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Intermolecular Copigmentation and Color Stabilization in the Violet Blue Flowers of Tall Bearded Iris Cultivar

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Abstract To reveal the violet blue color expression in the tall bearded iris cultivar 'Victoria Falls', colorimetric value, absorption spectra, quantitative analysis of phenolic compounds, and pH value were measured using falls and standards of the flowers. Higher lightness and lower chroma values were shown in standards than those of falls. Absorption spectral curves of the intact falls and standards are essentially the same, i.e. λmax at 578.9 nm and 580.2 nm, respectively. Copigment index [total *C*-glycosylflavone (TF) contents per total anthocyanin (TA) contents] in each part were calculated as 9.4 to 12.8 from the quantitative analysis. Total *C*-glycosylxanthone (TX) contents per TA value were also calculated as 3.1 to 4.1, respectively. Based on these results, *in vitro* examination using isolated anthocyanin, violanin, *C*-glycosylflavone, swertisin 2″-*O*-arabinoside, and *C*-glycosylxanthone, mangiferin, was performed. Degradation rate of the optical density of these solutions was also measured. Bathochromic shift of 34.3 and 37.8 nm was observed by the addition of 0.3 and 0.9 mM swertisin 2″-*O*-arabinoside to 0.1 mM violanin solution. Bathochromic shift of λmax 5.2 nm by the addition of 0.3 mM mangiferin to violanin solution was observed. Furthermore, we were reappeared the absorption spectral curve of the intact falls by the addition of 0.9 mM swertisin 2″-*O*-arabinoside and 0.3 mM mangiferin to 0.1 mM violanin solution, suggesting that the violet blue color expression and stability of the tall bearded iris cultivar is expressed by mixture of these compounds.

Key words: color expression, *C*-Glycosylflavone, *in vitro* reconstruction, mangiferin, violanin.

Introduction

Flower coloration containing anthocyanin is influenced by several factors such as copigmentation, metal complexation and the vacuolar pH values of epidermal cell (Yoshida *et al.*, 2009; Iwashina, 2015). Of these factors, intermolecular copigmentation, a natural process based on non-covalent (supramolecular) complex (Trouillas *et al.*, 2016), is known as bluing effect (bathochromic shift) and color stabilization in some flowers. It is known that blue to bluish-purple flower colors of Dutch iris (*Iris* × *hollandica* Hort. ex Todd.) and Japanese garden iris (*Iris ensata* Thunb.) occur by intermolecular copigmentation between anthocyanins and *C*-glycosylflavones (Asen *et al.*, 1970; Yabuya *et al.*, 1997, 2000; Mizuno *et al.*, 2013). In the case of Dutch iris, it was demonstrated using *C*-glycosylflavone, swertisin 2″-*O*-(4‴-acetylrhamnoside) and delphinidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (violanin). In Japanese garden iris, it was reported that *C*-glycosylflavone, isovitexin is caused the intermolecular copigmentaion with anthocynins such as malvidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-5-*O*-glucoside (ensatin), petunidin 3-*O*-[(4‴-*p*-coumaroylrhamnosyl)-(1→6)-glucoside]-

5-*O*-glucoside (petanin). In addition, it has been revealed that their bluing effect depend on the amount and structure of these compounds (Asen *et al.*, 1971, 1972; Yabuya *et al.*, 1997).

Tall bearded iris (*Iris* × *germanica* L.) is one of the ornamental plants in the genus *Iris* as well as Dutch iris and Japanese garden iris. The iris grows to more than 70 cm tall and have glamorous flower. Anthocyanin, violanin, six *C*-glycosylflavones such as swertisin 2″-*O*-arabinoside, swertiajaponin 2″-*O*-arabinoside, schaftoside, isoschaftoside, swertiajaponin and swertisin 2″-*O*-glucoside, and *C*-glycosylxanthone, mangiferin, were isolated from the violet blue flower cultivar 'Victoria Falls' (Mizuno *et al.*, 2012). However, it is not clear whether those are correlated to the flower color.

In this study, we performed the measurement of colorimetric value, absorption spectra, quantitative analysis, and pH value of falls and standards of the tall bearded iris cultivar 'Victoria Falls'. Furthermore, based on these results, *in vitro* examination using isolated anthocyanin, *C*-glycosylflavone and *C*-glycosylxanthone was performed.

Materials and Methods

Plant materials

The tall bearded iris (*Iris* × *germanica*) cultivar 'Victoria Falls' was cultivated in the Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan. Flowers were collected at full-bloom stage in May 2019 (Fig. 1). Their fresh falls and standards were used as plant materials. The flower color values were recorded by the Royal Horticultural Society (R.H.S.) color chart and a color reader (CR-10, Konica Minolta Sensing Inc., Japan) as CIE$L^*a^*b^*$ (Gonnet, 1995, 1998).

