Fundamental studies on the breeding of flower colors in *Iris* species

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Summary

Out of the various *Iris* species, the Japanese garden iris (*I. ensata* var. *ensata*), Dutch iris (*I. hollandica*), and tall bearded iris (*I. germanica*) are well known as garden species. *I. ensata* has been extensively developed as an ornamental species in Japan. In addition to being used as a garden plant, it is also used as a cut flower and potted plant. This species produces purple, reddish purple, bluish purple, light purple, pink, and white flower colors due to flavonoid pigments; the main component of the pigments is anthocyanin. In spite of these variations, *I. ensata* does not exhibit blue, red, yellow, and orange colors.

Anthocyanins comprise an anthocyanidin linked with sugar residues and occasionally with acyl groups. In the biosynthetic pathway of anthocyanins, the unstable anthocyanidins are usually stabilized first through glucosylation at the 3-position by UDP-glucose: anthocyanidin 3-*O*-glucosyltransferase (3GT). The pathway leading to anthocyanidin 3-glucoside (3G) is commonly conserved among plant species. Anthocyanidin 3Gs are further modified by glycosylation, acylation, and methylation, and these modification patterns vary among plant species. Among these

modifications, glycosylation and acylation are important for increasing their stability and water solubility; however, most of the cloned glycosyltranseferase and acyltransferase cDNAs are from dicotyledones, and there is limited knowledge regarding the molecular biology and biochemistry of monocotyledonous glycosyltranseferases and acyltransferases as described in Chapter 1. In particular, no reports have been published on the characterization of the enzymes and genes involved in the anthocyanin biosynthetic pathway of *Iris* species.

The objective of this study was the characterization of anthocyanins, biosynthetic enzymes, and genes in *Iris* species, to promote the breeding of diverse flower colors. In order to identify the anthocyanins that can be used for breeding, anthocyanins of the outer perianths in 14 species of *Iris* were analyzed by high performance liquid chromatography (HPLC) procedures, and the enzymatic properties of anthocyanin 5-*O*-glucosyl- transferase (5GT) and anthocyanin 3-*p*-coumaroyltransferase (3AT) were investigated in *I. ensata*. Furthermore, the cDNAs encoding 5GT and 3AT were successfully isolated from a cDNA library constructed from flower buds of *I. hollandica*, and their functional expressions were examined. The results obtained are summarized in the following 5 sections.

1. Characterization of anthocyanins in *Iris* species

The anthocyanins of 262 cultivars (lines) of *I. ensata* were analyzed by HPLC, and these plants were classified into 29 types of major anthocyanins.

Out of these, the 21 new types that were identified were, petunidin 3*p*CRG5G - delphinidin 3*p*CRG5G, delphinidin 3*p*CRG5G - petunidin 3*p*CRG5G, cyanidin 3*p*CRG5G - peonidin 3*p*CRG5G, delphinidin 3RG - petunidin 3*p*CRG5G, delphinidin 3*p*CRG5G - delphinidin 3RG, petunidin 3*p*CRG - petunidin 3RG, delphinidin 3RG - delphinidin 3*p*CRG, petunidin 3RG5G - malvidin 3RG5G, malvidin 3RG5G - peonidin 3RG5G, peonidin 3RG5G - cyanidin 3RG5G, peonidin 3RG5G - cyanidin 3G, petunidin 3RG - malvidin 3RG, petunidin 3RG - delphinidin 3RG, peonidin 3RG - cyanidin 3RG, petunidin 3G - delphinidin 3G, petunidin 3*p*CRG, peonidin 3RG5G, cyanidin 3RG5G, petunidin 3RG, delphinidin 3RG, and malvidin 3*p*CRG5G - peonidin 3*p*CRG5G - petunidin 3*p*CRG5G. Among these new types, cyanidin 3RG5G was the anthocyanin most useful for the breeding of red flowers in *I. ensata.*

Among 13 *Iris* species, excluding *I. ensata,* delphinidin 3CRG5G from *I. sanguinea* and *I. germanica* and delphinidin 3-*cis*-*p*CRG5G from *I. milesii* were the first to be identified as new anthocyanins in this genus.

