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Summary The flower colors of 15 cultivars and a line in Japanese garden iris, *Iris ensata* Thunb. were examined by CIELAB scatter diagram. The cultivars classified as anthocyanin types, malvidin 3RGac5G-petunidin 3RGac5G, malvidin 3RG5G-petunidin 3RG5G and peonidin 3RGac5G showed color distribution from purple to red-purple, while the cultivar and line in peonidin 3RG5G and peonidin 3RG5G-cyanidin 3RG5G types were purplish red to red. This indicates that cyanidin 3RG5G and peonidin 3RG5G are good anthocyanin source for the breeding of red flower color. The anthocyanins of 83 cultivars, 13 lines and 5 wild forms of *I. ensata* were analyzed by HPLC, and these plants were classified into 17 types of major anthocyanins. Among these, 7 new types, delphinidin 3RGac5G-delphinidin 3RG, delphinidin 3RG-petunidin 3RGac5G, petunidin 3G-delphinidin 3G, petunidin 3RGaG, peonidin 3RG5G, cyanidin 3RG5G and malvidin 3RGac5G-peonidin 3RGac5G-petunidin 3RGac5G were detected, and a novel anthocyanin source of red flower, cyanidin 3RG5G was obtained for the first time.

Key words *Iris ensata*, Anthocyanin, HPLC analysis, Cyanidin 3RG5G, Flower color breeding.

Japanese garden iris, *Iris ensata* Thunb. is one of the important ornamental species in Japan, and produces purple, red-purple, blue-purple, light-purple, pink and white flower colors due to flavonoid pigments, the main components of which are anthocyanins. In spite of such variation, this species lack flower colors such as blue, red, yellow and orange, and development of new color cultivars is essential for the detection of novel pigments.

The anthocyanins of the outer perianths in *I. ensata* and its wild forms have been studied by Hayashi (1940), Hayashi *et al.* (1978), Ishikura and Yamamoto (1978), Yabuya (1991), Yabuya *et al.* (1994) and Yabuya *et al.* (2001). Recently, the cultivars and wild forms of this species have been classified into 16 types, malvidin 3-(p-coumaroyl)-rutinosido-5-glucoside (malvidin 3RGac5G)-petunidin 3RGac5G, petunidin 3RGac5G-malvidin 3RGac5G, petunidin 3RGac5G-delphinidin 3RGac5G, delphinidin 3RGac5G-petunidin 3RGac5G, cyanidin 3RGac5G-peonidin 3RGac5G, delphinidin 3-rutinoside (delphinidin 3RG)-delphinidin 3-(p-coumaroyl)-rutinoside (delphinidin 3RGac), malvidin 3-rutinosido-5-glucoside (malvidin 3RG5G)-petunidin 3RG5G, petunidin 3RG5G-malvidin 3RG5G, malvidin 3RG5G-peonidin 3RG5G, peonidin 3RG5G-cyanidin 3RG5G, peonidin 3RG-cyanidin 3RG, malvidin 3RGac5G, petunidin 3RGac5G, delphinidin 3RGac5G, peonidin 3RGac5G and cyanidin 3RGac5G according to the order of major anthocyanins (Yabuya 1991, Yabuya *et al.* 1994, 2001). Among these types, delphinidin 3RGac5G was useful for the breeding of blue flower color (Yabuya 1991) and a breeding strategy leading to copigmentation of this anthocyanin with isovitexin was proposed by Yabuya *et al.* (1997). However, the anthocyanins and flower colors of *I. ensata* have not yet been fully characterized.

In this study, color variation and anthocyanins of outer perianths in *I. ensata* were analyzed by spectrophotometer and high-performance liquid chromatography (HPLC), and cyanidin 3RG5G...
type was detected as a novel anthocyanin source of red flower for the first time.

Materials and methods

Plant materials
Eighty three cultivars, 13 lines and 5 wild forms of *Iris ensata* Thunb. var. *ensata* (Makino) Nakai (=*I. kaempferi* Sieb. var. *hortensis* Makino), were subjected to anthocyanin analysis (Table 1) and 15 cultivars and a line for flower color measurement (Fig. 1). These plants were grown in the experimental garden of Miyazaki University, Japan. Fully expanded flowers were harvested to measure their flower color and anthocyanins.

Flower color measurement
Two chromatic components *a* and *b* of the CIELAB color coordinate of three outer perianths of each plant material were measured with a spectrophotometer NF333 (NIPPON DENSHOKU, Japan). The CIELAB is a colorimetric system, ordained by CIE (Commission Internationale de l’Eclairage) in 1976, and *a* and *b* indicate chromaticity.

HPLC analysis of anthocyanins
Anthocyanin samples for HPLC analysis of each plant material was prepared, as per the method described by Yabuya (1987). The HPLC analysis was performed using Shimadzu LC-9A liquid chromatograph equipped with a STR ODS-II column (150×4.6 mm, Shimadzu, Japan) at 35°C under Shimadzu CTO-10A column oven. The anthocyanin samples were separated by the gradient elution: in 20 min linear 0 to 40% B in A+B and in 20 min 40% B in A+B (A, 10% acetic acid and 0.1% phosphoric acid in water; B, 50% acetonitrile in water), and flow-rate was 1 ml min⁻¹. Anthocyanins were detected at 540 nm with Shimadzu SPD-10A UV-VIS detector. Retention times and quantitative calculations of the anthocyanin peaks were obtained with C-R6A Shimadzu Chromatopac.