Absorption spectra of fresh flower

The fully expanded falls and standards were used for the measurement of visible absorption spectra (400–700 nm) with UV-2600 spectrophotometer (Shimadzu, Kyoto) which attached integrated sphere ISR-2600 (Shimadzu).

Fig. 1. Tall bearded iris cultivar 'Victoria Falls'. The downward-curving petals are called as falls. The upright petals are called as standards.

Authentic samples

Authentic violanin (Fig. 2A) was obtained from the flowers of *I. sanguinea* Hornem. (Mizuno *et al.*, 2018). Authentic swertisin 2″-*O*-arabinoside (Fig. 2B) and mangiferin (Fig. 2C) were obtained from the flowers of tall bearded iris cultivar 'Victoria Falls' (Mizuno *et al.*, 2012). These compounds were checked by HPLC before use as the standards of quantitative analysis and *in vitro* examination.

Quantitative evaluation of anthocyanin, C-glycosylflavone and C-glycosylxanthone

Total anthocyanin (TA), total *C*-glycosylflavone (TF) and total *C*-glycosylxanthone (TX) contents were measured and calculated by HPLC and LC-Solution software (Shimadzu) from simple linear regression using violanin for TA contents at 530 nm, swertisin 2″-*O*-arabinoside for TF contents at 350 nm, and mangiferin for TX contents at 350 nm. Each TA, TF and TX contents were revealed as nmol mg^{-1} fresh weight.

HPLC analyses were performed with Shimadzu HPLC systems equipped with a SPD–20A UV–Vis detector using InertSustain C18 (I.D.

Table 1. Chromaticity in the falls and standards of tall bearded iris cultivar 'Victoria Falls'

Flower parts	RHSCC	Chromaticity			Chroma (C)	Hue angle (h°)
		L^*	a^*	b^*		
Falls	VioletBlue 94C	47.1 ± 1.8	17.9 ± 0.8	− 35.2 ± 0.7	39.5	297.0
Standards	VioletBlue 94D	56.3 ± 1.9	12.0 ± 1.0	− 29.0 ± 1.2	31.4	292.4

The measurement was performed triplicate (n = 3 ± SE).

The values of chroma (C) and hue angle ($h°$) were calculated as fallows. $C^* = (a^{*2} + b^{*2})^{1/2}$ and $h = \tan^{-1}(b^*/a^*)$.

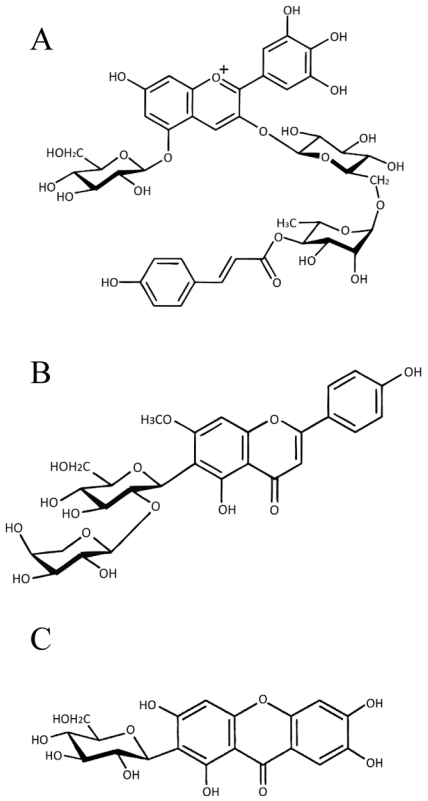

Fig. 2. Structures of violanin (A), swertisin 2″-*O*-arabinoside (B) and mangiferin (C).

10 × 250 mm, GL Sciences Inc., Tokyo), at a flow-rate of 1.0 ml min^{-1}. 5% HCOOH in H_2O (solvent A) and 5% HCOOH in 90% MeCN (solvent B) were used as the mobile phase under gradient elution conditions. The gradient solution program is as follows: a linear gradient from 10% to 50% solvent B for 35 min, 50% solvent B for 10 min.

pH measurement

The pH values of pressed juice from the falls and standards were measured using a twin pH meter AS-212 (HORIBA, Ltd., Tokyo). The measurements were performed in triplicate.

In vitro examination of intermolecular copigmentation

Purified violanin, swertisin 2″-*O*-arabinoside and mangiferin were dissolved in citric phosphate buffer (pH 5.2). The proportion of these compounds was decided based on the result of quantitative analysis of the falls. Visible absorption spectra of these solutions were measured using UV-2600 spectrophotometer at 0, 10 and 30 min after dissolution.

