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Anthocyanin-flavonol Copigmentation in Bluish Flowers of *Gladiolus* × *grandiflora* Cultivar

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Abstract The *in vitro* identification of copigmentation was carried out using anthocyanin, malvidin 3,5-di-*O*-glucoside, and three flavonol glycosides, kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside. They are major pigments of *Gladiolus* × *grandiflora* cultivar 'Ariake'. Anthocyanin brought about copigmentation, i.e., the bathochromic shift (bluing effect) of visible λmax due to increased concentrations of kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside, and 14–30 and 12–29 nm were estimated as the magnitude (Δλmax), respectively. However, kaempferol 3-*O*-sophoroside was not occurred the bathochromic shift of malvidin 3,5-di-*O*-glucoside. These results showed that bluing effect on the flower color of the bluish cultivar 'Ariake' caused at least in part by the copigmentation between anthocyanin and two flavonol glycosides, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside.

Introduction

The flower colors, especially purple to blue, due to anthocyanins are well known to be influenced by intermolecular copigmentation (e.g., Yazaki, 1976; Takeda *et al.*, 1993; Yabuya *et al.*, 1997; Mizuno *et al.*, 2015), intramolecular copigmentation (e.g., Terahara *et al.*, 1990; Figueiredo *et al.*, 1999; Sakaguchi *et al.*, 2013), metal complexation (Takeda *et al.*, 2005; Shiono *et al.*, 2005, 2008; Yoshida *et al.*, 2006), and the vacuolar pH values of epidermal flower cells (Yoshida *et al.*, 1995; Fukuda-Tanaka *et al.*, 2000; Takeda *et al.*, 2010).

Gladiolus × *grandiflora* cultivars are widely grown all over the world as popular flowers. Flower anthocyanins and their distribution in many *Gladiolus* cultivars have been reported by Takemura *et al.* (2008). Their anthocyanins were isolated from the flowers of six selected cultivars and identified as 3-*O*-rutinoside-5-*O*-glucosides of cyanidin, malvidin, pelargonidin and peonidin, and 3,5-di-*O*-glucosides of petunidin, malvidin, pelargonidin, cyanidin and peonidin, and pelargonidin 3-*O*-rutinoside and malvidin 3-*O*-glucoside (Takemura *et al.*, 2008).

On the other hand, some flavonol glycosides were isolated from the flowers of *Gladiolus* cultivars and identified as kaempferol 3-*O*-sophoroside, 3-*O*-glucoside, 3-*O*-rutinoside and 3-*O*-glucosyl-(1→2)-rhamnoside, quercetin 3-*O*-rutinoside, and myricetin 3-*O*-rutinoside, 3-*O*-glucoside and 3-*O*-rhamnoside (Takemura and Iwashina, 2018). As minor flavonols, kaempferol 3-*O*-pentosylglucoside, 3-*O*-rhamnosylrhamnosylglucoside and 3-*O*-glucosylrhamnoside, laricitrin 3-*O*-diglucoside, 3-*O*-rhamnosylglucoside and 3-*O*-rhamnosylhexoside, and syringetin 3-*O*-rhamnosylglucoside were also found (Takemura and Iwashina, 2018).

Fig. 1. *Gladiolus* × *grandiflora* cultivar 'Ariake'.

Of their *Gladiolus* cultivars, 'Ariake' may be one of the most bluish flower cultivars (Fig. 1). As major anthocyanin, malvidin 3,5-di-*O*-glucoside has been isolated from 'Ariake', together with two minor malvidin glycosides (Takemura *et al.*, 2005). Three major flavonols, kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside were also found in this cultivar, together with other kaempferol, quercetin, myricetin, laricitrin and syringetin 3-*O*-glycosides (Takemura *et al.*, 2005; Takemura and Iwashina, 2018).

In this survey, the *in vitro* identification of copigmentation was carried out using anthocyanin, malvidin 3,5-di-*O*-glucoside, and three flavonol glycosides, kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside which are major pigments in 'Ariake' flowers.