Identification of anthocyanins was carried out by comparison with known samples using λmax and co-chromatography as described by Yabuya et al. (1994). The known samples of anthocyanins such as malvidin 3RG5G, malvidin 3RGac5G, petunidin 3RG5G, petunidin 3RGac5G, delphinidin 3RG, delphinidin 3RG5G, delphinidin 3RGac5G, peonidin 3RG5G, peonidin 3RGac5G and cyanidin 3RG5G were obtained by the methods of Yabuya (1991) and Yabuya et al. (1994). Petunidin 3-glucoside (petunidin 3G), delphinidin 3G and petunidin 3RGac were kindly provided by Dr. M. Yamaguchi.

Results and discussion
The distributions of 15 cultivars and a line in *I. ensata* with respect to flower color on CIELAB scatter diagram are shown in Fig. 1. The cultivars classified as anthocyanin types, malvidin 3RGac5G-petunidin 3RGac5G, malvidin 3RG5G-petunidin 3RG5G and peonidin 3RGac5G show a distribution from purple to red-purple. On the other hand, the cultivar and line in peonidin 3RG5G and peonidin 3RG5G-cyanidin 3RG5G types are distributed from purplish red to red. This shows that cyanidin 3RG5G and peonidin 3RG5G are good anthocyanin source for the breeding of red flower color.

The anthocyanins of 83 cultivars, 13 lines and 5 wild forms of *I. ensata* were analyzed by HPLC, and these plants were classified into 17 types of major anthocyanins as shown in Table 1. In this study, 11 anthocyanins (Table 1) were regarded as the major anthocyanins, which accounted for more than 20% of total anthocyanins detected in each plant. Among 17 types of major anthocyanins, 7 types such as delphinidin 3RGac5G-delphinidin 3RG, delphinidin 3RG-petunidin 3RGac5G and delphinidin 3RG-petunidin 3RGac5G...
Fig. 1. Distribution of 16 anthocyanin type cultivars (line) in Japanese garden iris by flower colors based on their CIELAB coordinates. $a^*$ and $b^*$ see Materials and methods.
3RGac5G, petunidin 3G-delphinidin 3G, petunidin 3RGac, peonidin 3RG5G, cyanidin 3RG5G and malvidin 3RGac5G-peonidin 3RGac5G-petunidin 3RGac5G were obtained as new types (Fig. 2).

Among these new types, cyanidin 3RG5G is worthy of remark because this type is useful for the creation of a novel flower color in *I. ensata*. According to Davies and Schwinn (1997), most of the red colors seen in flowers are based on pelargonidin or cyanidin type anthocyanins. To our knowledge, however, no pelargonidin type anthocyanins have been detected in *I. ensata*, and cyanidin 3RG5G is worthy of remark because this type is useful for the creation of a novel flower color in *I. ensata*. According to Davies and Schwinn (1997), most of the red colors seen in flowers are based on pelargonidin or cyanidin type anthocyanins.
din 3RG5G produced the lowest value of $\lambda_{\text{max}}$ among cyanidin and peonidin type anthocyanins (Yabuya et al. 2001). In *I. ensata*, therefore, cyanidin 3RG5G is the most suitable anthocyanin for breeding of red flower, and the cyanidin 3RG5G type cultivar ‘Miyakei 4’ is the best available gene source, although this cultivar exhibits pink flowers.

A monoglucoside type of anthocyanins, petunidin 3G-delphinidin 3G were first detected in *I. ensata*, and the cultivar ‘Togenochaya’. In this cultivar, the anthocyanin type exhibits pale dark red-purple flowers. The petunidin 3RGac type cultivars, ‘Inishienosato’ and ‘Nokibanoume’ show dark red-purple flowers and the cultivar ‘Shohu’ grayish red-purple. Moreover, the peonidin 3RG-cyanidin 3RG type cultivar ‘Giondaiko’ produces pale dark magenta flowers and the delphinidin 3RG-delphinidin 3RGac cultivar ‘Shibukake’ grayish purple (Yabuya et al. 2001). Yabuya (1991) reported that malvidin 3RGac5G-petunidin 3RGac5G type of *I. ensata* is the basic (wild) one for the types of major anthocyanins, petunidin 3G-delphinidin 3G, petunidin 3RGac, peonidin 3RG-cyanidin 3RG and delphinidin 3RG-delphinidin 3RGac were regarded as variants for 5-glucosylation of anthocyanins. The detection of these variants is of interest, because they have common characters of dark or grayish tones for flower colors.

As described previously by Yabuya et al. (2001), the $\lambda_{\text{max}}$ values of cyanidin and peonidin type anthocyanins were anthocyanidin 3RG5G<3RG<3RGac5G. This indicates that acylation of anthocyanidin 3RG5G by $p$-coumaric acid reduced reddening effect, while 5-glucosylation of anthocyanidin 3RG promoted. Thus, it is proposed that 5-glucosylation of anthocyanins promoted reddening effect, and the deletion of the 5-glucosylation induces dark or grayish tones for flowers of *I. ensata*. 

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**Fig. 2.** The representative HPLC chromatograms of malvidin 3RGac5G (peak No. 5)-petunidin 3RGac5G (peak No. 4) (A: ‘Nessanomai’), petunidin 3G (peak No. 3)-delphinidin 3G (peak No. 2) (B: ‘Togenochaya’), petunidin 3RGac (peak No. 6) (C: ‘Inishienosato’) and cyanidin 3RG5G (peak No. 1) (D: ‘Miyakei 4’) types in Japanese garden iris.
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References


