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Rapid Report

Molecular and Biochemical Characterization of Three Anthocyanin Synthetic Enzymes from *Gentiana triflora*

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Full length cDNA clones of flavonoid 3',5'-hydroxylase, dihydroflavonol 4-reductase and flavonoid 3-glucosyltransferase were cloned from petals of *Gentiana triflora*. Their sequences were homologous to counterparts from other plants. Flavonoid 3',5'-hydroxylase and flavonoid 3-glucosyltransferase were enzymatically characterized by expressing cDNAs in heterologous expression systems.

Key words: Anthocyanin — Cloning — Flavonoid — Gene expression — Gentian (*Gentiana triflora*).

Anthocyanins are a class of coloured flavonoid which are the major pigments in flowers. They confer pink, red, mauve and blue to flowers and attract pollinators. The anthocyanin biosynthetic pathway has been well established and this field has been reviewed (Martin and Gerats 1992, Holton and Cornish 1995). Most of the structural genes and a few regulatory genes involved in the pathways have been cloned from several plants. This has lead to a greater understanding of the pathway and its regulation (Forkman 1993, Holton and Cornish 1995). Cloning of these genes has also attracted industrial interest because it is possible to modify flower colours with genetic engineering (Holton and Tanaka 1994, Holton and Cornish 1995).

The synthetic pathway to anthocyanidin 3-glucosides is shown in Figure 1. The first key enzyme of flavonoid biosynthesis is chalcone synthase which catalyzes the condensation reaction to yield 4,2',4',6'-tetrahydroxychalcone. The chalcone is isomerized to naringenin (5,7,4'-trihydroxyflavanone), by chalcone isomerase. Hydroxylation of naringenin at the 3 position by flavanone 3-hydroxylase leads to the formation of dihydrokaempferol (DHK). DHK is subsequently hydroxylated to dihydroquercetin (DHQ) and to dihydromyricetin (DHM) by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3',5'H), respectively (Fig. 1). These two hydroxylases belong to the Cyt P-450 superfamily. Both are key enzymes determining flower colour because they hydroxylate the B-ring of the dihydroflavonols and this hydroxylation eventually determines the structure of the anthocyanidin and so flower colour (Holton and Tanaka 1994). Dihydroflavonol 4-reductase (DFR) catalyzes the reduction of dihydroflavonols to leucoanthocyanidins. Leucoanthocyanidins are converted to anthocyanidins by anthocyanidin synthase and then to the anthocyanidin 3-glucosides by UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT). Flavones and flavonols are derived from flavanone by flavone synthase and from dihydroflavonol by flavonol synthase, respectively. Anthocyanidin 3-glucosides are further modified in species specific ways with glycosylation, methylation and acylation reactions (Heller and Forkman 1993).

Gentiana triflora mainly produces blue flowers and is one of major cut flowers in Japan. Its closely related species, *Gentiana makinoi*, also produces blue flowers which contain a novel anthocyanin, gentiodelphin (delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-caffeoyl-β-D-glucoside)-3'-(6-*O*-caffeoyl-β-D-glucoside)) (Goto et al. 1982). Yoshida et al. (1992) suggested that the two caffeic acids in gentiodelphin play a critical role in stabilization of gentiodelphin by intramolecular sandwich-type stacking of the two caffeic acids. To understand the biosynthesis of gentiodelphin in terms of molecular biology and biochemistry, we are extensively studying the pathway. We report here the molecular cloning of three genes in the pathway, F3',5'H, DFR and 3GT, and the biochemical characterization of F3',5'H and 3GT from *Gentiana triflora*. The main anthocyanin in this species is also gentiodelphin (Hosokawa et al. 1996, Y. Fukui, unpublished).

Plant materials and chemicals—Fresh and pigmented petals were collected from commercially available *Gentiana triflora* Pall. var. *japonica* Hara cut flowers at a pre-anthesis stage. Flavonoids were purchased from Extrasynthese or were from in-house stock.

Standard molecular procedures—Molecular procedures used were as described previously (Sambrook et al. 1989, Brugliera 1994) unless specified. MacVector (Kodak) was used to calculate molecular weights and isoelectric

Abbreviations: DFR, dihydroflavonol 4-reductase; DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin; 3GT, UDP-glucose:flavonoid 3-glucosyltransferase; F3'H, flavonoid 3'-hydroxylase; F3',5'H, flavonoid 3',5'-hydroxylase.

