the first product with C<sub>15</sub> skeleton should be chalcones and not flavanones and 2) in the dominant genotype, common flavones, flavonols, and anthocyanins could be synthesized from this intermediate flavanone.

In cyclamen Seyffert (10) found a large amount of flavonols in the creamish flowered cultivars, and Van Bragt (12) suggested the possibility for the breeding of yellow-flowered cyclamen with the production of yellow flavonol glycosides, although he had not yet succeeded in the realization of true yellow-flowered cyclamen in his line of breeding. In our experiment, however, we discovered the occurrence of a chalcone which is, along with aurone, one of the most intensive agents for yellow flower color among various flavonoids.

Although in the plant kingdom the carotenoids are the major yellow pigments, both in color intensity and distribution, in some species, flavonoids contribute to the yellow flower colors. In this context, Harborne (4) gave a special emphasis on the production of the following flavonoids, namely the production of anthocolor pigments (chalcones and aurones), flavonols with unusual hydroxylation (e.g., 8-hydroxyquercetin and 6-hydroxyquercetin), glycosylation (e.g., quercetin 7- and 4'-glucoside), and methylation (e.g., quercetin 3'-methyl ether and myricetin dimethyl ether); and anthocyanins with unusual hydroxylation (3-deoxyanthocyanin).

Instead of higher oxidized flavonols and anthocyanins, our yellow-flowered mutant cyclamen accumulated a large amount of chalcone similar to the recessive mutant of *Callistephus chinensis*. However, the mutant cyclamen has a weak genetical leakage, that of synthesizing trace amounts of flavonols. We are currently investigating the mode of inheritance of the yellow pigment and the pathway of chalcone biosynthesis in this mutant to better understand how this pigment is produced and transmitted to its progenies. Simultaneously, we hope to create new cyclamen cultivars with deep yellow flowers.

#### Literature Cited

1. Bryson, J.L. and T.J. Mitchell. 1951. Improved spraying reagents for the detection of sugars on paper chromatograms. *Nature* 167: 864.

2. Harborne, J.B. 1959. The chromatography of the flavonoid pigments. *J. Chromatog.* 2: 581-604.
3. Harborne, J.B. 1967. Comparative biochemistry of the flavonoids. p.79. Academic press, London and New York.
4. Harborne, J.B. 1976. Functions of flavonoids in plants. p. 736-778. In: T.W. Goodwin (ed.). Chemistry and biochemistry of plant pigments. Academic press, London.
5. Karrer, P. and R. Widmer. 1927. Untersuchungen über Pflanzenfarbstoffe. I. Über die Konstitution einiger Anthocyanidine. *Helv. Chim. Acta* 10: 5-23.
6. Kuhn, B., G. Forkman and W. Seyffert. 1978. Genetic control of chalcone-flavanone isomerase activity in *Callistephus chinensis*. *Planta* 138: 199-203.
7. Miyajima, I., I. Doi and T. Kage. 1990. Floral pigment and flower color expression in the petals of cyclamen. *Sci. Bul. Fac. Agr., Kyushu Univ.* 44: 83-89. (In Japanese).
8. Robinson, G.M. and R. Robinson. 1934. A survey of anthocyanins. IV. *Biochem. J.* 28: 1712-1720.
9. Seyffert, W. 1955. Über die Wirkung von Blütenfarbgenen bei Cyclamen. *Z. Vererbungslehre* 87: 311-334.
10. Seyffert, W. 1955. Die Vererbung der Blütenfarben bei hemiploiden Cyclamen. *Züchter* 25: 275-287.
11. Van Bragt, J. 1958. Flower colour pigments in *Cyclamen*. *Proc. Koninkl. Nederland. Akad. Wetenschap.* C61: 448-453.
12. Van Bragt, J. 1962. Chemogenetical investigations of flower colours in *Cyclamen*. *Meded. Landbouwhogeschool. Wageningen* 62(4): 1-43.
13. Werckmeister, P. 1954. Papierchromatographische Untersuchungen an Anthozyanen und chymochromen Begleitstoffen zur Frage der Blütenfarbenzüchtung. *Züchter* 24: 224-242.
14. Yamaguchi, M. 1989. Basic studies on the flower color breeding of carnations (*Dianthus caryophyllus* L.) *Bul. Fac. Hort. Minamikyushu Univ.* 19: 15-16. (In Japanese).

## 黄花シクラメンの黄色発現に関与する 主要色素の同定

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### 摘 要

二倍体白花シクラメン品種の内婚系統からみつかった黄花シクラメンについて、花卉の黄色発現に最も関与していると思われる主要色素を単離精製し、薄層クロマトグラフィー、高速液体クロマトグラフィーおよびスペクトル特性の諸点から検討した結果、カルコノナリンゲニン-2'-グルコシド(イソサリプルポシド)と

同定した。

本植物は *Callistephus chinensis* において見出されたカルコン-フラバノンイソメラーゼをもたない変異体と同様、劣性突然変異体と思われた。さらに本色素を蓄積することで濃黄色シクラメン品種作出の可能性に関して考察した。