2. Characterization of 5GT in *I.ensata* flowers

Enzyme extracts obtained from the flower buds of the cyanic cultivar (malvidin 3*p*CRG5G – petunidin 3*p*CRG5G type) "Hanamagaki" catalyzed the transfer of the glucosyl moiety from UDP-glucose to the 5-position of anthocyanidin 3RG to form the anthocyanidin 3RG5G, but not to the anthocyanidin 3G and 3*p*CRG. In addition to the characterization of the 5GT,

the activities of this enzyme were also examined for various cyanic and acyanic cultivars. Specific activities among cyanic cultivars were rather difficult to correlate with flower color, except for cv. Zamanobi, in which the activity was less than 20% when compared with others. On the other hand, 3 out of the 5 acyanic cultivars examined exhibited considerably high specific activities. Interestingly, the gene encoding 5GT is expressed independent of the other genes for anthocyanin biosynthesis.

3. Characterization of 3AT in the flowers of *I. ensata*

Enzyme extracts obtained from the flower buds of the cyanic cultivar "Hanamagaki" catalyzed the transfer of the *p*-coumaroyl moiety from *p*-coumaroyl-CoA to the 3-position of anthocyanidin 3RG and 3RG5G to form the anthocyanidin 3*p*CRG and 3*p*CRG5G, but not to anthocyanidin 3G. The activities of 3AT were also examined for various cyanic and acyanic cultivars. Specific activities among cyanic cultivars were within the wide range of ca. 1.7×10^{-2} and 1.9×10^{-1} pkat μg^{-1} that were rather difficult to relate to the anthocyanin contents of major anthocyanins. Two of the 5 acyanic cultivars examined exhibited considerably high specific activities, though the specific activity for cv. Yukitsubame with malvidin 3RG as a substrate was extremely low. As in the case of 5GT, the gene encoding 3AT is also expressed independent of the other genes for anthocyanin biosynthesis. As shown in Fig. 3-1, the pathway leading to the formation of end products from anthocyanidin 3RG during anthocyanin biosynthesis in flowers of *I. ensata*

was proposed.

4. Isolation and characterization of *5GT* cDNA clone in *I. hollandica*

A putative full-length cDNA encoding 5GT was isolated from a cDNA library constructed from the flower buds of *I. hollandica* by screening using a cDNA clone of anthocyanidin 3-*O*-glucosyltransferase (3GT) from *Antirrhinum majus* as a probe. The cDNA encoded an open reading frame (ORF) of 463 amino acids with a calculated molecular mass of 50,100 Da. Heterologous expression of the cDNA in *Escherichia coli* demonstrated that it encoded 5GT. This is the first report of *5GT* gene cloning from monocotyledonous plants. A molecular phylogenetic tree analysis of the amino acid sequences of glycosyltransferases from various plants showed that Ih5GT is a member of the 5GT group; however, it is distant from the dicot subgroup.

5. Isolation and characterization of *3AT* cDNA clone in *I. hollandica*

A cDNA contig clone *Ih3AT* encoding 3AT was successfully isolated from a cDNA library by PCR-based screening. *Ih3AT* encoded an ORF of 429 amino acids with a calculated molecular mass of 47,677 Da. Characterization of the enzymatic properties using the recombinant Ih3AT protein confirmed that *Ih3AT* cDNA encoded 3AT that catalyzes the transfer of *p*-coumaroyl moiety from *p*-coumaroyl-CoA to anthocyanidin 3RG5G to form anthocyanidin 3*p*CRG5G. This is the first report on the cloning of *AAT* gene from a monocot

plant.

Finally, the breeding for flower colors in *Iris* species, particularly of *I. ensata*, was discussed based on the results described above.