

## 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  $\lambda_{\text{max}}$  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 ( $\Delta\lambda_{\text{max}}$ ), 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 'Violetta' 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,  $^1\text{H}$  and  $^{13}\text{C}$  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.  $6.0 \times 150$  mm) (Senshu Scientific Co. Ltd., Tokyo), eluent, MeCN/H<sub>2</sub>O/HOAc/H<sub>3</sub>PO<sub>4</sub> (6:83:8:3)], and absolute values ( $4.16 \times 10^{-3}$  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.  $6.0 \times 150$  mm), eluent, MeCN/H<sub>2</sub>O/H<sub>3</sub>PO<sub>4</sub> (22:78:0.2)]. Absolute values (kaempferol 3-*O*-sophoroside,  $3.78 \times 10^{-2}$  mol/l, kaempferol 3-*O*-rutinoside,  $1.46 \times 10^{-2}$  mol/l, and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside,  $6.40 \times 10^{-2}$  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

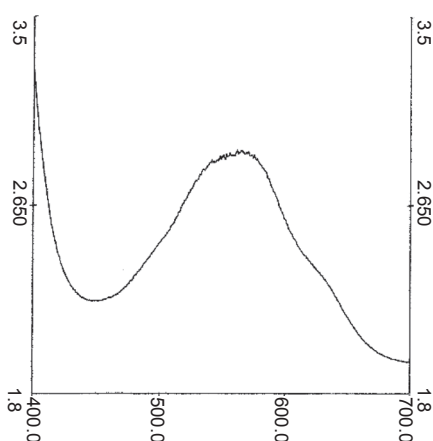
| Flavonols                                         | Malvidin 3,5-di- <i>O</i> -glucoside (nm) |                  |                        |           | after 1 day      |                        |           |
|---------------------------------------------------|-------------------------------------------|------------------|------------------------|-----------|------------------|------------------------|-----------|
|                                                   | $2 \times 10^{-4}$ (M)                    | $\lambda_{\max}$ | $\Delta\lambda_{\max}$ | intensity | $\lambda_{\max}$ | $\Delta\lambda_{\max}$ | intensity |
| Kaempferol 3- <i>O</i> -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- <i>O</i> -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- <i>O</i> -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*  $\times$  *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 ( $2 \times 10^{-4}$  M) and kaempferol 3-*O*-sophoroside, kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside ( $4 \times 10^{-4}$  M,  $6 \times 10^{-4}$  M and  $10 \times 10^{-4}$  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.

### 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  $\lambda_{\max}$  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

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

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  $\lambda_{\max}$  due to increased concentrations of kaempferol 3-*O*-rutinoside and kaempferol 3-*O*-glucosyl-

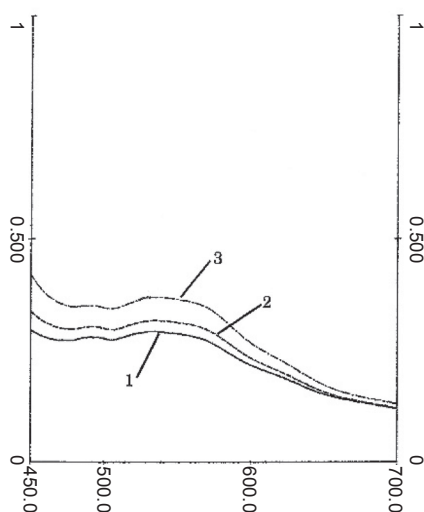


Fig. 3. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and kaempferol 3-*O*-sophoroside in  $\text{CH}_3\text{COOK}$  buffer. **1** = malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-sophoroside (1:2), **2** = malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-sophoroside (1:3) and **3** = malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-sophoroside (1:5). pH 4.8.