Result and Discussion

The values of RHS color chart and colorimetric values were shown in Table 1. Higher L^* value and lower C^* value of the standards were revealed, meaning that the standards are more lightness than the falls. RHS color chart and the value of hue angle, i.e. 297.0° for the falls and 292.4° for the standards, revealed that the flower color is violet blue.

Visible absorption spectra of the intact falls and standards of 'Victoria Falls' were shown by solid and dashed lines in Fig. 3. The λmax of each part was shown at 578.9 and 580.2 nm. Two shoulders at 544.4 and 636.0 nm, and at 544.4 and 635.4 nm were also observed, respectively. These absorption spectral curves were revealed as essentially the same to each other.

TA, TF and TX contents in the falls and standards of 'Victoria Falls' were shown in Table 2.

Table 2. Quantitative analysis of anthocyanin and *C*-glycosylflavone and *C*-glycosylxanthone in the flower of the tall bearded iris cultivar 'Victoria Falls'

Flower parts	Amount of three phenolic compounds (nmol mg^{-1})			TF/TA ratio	TX/TA ratio
	TA*	TF**	TX***		
Falls	1.50	14.09	4.71	9.4	3.1
Standards	1.18	15.06	4.83	12.8	4.1

*TA = Total anthocyanin contents.

**TF = Total *C*-glycosylflavone contents.

***TX = Total *C*-glycosylxanthone contents.

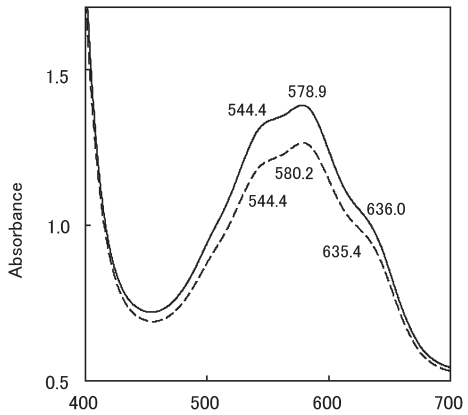

Fig. 3. Visible absorption spectra of the intact falls (solid line) and standards (dashed line).

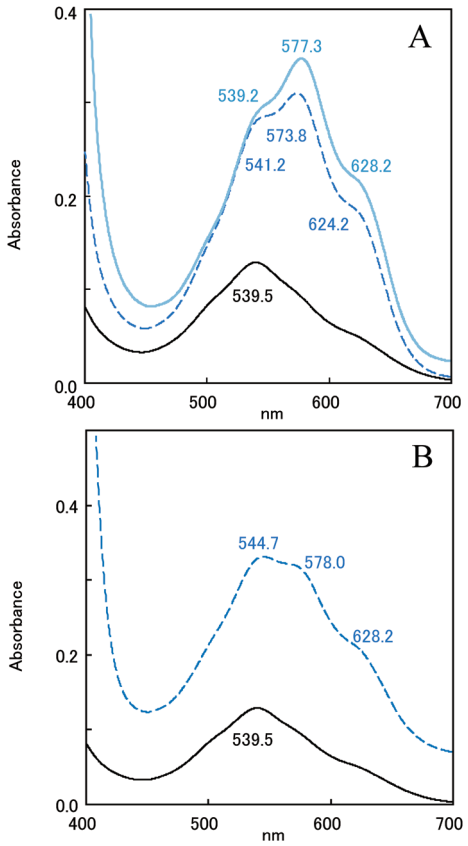

Fig. 4. Effect of swertisin 2″-*O*-arabinoside (A) and mangiferin (B) on the visible absorption spectra of violanin. Concentration of swertisin 2″-*O*-arabinoside: 0 (solid line), 0.3 (blue dashed line) and 0.9 (blue bold line) mM, and mangiferin are 0 (solid line) and 0.3 mM (blue dashed line).

The ratios of TA contents per TF contents and TA contents per TX contents were calculated as TF/TA values and TX/TA values (Table 2). The value of TF/TA was used for index of bluing effect in some flower color (Wang *et al.*, 2001; Uddin *et al.*, 2002). TA contents of the falls were higher than those of the standards. TF and TX contents were generally similar to each other. TF/TA and TX/TA values were higher in the standards than those of the falls.

