A molecular genetic study on anthocyanin biosynthesis of *Iris hollandica*

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Summary

Anthocyanins consist of an anthocyanidin (as chromophore) linked with sugar residues and occasionally with acyl groups. In anthocyanin biosynthesis, the unstable anthocyanidins are stabilized first by 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 and has been studied in detail (Fig. 1-2). Anthocyanidin 3Gs are further modified by glycosylation, acylation and methylation, and these modifications produce a wide variety of anthocyanins. As a result, anthocyanins express a wide range of flower colors such as pink, red, orange, scarlet, purple, blue and so on. To promote flower color breeding, therefore, detailed knowledge of anthocyanin biosynthesis is needed. In this study, molecular genetic characterization of anthocyanin biosynthesis in *Iris hollandica* was conducted, and the results obtained are summarized as follows.

1. Cloning of anthocyanin biosynthetic genes and their expression analyses

Chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*) and *3GT* cDNA clones (*IhCHS*, *IhF3H1*～*3*, *IhDFR* and *Ih3GT*) were isolated from a cDNA library of flower buds from *I. hollandica* cv. Blue Diamond by screening with *CHS*, *F3H* and *3GT* cDNA clones of *Antirrhinum majus* and a *DFR* cDNA fragment of *I. hollandica*. In addition, an anthocyanidin synthase (*ANS*) cDNA

clone (*IhANS*) was isolated from random sequencing of the cDNA library, and a chalcone isomerase (*CHI*) cDNA fragment (*pIhCHI*) was obtained from PCR using EST information of this species. The characterization of these cDNA clones was summarized in Table 2-1. From multiple alignment and molecular phylogenetic tree analyses of the deduced amino acid sequences of these cDNA clones, it was regarded that they encode each anthocyanin biosynthetic enzyme.

At different developmental stages of outer perianths in the bluish purple cultivar, 'Blue Diamond' of *I. hollandica*, expression of eight cDNA clones such as *IhCHI*, *IhF3H*, *IhDFR*, *IhANS*, *Ih3GT*, *Ih5GT* and *Ih3AT* were analyzed by relatively quantitative RT-PCR analysis, and were compared with accumulation of anthocyanins and flavones. In different flower organs of the cultivars, 'Blue Diamond' (bluish purple pistils, inner and outer perianths), 'Surprise' (white outer perianths and pistils, pale purple inner perianths) and 'White Wedgewood' (white pistils, inner and outer perianths) at anthesis, expression of these genes were also analyzed and were compared with accumulation of anthocyanins and flavones. In addition, the cause of whitening in pistils, inner and outer perianths of 'Surprise' and 'White Wedgewood' was pursued from expression analyses of the cDNA clones. The expression patterns of these genes corresponded to accumulation of anthocyanins at different developmental stages of outer perianths in 'Blue Diamond'. This suggested that anthocyanin biosynthesis in this cultivar is regulated at the transcriptional level. On the contrary, in outer perianths of 'Blue Diamond', accumulation of flavones decreased with increasing accumulation of anthocyanins. 'Blue Diamond' showed the highest accumulation of anthocyanins among the three cultivars, and its outer perianths showed the highest accumulation of anthocyanins among the flower organs. On the other hand, each anthocyanin biosynthetic gene of 'Blue Diamond', 'Surprise' and 'White Wedgewood'

was highly expressed in pistils, inner and outer perianths, although they were expressed in anthers at low level. Furthermore, the gene expression analyses showed that white pistils, inner and outer perianths of 'White Wedgewood' and white outer peianths and pistils of 'Surprise' were caused by the defect or remarkable reduction of expression of *DFR* genes.

2. Functional expression analyses of anthocyanin biosynthetic genes

Flavanone 3-hydroxylase (F3H) is categorized into nonheme-Fe(II) iron and 2-oxoglutarate-dependent dioxygenase family, which requires Fe(II), 2-oxoglutarate, molecular oxygen, and ascorbate for its oxygenation activity. In this study, three cDNA clones, *IhF3H1*, *IhF3H2* and *IhF3H3* encoding F3H were isolated, and substrate specificity of their recombinant F3Hs was examined for various flavanones. The soluble crude protein extracts of *Escherichia coli* expressing IhF3H1, IhF3H2 and IhF3H3 were subjected to flavanone 3-hydroxylation assays in the presence of naringenin as a substrate and 2-oxoglutarate, ascorbate and $FeSO_4$ as cofactors. The heterologous expression demonstrated that each *IhF3H* cDNA encodes functional F3H which catalyzed 3-hydroxylation from naringenin to dihydrokaempferol. Furthermore, substrate specificity of IhF3H1 and IhF3H2 which showed high 3-hydroxylation activity for naringenin, were also examined using various flavanones. IhF3H1 and IhF3H2 exhibited broad substrate specificity for flavanones. These enzymes showed 2, 3-dihydroflavone, sakuranetin (7-methylated naringenin) and eriodictyol to be no or very little functional as substrates. On the contrary, homoeriodictyol (3'-methylated eriodictyol) and liquiritigenin (5-deoxiflavanone) exhibited much higher activity than naringenin for 3-hydroxylation reaction of IhF3Hs. In particular, the latter was the best substrate for the activity of IhF3Hs, and the activity of IhF3Hs for this substrate

was 1.83-2.82 folds higher than that of naringenin. These results suggested that the activity of IhF3H1 and IhF3H2 is strongly dependent on the hydroxyl group of 7-position of flavanones.