Materials and Methods

Plant materials

Gladiolus × *grandiflora* cultivars 'Violetta' and 'Christmas' were cultivated in the nursery of the Tsukuba Botanical Garden, National Museum of Nature and Science, Tsukuba, Japan. Another cultivar 'Ariake' was cultivated in Ogawa Town, Ibaraki, Japan.

Extraction, isolation and identification of anthocyanin and flavonols

Malvidin 3,5-di-*O*-glucoside was extracted and isolated from the flowers of *Gladiolus* cultivar 'Violletta' according to Takemura *et al.* (2008). Three flavonols, kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside, and kaempferol 3-*O*-sophoroside and 3-*O*-rutinoside, were extracted and isolated from the flowers of *Gladiolus* cultivars 'Christmas' and 'Ariake' according to Takemura and Iwashina (2018). They were identified by UV-vis spectra, LC-MS, acid and alkaline hydrolyses, ^{1}H and ^{13}C NMR, and/or TLC and HPLC comparisons with authentic samples.

Quantitative analysis of anthocyanin and flavonols

Anthocyanin of the perianth was dissolved with 0.1%HCl–MeOH (1 ml) for one day. This solution was diluted to 100, 200 and 400 folds by addition of 0.1%HCl–MeOH. Absorption spectra (450–600 nm) of their solution were measured by HPLC [Pegasil ODS column (I.D. 6.0 × 150 mm) (Senshu Scientific Co. Ltd., Tokyo), eluent, $MeCN/H_2O/HOAc/H_3PO_4$ (6 : 83 : 8 : 3)], and absolute values (4.16×10^{-3} mol/l) were calculated by calibration curves. On the other hand, flavonols of the perianth were dissolved with MeOH (1 ml) and diluted to 100, 200 and 400 folds, and absorption spectra (220–500 nm) were measured by HPLC [Pegasil ODS column (I.D. 6.0 × 150 mm), eluent, $MeCN/H_2O/H_3PO_4$ (22 : 78 : 0.2)]. Absolute values (kaempferol 3-*O*-sophoroside, 3.78×10^{-2} mol/l, kaempferol 3-*O*-rutinoside, 1.46×10^{-2} mol/l, and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside, 6.40×10^{-2} mol/l) of their solution were calculated by calibration curves.

Table 1. The effect of concentration and copigmentation of malvidin 3,5-di-*O*-glucoside with three flavonol glycosides

Flavonols	Malvidin 3,5-di-*O*-glucoside (nm)				after 1 day		
	2×10^{-4} (M)	λmax	Δλmax	intensity	λmax	Δλmax	intensity
Kaempferol 3-*O*-sophoroside	0	532	—	0.24	532	—	0.23
	2	532	0	0.29	532	0	0.27
	3	532	0	0.32	532	0	0.31
	5	535	3	0.37	532	0	0.37
Kaempferol 3-*O*-rutinoside	0	532	—	0.24	532	—	0.23
	2	546	14	0.37	542	10	0.33
	3	556	24	0.42	542	10	0.39
	5	562	30	0.49	542	10	0.46
Kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside	0	532	—	0.24	532	—	0.23
	2	544	12	0.31	541	9	0.30
	3	558	26	0.40	543	11	0.33
	5	561	29	0.43	557	25	0.42
Mixture of three flavonols	0	532	—	0.24	532	—	0.23
	2.3 : 1.3 : 1.4	541	9	0.42	540	8	0.43

Absorption spectra of fresh perianths

Fully expanded bluish perianth of *Gladiolus*×*grandiflora* cultivar 'Ariake' was measured for visible absorption spectra by Shimadzu multipurpose spectrophotometer MPS-2000. To examine the absorption spectra of anthocyanin-flavonol copigmentation, malvidin 3,5-di-*O*-glucoside (2×10^{-4}M) and kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (4×10^{-4}M, 6×10^{-4}M and 10×10^{-4}M) were dissolved in 0.2M sodium acetate buffer solution (pH 4.8, 1.5 ml) and then mixed. After 5 min from mixing the anthocyanin and flavonol solution, the copigmentation absorption spectra were measured by the visible absorption with a Shimadzu multipurpose spectrophotometer MPS-2000.