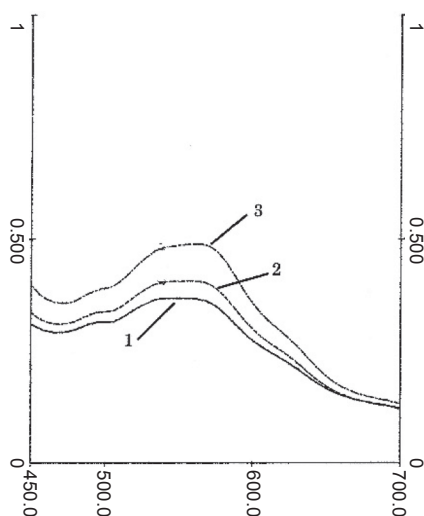


Fig. 4. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and kaempferol 3-*O*-rutinoside in  $\text{CH}_3\text{COOK}$  buffer. **1** = malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-rutinoside (1:2), **2** = malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-rutinoside (1:3) and **3** = malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-rutinoside (1:5). pH 4.8.

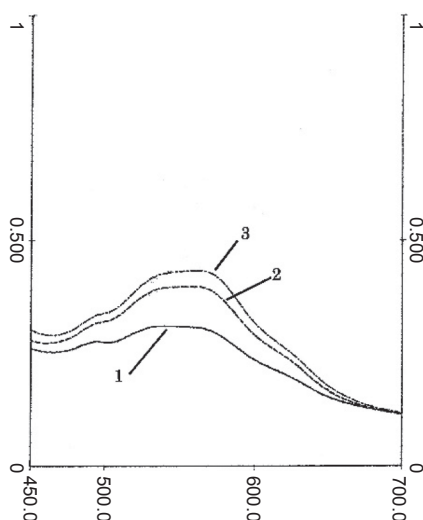


Fig. 5. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside in  $\text{CH}_3\text{COOK}$  buffer. **1** = malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-glucosyl-(1→2)-rhamnoside (1:2), **2** = malvidin 3,5-di-*O*-glucoside:kaempferol 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 ( $\Delta\lambda_{\text{max}}$ ), 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-caffeoyl-quinic acid and 3-*p*-coumaroyl-quinic acid similarly act as copigments. However, 5-caffeoyl-quinic 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

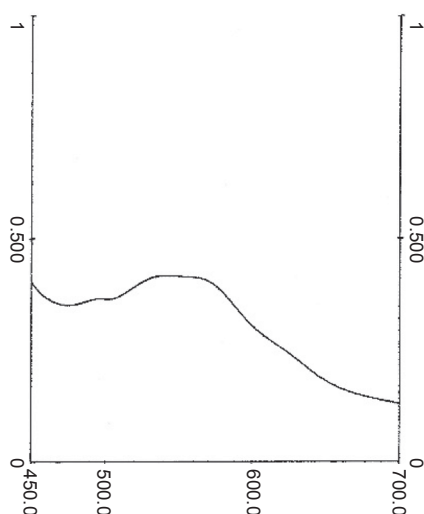


Fig. 6. UV-vis absorption spectral curve of mixture of malvidin 3,5-di-*O*-glucoside and three kaempferol 3-*O*-glycosides in  $\text{CH}_3\text{COOK}$  buffer. Malvidin 3,5-di-*O*-glucoside:kaempferol 3-*O*-sophoroside:kaempferol 3-*O*-rutinoside:kaempferol 3-*O*-glucosyl-(1 $\rightarrow$ 2)-rhamnoside (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', twelve flavonols except for kaempferol 3-*O*-rutinoside, 3-*O*-sophoroside and 3-*O*-glucosyl-(1 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 2)-rhamnoside attach each 1 mol of glucose and rhamnose. On the other hand, kaempferol 3-*O*-sophoroside attaches 2 mol glu-

cose.

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.