In anthocyanin biosynthesis, UDP-glucose: 3GT catalyzes the transfer of the glucosyl moiety from UDP-glucose to the 3-hydroxyl group of anthocyanidins, producing the first stable anthocyanins. In this study, a cDNA clone (*Ih3GT*) encoding 3GT was isolated, and the molecular mass of recombinant Ih3GT protein from *E. coli* was estimated to be ca. 50 kDa by Western blotting. This value corresponded to calculated molecular mass of Ih3GT (49,558 Da). Furthermore, characterization of the enzymatic properties using the recombinant protein showed that *Ih3GT* cDNA encodes 3GT. Anthocyanidins such as delphinidin, malvidin, peonidin, cyanidin and pelargonidin served as substrate to different extents, and malvidin was the best glucose acceptor, while pelargonidin was the worst one. In this study, the detection of the Ih3GT activity for pelargonidin is of very interest, because to my knowledge, no pelargonidin type anthocyanins have been detected in *Iris* species.

In plants, *O*-methyltransferases (OMTs) play an important role in methylation of secondary metabolites, especially flavonoids and other phenylpropanoids, and two cDNA clones, *IhOMT1* and *IhOMT2,* encoding OMTs were successfully isolated from a cDNA library of flower buds of *I. hollandica*. *IhOMT1* encodes an open reading frame (ORF) of 365 amino acids with calculated molecular mass of 40,193 Da and isoelectric point (pI) of 5.54, while *IhOMT2,* which shares 31.5% amino acid sequence identity with IhOMT1, encodes 369 amino acids with calculated molecular mass of 40,385 Da and pI of 5.50. In addition, the molecular masses of both recombinant IhOMT1 and IhOMT2 proteins were estimated to be about 40 kDa by protein gel blot analysis. Characterization of the enzymatic properties using the recombinant IhOMT1 protein

confirmed that *IhOMT1* cDNA encodes a *S*-adenosyl-*L*-methionine (SAM)-dependent COMT, which catalyzes the transfer of the methyl moiety from SAM to caffeic acid to form ferulic acid. Its optimum activity was observed at pH 7.5 to 8.0 and at 35 °C. This is the first report on the isolation and characterization of a *COMT* cDNA clone involved in the phenylpropanoid biosynthesis of Iridaceae plants. In contrast, IhOMT2 showed no activity in SAM-dependent assays for various phenylpropanoids.

3. Cloning of gDNAs encoding dihydroflavonol 4-reductase and their 5' flanking region sequences

As described above, white inner and outer perianths of 'White Wedgewood' and white outer perianths of 'Surprise' were the result of a defect or remarkable reduction of *DFR* gene expression. To clarify this phenomenon, *DFR* gDNAs and their 5' flanking region sequence of 'Blue Diamond' (BD), 'Surprise' (Su) and 'White Wedgewood' (WW) were cloned from nested-PCR and inverse PCR using the *IhDFR* cDNA clone. As a result, genomic DNA clones such as *gDFR-BD1* (1,968 bp) and *gDFR-BD2* (1,962 bp) for 'Blue Diamond', *gDFR-WW7* (1,957 bp), *gDFR-WW13* (1,969 bp) and *gDFR-WW15* (1,966 bp) for 'White Wedgewood' and *gDFR-Su1* (1,958 bp) and *gDFR-Su2* (1,960 bp) for 'Surprise' were isolated. These clones comprised 5 exons encoding an ORF of 1,086 bp and 4 introns. In addition, as abnormal sequences due to nonsense and frameshift mutations were not found in *gDFR* clones of 'White Wedgewood' and 'Surprise' in comparison with *gDFR* clones of 'Blue Diamond', 5' flanking region sequences of *DFR* gDNAs of 3 cultivars were cloned. Based on comparison among these sequences of the cultivars, it was found that *gDFR-BD5f2～4*, *gDFR-WW5f1～4* and *gDFR-Su5f1～3* contained insertion sequences (*rTih1*) of putative *Ty1-copia* like LTR (long terminal repeat) retrotransposons at 513 bp (BD and WW) or 488 bp (Su) upstream (promoter

region) from the initiation codon. This indicated that the defect or reduction of *DFR* gene expression in white pistils, inner and outer perianths of 'White Wedgewood' and the remarkable reduction of *DFR* gene expression in white pistils and outer perianths of 'Surprise' were caused by insertions (*gDFR*-WW5f1～4, *gDFR*-Su5f1～3) of *rTih1* into promoter regions of their *DFR* genes, and that bluish purple pistils, inner and outer perianths of 'Blue Diamond' was expressed by the *DFR* gene with *gDFR*-BD5f1 which lacks *rTih1*.

Finally, on the basis of the results obtained from this study, the breeding strategies of flower colors were discussed.