The nucleotide sequences reported in this paper have been submitted to DDBJ, EMBL and GenBank nucleotide sequence databases with accession numbers D85184, D85185 and D85186.

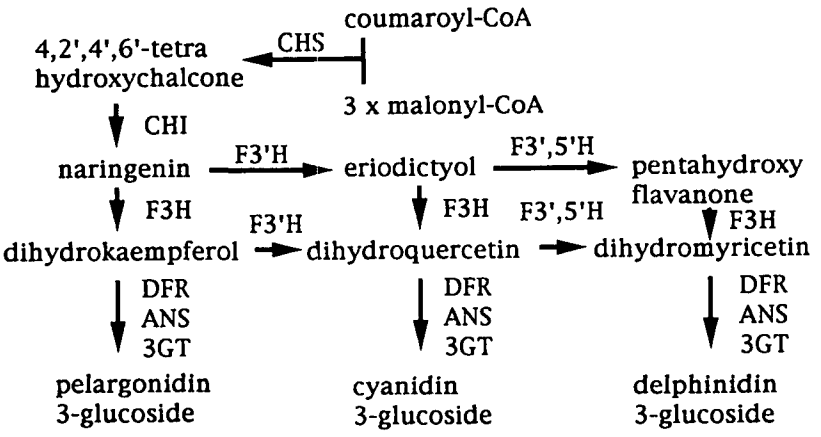

Fig. 1 Biosynthetic pathway of anthocyanin. Pentahydroxyflavanone and dihydromyricetin can be synthesized from naringenin and dihydrokaempferol, respectively, by a single enzyme, F3′,5′H. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:flavonoid 3-glucosyltransferase; F3′H, flavonoid 3′-hydroxylase; F3′,5′H, flavonoid 3′,5′-hydroxylase.

points of deduced amino acid sequences. Geneworks (IntelliGenetics) was used to compare the amino acid sequences.

Construction and screening of a cDNA library—A cDNA library was constructed with the directional ZAP-cDNA synthesis kit as described in Tanaka et al. (1995). Gentian F3′,5′H and 3GT cDNAs were obtained from the library screened with the Hf1 F3′,5′H cDNA of *Petunia hybrida* (Holton et al. 1993) and the 3GT *Antirrhinum majus* (Martin et al. 1991) as described previously (Tanaka et al. 1995). The DIG-DNA labeling and detection kit (Boeringer-Mannheim) were used following the manufacturer's instructions. A DNA fragment specific to gentian DFR cDNA was amplifiied using two oligonucleotides designed on the basis of conserved amino acid sequences of published DFRs as described by Helariutta et al. (1993). The obtained DNA fragment was used as a probe for library screening as described above.

Expression and enzymatic assay of F3′,5′H—The yeast expression system and related procedures are described by Ashikari et al. (1989). The vector containing gentian F3′,5′H cDNA was constructed by inserting the *Eco*RI and *Xho*I digested cDNA into pYE2211 digested with *Eco*RI and *Sal*I. The cDNA insert is regulated by a constitutive promoter, glyceraldehyde-3-phosphate dehydrogenase in this plasmid. A yeast transformant expressing gentian F3′,5′H was cultured in 10 ml of modified Burkholder's medium (Tanaka et al. 1977) containing 1% casamino acid (Difco) in a 50 ml flask at 30°C for 36 h with vigorous shaking. The cells were harvested by centrifugation and washed twice with 100 mM potassium phosphate, pH 8.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A). The washed cells were suspended in 500 μl of buffer A containing 10 μM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride in an Eppendorf tube and then destroyed with a mini-beadsbeader (BiospecProducts) for 2 min after adding 200 mg of glass beads (Sigma). The crude extract was recovered after centrifugation (11,000 × *g*, 10 min, 4°C) and subjected to enzymatic assay. The assay mixture contained 100 μl of the crude extract, 100 μl buffer A, 5 μl 50 mM NADPH and 5 μl substrates (2 mg ml^{-1}) dissolved in ethanol. The mixtures were extracted twice with 200 μl ethyl acetate after a 16-h reaction at 22°C. The recovered ethyl acetate was dried and the resultant pellet was dissolved in 50% acetonitrile containing 0.1% (v/v) TFA for HPLC analysis.