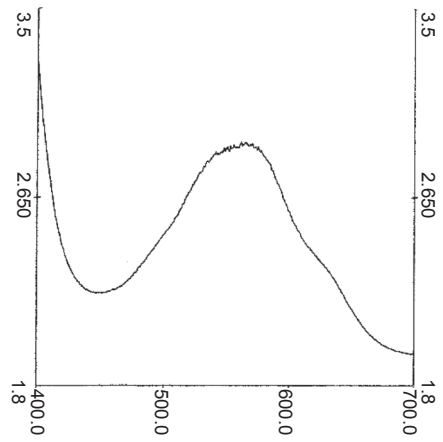

Fig. 2. UV-vis absorption spectral curve of intact bluish perianth of *Gladiolus*×*grandiflora* cultivar 'Ariake'.

Results and Discussion

The intact absorption spectral curve of the bluish perianth of cultivar 'Ariake' was shown in Fig. 2. Although absorption maxima of malvidin 3,5-di-*O*-glucoside is λmax 532 nm, that of the perianth appeared in 562 nm, showing the occurrence of the bathochromic shift of 30 nm. Moreover, since it has only one absorption maxima at 562 nm, and the absence of absorption maxima at 660–670 nm, this is apparently not due to metal complexing and pH (Saitô, 1967).

Copigment effects of three flavonol glycosides on absorption spectra of malvidin 3,5-di-*O*-glucoside are shown in Table 1 and Figs. 3, 4 and 5. As the results, malvidin 3,5-di-*O*-glucoside brought about copigmentation by the addition of flavonol glycosides, i.e., the bathochromic shift (bluing effect) in the visible λmax due to increased concentrations of kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-

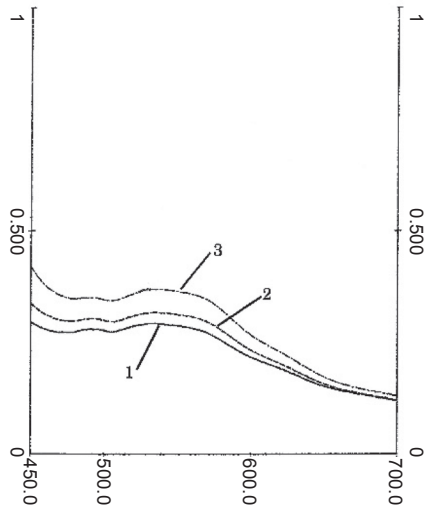

Fig. 3. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and kaempferol 3-*O*-sophoroside in CH_3COOK buffer. **1** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-sophoroside (1 : 2), **2** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-sophoroside (1 : 3) and **3** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-sophoroside (1 : 5). pH 4.8.

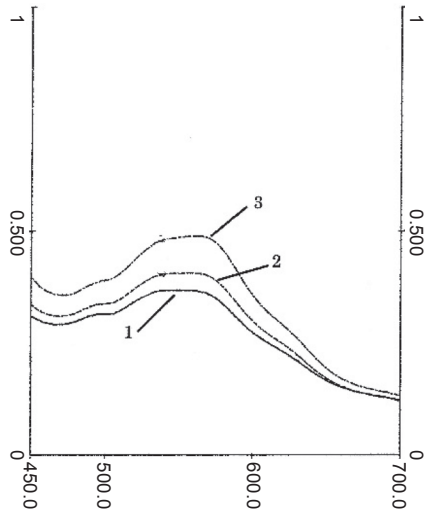

Fig. 4. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and kaempferol 3-*O*-rutinoside in CH_3COOK buffer. **1** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-rutinoside (1 : 2), **2** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-rutinoside (1 : 3) and **3** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-rutinoside (1 : 5). pH 4.8.