## References

- Asen, S., Stewart, R. N. and Norris, K. H. 1972. Co-pigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry* 11: 1139–1144.
- Figueiredo, P., George, F., Tatsuzawa, F., Toki, K., Saito, N. and Brouillard, R. 1999. New features of intramolecular copigmentation by acylated anthocyanins. *Phytochemistry* 51: 125–132.
- Fukuda-Tanaka, S., Inagaki, Y., Yamaguchi, Y., Saito, N. and Iida, S. 2000. Colour-enhancing protein in blue petals. *Nature* 407: 581.
- Mizuno, T., Uehara, A., Mizuta, D., Yabuya, T. and Iwashina, T. 2015. Contribution of anthocyanin-flavone copigmentation to grayed violet flower color of Dutch iris cultivar 'Tiger's Eye' under the presence of carotenoids. *Scientia Horticulturae* 186: 201–206.
- Saitō, N. 1967. Light absorption of anthocyanin-containing tissue of fresh flowers by the use of the opal glass transmission method. *Phytochemistry* 6: 1013–1018.
- Sakaguchi, K., Kitajima, J. and Iwashina, T. 2013. Acylated delphinidin glycosides from violet and violet-blue flowers of *Clematis* cultivars and their coloration. *Natural Product Communications* 8: 1563–1566.
- Shiono, M., Matsugaki, N. and Takeda, K. 2005. Structure of the blue cornflower pigment. *Nature* 436: 791.
- Shiono, M., Matsugaki, N. and Takeda, K. 2008. Structure of commelinin, a blue complex pigment from the blue flowers of *Commelina communis*. *Proceedings of the Japan Academy, Series B* 84: 452–456.
- Takeda, K., Fujii, A., Senda, Y. and Iwashina, T. 2010. Greenish blue flower colour of *Strongylodon macrobotris*. *Biochemical Systematics and Ecology* 38: 630–633.
- Takeda, K., Harborne, J. B. and Waterman, P. G. 1993. Malonylated flavonoids and blue flower colour in lupin. *Phytochemistry* 34: 421–423.
- Takeda, K., Yamashita, T., Takahashi, A. and Timberlake, C. F. 1990. Stable blue complexes of anthocyanin-aluminium-3-*p*-coumaroyl- or 3-caffeoyl-quinic acid involved in the bluing of *Hydrangea* flower. *Phytochemistry* 29: 1089–1091.
- Takeda, K., Osakabe, A., Saito, S., Furuyama, D., Tomita, A., Kojima, Y., Yamadera, M. and Sakuta, M. 2005.

- Components of protocyanin, a blue pigment from the blue flowers of *Centaurea cyanus*. *Phytochemistry* 66: 1607–1613.
- Takemura, T. and Iwashina, T. 2018. Flavonol glycosides and their distribution in the perianths of *Gladiolus* cultivars. *Bulletin of the National Museum of Nature and Science, Series B (Botany)* 45: 29–40.
- Takemura, T., Takatsu, Y., Kasumi, M., Marubashi, W. and Iwashina, T. 2005. Flavonoids and their distribution patterns in the flowers of *Gladiolus* cultivars. *Acta Horticulturae* 673: 487–493.
- Takemura, T., Takatsu, Y., Kasumi, M., Marubashi, W. and Iwashina, T. 2008. Anthocyanins of *Gladiolus* cultivars and their contribution to flower colors. *Journal of the Japanese Society for Horticultural Science* 77: 80–87.
- Terahara, N., Saito, N., Honda, T., Toki, K. and Osajima Y. 1990. Structure of ternatin A1, the largest ternatin in the major blue anthocyanins from *Clitoria ternatea* flowers. *Tetrahedron Letters* 31: 2921–2924.
- Yabuya, T., Nakamura, M., Iwashina, T., Yamaguchi, M. and Takehara, T. 1997. Anthocyanin-flavone copigmentation in bluish purple flowers of Japanese garden iris (*Iris ensata* Thunb.). *Euphytica* 98: 163–167.
- Yazaki, Y. 1976. Co-pigmentation and the color change with age in petals of *Fuchsia hybrida*. *Botanical Magazine, Tokyo* 89: 45–57.
- Yoshida, K., Kondo, T., Okazaki, Y. and Katou, K. 1995. Cause of blue petal colour. *Nature* 373: 291.
- Yoshida, K., Kitahara, S., Ito, D. and Kondo, T. 2006. Ferric ions involved in the flower color development of the Himalayan blue poppy, *Meconopsis grandis*. *Phytochemistry* 67: 992–998.