Expression and enzymatic assay of 3GT—The p*Trc*99a expression vector (Pharmacia) was used to express the 3GT cDNA. An oligonucleotide, AAACCATGGCACCCGTATCC, was synthesized to make an *Nco*I site containing the initiation codon of the cDNA, in order to subclone the cDNA to the vector using *Nco*I and *Xho*I sites. The cDNA insert is regulated under the inducible *trc* promoter in the plasmid. The transformant expressing the 3GT protein was cultured in 50 ml of 2 × YT media following the manufacturer's instruction. The harvested cells were washed with 30 mM Tris-HCl and 30 mM NaCl at pH 7.5 and suspended in 5 ml of the same buffer containing 4 mg of lysozyme. The suspension was frozen and thawed once, and sonicated. The crude enzyme extract was obtained after centrifugation (18,000 × *g*, 10 min, 4°C). The reaction mixture for the 3GT consisted of 20 μl of the crude extract, 10 μl 1 M Tris-HCl, pH 7.5, 10 μl 5 mM UDP-Glucose and 10 μl substrate (1.5 mg ml^{-1}) dissolved in 2-methoxyethanol. The mixture was kept at 25°C for 5 min and the reaction stopped by adding 50 μl of 13.8% (v/v) acetic acid in acetonitrile. After centrifugation at 11,000 × *g* for 10 min, the supernatant of the reaction was subjected to HPLC analysis.

HPLC analysis—HPLC analysis for F3′,5′H was carried out on a YMC ODS-A312 column (6 mm × 150 mm) with gradient elution, using a gradient of 18% acetonitrile and 0.1% TFA to 72% acetonitrile and 0.1% TFA over 10 min, followed by 72% acetonitrile and 0.1% TFA. The flow rate was 1 ml min^{-1} and the column temperature was 40°C. The detection of flavonoid compounds was carried out using a Gilson Model 116 at 290 nm and a Shimazu photodiode array detector SPD-M6A from 250 nm to 400 nm. HPLC analysis for 3GT was carried out with the same column with gradient elution, using a gradient condition of 4.5% acetonitrile and 0.1% TFA to 72% acetonitrile and 0.1% TFA over 6 min following 4 min of 4.5% acetonitrile and 0.1% TFA. The flow rate was 1 ml min^{-1} and the column temperature was 40°C. Compounds were identified by comparison to the retention times and spectral patterns (250 nm to 600 nm) of known standards.

Isolation of cDNA for F3′,5′H—Scores of positive plaques were identified after screening approximately 300,000 clones. Twelve plaques were randomly chosen, isolated and sequenced. All of them showed good homology to the petunia Hf1 cDNA. The longest clone was sequenced and expressed in yeast. The deduced protein consists of 516 amino acid residues and the calculated molecular weight and isoelectric point were 58,084 and 9.38, respectively. The amino acid sequence is shown in Figure 2. Comparison of the deduced amino acid sequence of gentian F3′,5′H to other reported F3′,5′Hs shows a high degree of identity (Fig. 2). Identities to *Eustoma russellianum* (lisianthus) (Kikuchi et al. 1993), petunia *Hf1*, petunia *Hf2* (Holton et al. 1993), *Solanum melongena* (egg plant) (Toguri et al. 1993), and *Campanula medium* (Kikuchi et al. 1993) F3′,5′Hs were 77%, 74%, 73%, 68% and 62%, respectively. *Gentiana* and *Eustoma* belong to the same family, Gentianaceae, and the identity of the two F3′,5′Hs is higher. *Campanula* F3′,5′H has some insertions in its amino acid sequence and so is less similar. The identities to other plant Cyts P-450 were less than 40%. These results supported the idea that the F3′,5′Hs form a family (CYP75) in the Cyt P-450 superfamily (Toguri et al. 1993).