For *in vitro* examination, the pH value of buffer solution was decided as 5.2 from the result of pressed juice, which is weakly acidic and the same value with that of Dutch iris (Mizuno *et al.*, 2013). The results of *in vitro* examination using purified violanin in addition of swertisin 2″-*O*-arabinoside or mangiferin at 10 min after dissolution were shown in Figs. 4A and 4B, and Table 3. Absorption maxima of buffer solution containing 0.1 mM violanin were revealed as λmax 539.5 nm. On the other hand, those of buffer solution of mixture of 0.1 mM violanin and

Table 3. Intermolecular copigmentation and color stability of violanin and swertisin 2″-*O*-arabinoside or mangiferin under buffer solution*

Anthocyanin (0.1 mM)	Copigment compounds (mM)		λmax (nm)	Δλmax (nm)	Degradation rate of the optical density (%)**
Violanin	Swertisin 2″-*O*-arabinoside	0	539.5	—	95.7
		0.3	573.8	34.3	80.4
		0.9	577.3	37.8	63.2
	Mangiferin	0.3	544.7	5.2	84.0

*Absorption spectra were measured at 10 min after dissolution.

**The values were determined by the degradation rate of optical density of 30 min after dissolution when the optical density of 0 min is 100%.

0.3 or 0.9 mM swertisin 2″-*O*-arabinoside were shown as λmax 573.8 and 577.3 nm, respectively. They were occurred bathochromic shift (Δ λmax) at 34.3 and 37.8 nm, respectively. When the optical density of 0 min after dissolution is 100%, degradation rate of optical density of 0, 0.3 and 0.9 mM were shown to be 95.7%, 80.4% and 63.2%, respectively (Table 3).

The absorption maxima of the mixture solution of 0.1 mM violanin and 0.3 mM mangiferin were revealed as λmax 544.7 nm. Degradation rate of optical density of 30 min after dissolution was shown to be 84.0% (Table 3). It was revealed that Δ λmax value of the mixture solution of 0.1 mM violanin and 0.3 mM swertisin 2″-*O*-arabinoside was larger than that of the mixture solution of 0.1 mM violanin and 0.3 mM mangiferin. Furthermore, the value of degradation rate of optical density was higher in 0.1 mM violanin and 0.3 mM mangiferin solution than that of 0.1 mM violanin and 0.3 mM swertisin 2″-*O*-arabinoside.

To reconstruct the color expression of the falls under *in vitro* condition, we mixed 0.1 mM violanin, 0.9 mM swertisin 2″-*O*-arabinoside and 0.3 mM mangiferin under buffer solution (Fig. 4). Absorption maxima were observed at λmax 577.9 nm with shoulders at 539.8 and 629.4 nm. These absorption curves were essentially the same with those of the intact falls (Fig. 2). Furthermore, degradation rate of optical density of 30 min after dissolution was revealed as to be 59.3%.

In this study, we confirmed the effect of *C*-glycosylflavons on the flower color and color stability. It was shown that *C*-glycosylflavones are present as intermolecular copigementation substance in the tall bearded iris cultivar, as well as Dutch iris and Japanese garden iris (Asen *et al.*, 1970; Yabuya *et al.*, 1997, 2000; Mizuno *et al.*, 2013). Furthermore, we examined the influence of mangiferin on the flower color. Thus, it was shown that mangiferin is slightly shifted the absorption maxima of violanin.

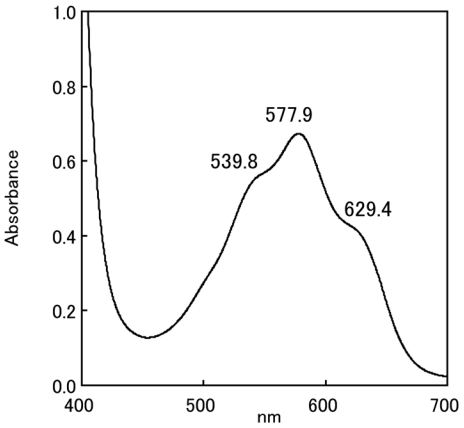

Fig. 5. Flower color reconstruction under *in vitro* condition from the falls of tall bearded iris 'Victoria Falls'. 0.1 mM violanin, 0.9 mM swertisin 2″-*O*-arabinoside and 0.3 mM mangiferin were dissolved in buffer solution (pH 5.2).

Mangiferin is widely distributed in plants (Richardson, 1983). The compound is known as one of antioxidant substance (Dar *et al.*, 2005). In the genus *Iris* section *Iris* species, including tall bearded iris, mangiferin and other xanthones

are present in the leaves and flowers (Bate-Smith and Harborne, 1963; Ashtakala and Forward, 1971; Williams *et al.*, 1997). This study suggested that mangiferin slightly contribute to the flower color expression and stability in some *Iris* species along with *C*-glycosylflavones.

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