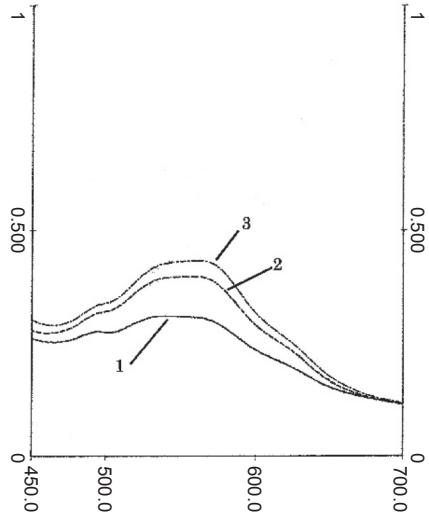

Fig. 5. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside in CH_3COOK buffer. **1** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (1 : 2), **2** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (1 : 3) and **3** = malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (1 : 5). pH 4.8.

(1→2)-rhamnoside, and 30 and 29 nm were estimated as the magnitude ($\Delta\lambda$max), respectively (Table 1, Figs. 4 and 5). On the other hand, the bathochromic shift did not occur by the addition of kaempferol 3-*O*-sophoroside (Table 1 and Fig. 3). In the case of *Hydrangea macrophylla* (Thunb. ex Murray) Ser. flowers, 3-caffeoylquinic acid and 3-*p*-coumaroyl-quinic acid similarly act as copigments. However, 5-caffeoylquinic acid (chlorogenic acid) obstructs copigment activity (Takeda *et al.*, 1990). Moreover, it was shown that although isovitexin (apigenin 6-*C*-glucoside) strongly acts as copigment, vitexin (apigenin 8-*C*-glucoside) weakly affect against cyanidin 3,5-di-*O*-glucoside (Asen *et al.*, 1972).

We calculated the ratio of malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-sophoroside : kaempferol 3-*O*-rutinoside : kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside in cultivar 'Ariake' perianth

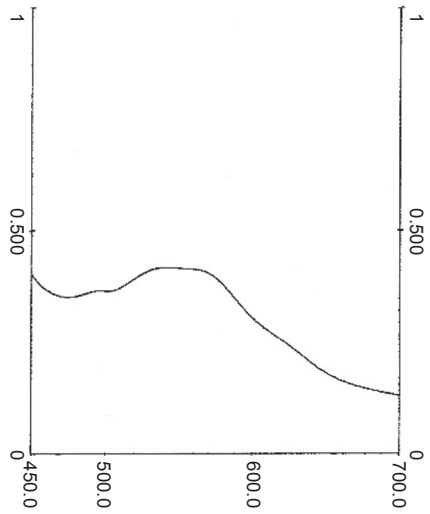

Fig. 6. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and three kaempferol 3-*O*-glycosides in CH_3COOK buffer. Malvidin 3,5-di-*O*-glucoside : kaempferol 3-*O*-sophoroside : kaempferol 3-*O*-rutinoside : kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (1 : 2.3 : 1.3 : 1.4). pH 4.8.

by calibration curve, and determined as 1 : 2.3 : 1.3 : 1.4. However, the bathochromic shift of this mixture on absorption spectra of malvidin 3,5-di-*O*-glucoside did not agree with that of fresh perianth (Fig. 6). In *Gladiolus* cultivar 'Ariake', tweleve flavonols except for kaempferol 3-*O*-rutinoside, 3-*O*-sophoroside and 3-*O*-glucosyl-(1→2)-rhamnoside were contained in the perianths. As the results, it was presumed that other flavonol glycosides also cause or obstruct the copigmentation in the perianths. When kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside were added in malvidin 3,5-di-*O*-glucoside solution, their absorption maxima and spectral curves were similar to that of fresh perianth, showing that the bluish color of 'Ariake' flowers is due to copigmentation with their flavonols. However, kaempferol 3-*O*-sophoroside was not occurred the copigmentation. Kaempferol 3-*O*-rutinoside and 3-*O*-glucosyl-(1→2)-rhamnoside attach each 1 mol of glucose and rhamnose. On the other hand, kaempferol 3-*O*-sophoroside attaches 2 mol glucose.

As the results, it was presumed that although kaempferol 3-*O*-rhamnosylglucoside and 3-*O*-glucosylrhamnoside are occurred the copigmentation, kaempferol 3-*O*-sophoroside, which attach 2 mol glucose but not each 1 mol of glucose and rhamnose, obstruct the copigmentation in *Gladiolus* cultivar flowers.

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