Biochemical characterization of recombinant gentian F3′,5′H—Recombinant gentian F3′,5′H was produced in the yeast expression system. The capacity to act on flavonoids is summarized in Table 1. The enzyme catalyzed hydroxylation of naringenin to eriodictyol (5,7,3′,4′-tetrahydroxyflavanone), eriodictyol to 5,7,3′,4′,5′-pentahydroxyflavanone, DHK to DHQ, DHQ to DHM and apigenin to luteolin. These results confirm that the cloned cDNA encodes a F3′,5′H with a broad substrate specificity. *Verbena hybrida* and petunia F3′,5′Hs (Stotz and Foreman 1982 and Holton et al. 1993, respectively) were reported to hydroxylate flavanones and dihydroflavonols. Interestingly petunia *Hf2* cDNA expressed in the same yeast system did not hydroxylate apigenin (Y. Tanaka, unpublished). Gentian F3′,5′H may therefore have broader substrate specificity than other F3′,5′Hs. F3′H, which is another P-450 enzyme hydroxylating the B-ring, has even broader substrate specificity. *Z. mays* F3′H can hydroxylate a flavonol as well as a flavanone, a dihydroflavonol and a flavone (Larson and Bussard 1986).

Isolation of cDNA for DFR—Many positive plaques were identified from the library of approximately 300,000 clones. Twelve dozen plaques were chosen, isolated and sequenced. All of them showed good homology to the reported DFR cDNAs. The longest clone was sequenced completely. The deduced protein has 359 amino acid residues and calculated molecular weight and the isoelectric point was 40,138 and 4.97, respectively. The amino acid sequence is shown in Figure 3. Comparison of the deduced amino acid sequences of reported DFR showed that the sequences were well conserved except at the beginning and end of the sequences.

Gentian DFR is more closely related to DFRs from dicots than to those from monocots. Comparison of

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                                                  ***** ***  *** *** ****  * ** ***  **** *  *   **
(1)MSPIYTTLTLHLATALFLFFHVQKLVHYLHGKATGHRCRRLPPGPTGWPILGALPLLGNMPHVTFANMAKKYGSVMYLKVGSHGLAIAST

   * ** ***** * *******  * *  ** * ***** *** *******   * ** ***  *  *   * * **  *
(91)PDAAKAFLKTLDLNFSNRPPNAGATHLAYNAQDMVFAHYGPKWKLLRKLSNLHMLGGKALENWADVRKTELGYMLKAMFESSQNNEPVMI

    *** *****     ** * *        **  ** ***  ** ********   **** **   **   **** **      *
(181)SEMLTYAMANMLSQVILSRRVFNKKGAKSNEFKDMVVELMTSAGYFNIGDFIPSIGWMDLQGIEGGMKRLHKKFDVLLTRLLDDHKRTSQ

    **  *****          *  *   * *****  ********** ***** *          *  ***  **  **    **  ***
(271)ERKQKPDFLDFVIANGDNSDGERLNTDNIKALLLNLFTAGTDTSSSIIEWALAELLKNRTLLTRAQDEMDRVIGRDRRLLESDIPNLPYL

    ***** **************          *  *** *** ********** **        * ***     **  *** **********
(361)QAICKETFRKHPSTPLNLPRNCIRGHVDVNGYYIPKGTRLNVNIWAIGRDPSVWGDNPNEFDPERFLYGRNAKIDPRGNHFELIPFGAGR

    ***** * *   ******** *** * *       ** * ** **** *** * * ***    *
(451)RICAGTRMGILLVEYILGTLVHSFDWKLGFSEDELNMDETFGLALQKAVPLAAMVIPRLPLHVYAP
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Fig. 2 The deduced amino acid sequence of F3′,5′H from *Gentiana triflora*. Amino acid residues conserved in reported F3′,5′Hs are asterisked. See text for details.

Table 1 Hydroxylation of flavonoids by gentian F3′,5′H expressed in yeast

Substrate	Product	Amount of product (pmol (mg protein)$^{-1}$ h^{-1})
Flavanone		
Naringenin	Eriodictyol	18
Eriodictyol	Pentahydroxyflavanone	7.8
Dihydroflavonol		
Dihydrokaempferol	Dihydroquercetin	10
Dihydroquercetin	Dihydromyricetin	27
Flavone		
Apigenin	Luteolin	9.0
Flavonol		
Kaempferol	No products were detected	

The reaction mixture contained 1.6 mg of crude protein extracted from yeast expressing gentian F3′,5′H. Each product was identified from its retention time and absorption spectrum and quantified by HPLC analysis. The host yeast did not have any capacity to act on these substrates.

gentian DFR amino acid sequence to *Gerbera hybrida* (Helariutta et al. 1993), *Petunia hybrida* (Beld et al. 1989, Huit et al. 1994), *Callistephus chinensis* (Chinese aster) (Min et al. 1995 the accession number: Z67981), *Vitis vinifera* (grape) (Sparvoli et al. 1994), *Lycopersicon esculentum* (tomato) (Bongue-Bartelsman et al. 1994), *Rosa hybrida* (Tanaka et al. 1995), *Arabidopsis thaliana* (Shirley et al. 1992), *Dianthus caryophyllus* (carnation) (Min et al. 1995 Z67983), *Antirrhinum majus* (Beld et al. 1989), *Zea mays* (Schwartz-Sommer et al. 1987) and *Hordeum vulgare* (Barley) (Kristiansen and Rohde 1991) gave identities of 71%, 70%, 69%, 66%, 66%, 64%, 61%, 61%, 58%, 57% and 57%, respectively. The reason for the low identity to *Antirrhinum* DFR relative to other dicot DFRs is that *Antirrhinum* DFR has an extra sequences in its C-terminus.

DFR is proposed to be part of a superfamily also containing mammalian 3β-hydroxysterol dehydrogenase and other proteins. Some strictly conserved amino acid residues are observed in the family (Baker and Blasco 1992). These residues are also conserved in gentian DFR as shown in Figure 3 and are likely to be essential for function of these proteins.

The anthocyanins in gentian flowers are derivatives of cyanidin and delphinidin (Hosokawa et al. 1995, 1996, Y. Fukui, unpublished). Pelargonidin derivatives have not been reported so far. DFR plays an important role in determination of flower colour. *Petunia hybrida* does not produce significant amount of pelargonidin because its DFR can barely reduce DHK (Forkman and Ruhnau 1987). Similarly, gentian DFR may also not be able to reduce DHK and this could be a reason why no pelargonidin has been isolated from gentian flowers. Biochemical characterization of gentian DFR is in progress in our laboratory.

Isolation of cDNA for 3GT—Many positive plaques were identified after the screening. Twelve plaques were randomly chosen, isolated and sequenced. Many of them showed good homology to *Antirrhinum* 3GT (Martin et al. 1991) and the longest clone was sequenced. The deduced protein has 453 amino acid residues and calculated molecular weight and isoelectric point of 50,005 and 5.18, respectively. The amino acid sequence is shown in Figure 4.

Comparison of the deduced amino acid sequence of gentian 3GT to other 3GTs revealed a high degree of identi-

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                *  *  *         *                                                         *  *
(1)MEGGILSNATTVCVTGASGYIGSWLAMRLLERGYTVRATVRDPGNLKKVQHLLELPKASTNLTLLKADLTEEGSFDEAIHGCHGVFHVAT

                       *
(91)PMDFESKDPKNEVIKPTIDGFLSIIRSCVKAKTVKKLVFTSSAGTVDVQEQQKPVYDENDWSDLDFINSTKMTGWMYFVSKILAEKAAWE

                                                                             *
(181)VTKANDIGFISIIPTLVVGPFITTTFPPSLITALSLITGNEAHYGIIKQGQFVHLDDLCEAHIFXYEHPEAEGRYICSSHDTTIHDLAKM

(271)IRQNWPEYYIPTKLKGIDEDIPVVSFSSNKLIDLGFQYKYTLEDMFRGAIDTCKEKRMLPLSIGHQKESTDPEVDEVVKEMELIQDSLD
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Fig. 3 The deduced amino acid sequence of DFR of *Gentiana triflora*. Amino acid residues conserved in the 3β-hydroxysteroid dehydrogenase/DFR superfamily (Baker and Blasco 1992) are asterisked.

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(1)MSPVSHVAVLAFPFGTHAAPLLTLVNRLAASAPDIIFSFFSTSSSITTIFSPTNLISIGSNIKPYAVWDGSPEGFVFSGNPREPIEYFLN

(91)AAPDNFDKAMKKAVEDTGVNISCLLTDAFLWFAADFSEKIGVPWIPVWTAASCSLCLHVYTDEIRSRFAEFDIAEKAEKTIDFIPGLSAI

(181)SFSDLPEELIMEDSQSIFALTLHNMGLKLHKATAVAVNSFEEIDPIITNHLRSTNQLNILNIGPLQTLSSSIPPEDNECLKWLQTQKESS

                                                    * *   *    *          *
(271)VVYLSFGTVINPPPNEMAALASTLESRKIPFLWSLRDEARKHLPENFIDRTSTFGKIVSWAPQLHVLENPAIGVFVTHCGWNSTLESIFC

              ***   *
(361)RVPVIGRPFFGDQKVNARMVEDVWKIGVGVKGGVFTEDETTRVLELVLFSDKGKEMRQNVGRLKEKAKDAVKANGSSTRNFESLLAAFNK

(451)LDS
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Fig. 4 The deduced amino acid sequence of 3GT of *Gentiana triflora*. Amino acid residues conserved in glycosyltransferases (Brugliera et al. 1994) are asterisked. See text for details.

ty. Identities to *A. majus* (Martin et al. 1991), *Solanum melongena* (Toguri et al. X77369), *Z. mays* (Furtek et al. 1988, Ralston et al. 1988) and *H. vulgare* (Wise et al. 1990) were 52%, 46%, 32% and 31%, respectively. Gentian 3GT is clearly more homologous to dicot 3GTs than to monocot ones. The gentian 3GT amino acid sequence is similar to *Manihot esculentla* (cassava) glucosyl transferase clones (Hughes and Hughes 1994). A cassava clone (pCGT7) was not full length but has the identity is 49% in the sequenced region. Gentian 3GT has a lower degree of identity to glucosyltransferases in the database and even to flavonoid 3-rhamnosyltransferase (13%) (Brugliera et al. 1994). It has been suggested that some amino acid residues are conserved in a wide range of glycosyltranserases (Brugliera et al. 1994), and all but one of such residues are also conserved in gentian 3GT (Fig. 4).

Table 2 Glucosylation of flavonoids by gentian 3GT expressed in *E. coli*

Substrate	Amount of product (nmol (mg protein)$^{-1}$ min^{-1})
Anthocyanidin	
Pelargonidin	22
Cyanidin	35
Delphinidin	57
Flavonol	
Kaempferol	2.5
Quercetin	3.7
Myricetin	4.0
Dihydroflavonol	
Dihydrokaempferol	0
Dihydroquercetin	0
Flavone	
Apigenin	0
Anthocyanin	
Delphinidin-3-glucoside	0

The reaction mixture contained 120 μg of crude protein extracted from *E. coli* expressing gentian 3GT. Each product was identified from its retention time and absorption spectrum and quantified by the HPLC analysis. The host did not convert any of these substrates.

Biochemical characterization of recombinant gentian 3GT—Gentian 3GT was expressed in *E. coli*, and its substrate specificity determined using various flavonoids, as summarized in Table 2. Gentian 3GT converts anthocyanidins much more efficiently than flavonols. It also tends to glucosylate more efficiently anthocyanidins and flavonols with more hydroxyl groups. It does not modify dihydroflavonols at all. Flavone and delphinidin 3-glucoside that did not have 3-hydroxyl group were not modified either. Gentian 3GT is suggested to be a UDP glucose: anthocyanidin 3-*O*-glucosyltransferase because the 3GT prefers anthocyanidins to flavonols and gentian flowers does not contain significant amount of flavonols if any (Y. Fukui, unpublished). Although 3GTs have been generally reported to have broader substrate specificity and can glucosylate anthocyanidins, dihydroflavonols and flavonols (reviewed by Heller and Forman 1993), each 3GT has its preferable substrates; grape contains a 3GT which catalyzes anthocyanidins but not flavonols (Do et al. 1995) and red cabbage seedlings contain a 3GT which modifies flavonols more efficiently than anthocyanidin (Sun and Hrazdina 1991).

All of the structural and functional results obtained here are similar to the results obtained for the same genes isolated from other species in the anthocyanin pathway. This suggests that the biosynthesis pathway to anthocyanidin 3-glucosides in *Gentian triflora* is essentially the same as the pathway shown in Figure 1. The successful expression of genes involved in the anthocyanin pathway in heterologous systems would provide a good tool to characterize the enzymes in the pathway.

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References

Ashikari, T., Kiuchi, N., Tanaka, Y., Shibano, Y., Amachi, T. and Yoshizumu, H. (1989) Direct fermentation of raw corn to ethanol by yeast transformants containing a modified *Rhizopus* glucoamylase gene. *Appl. Microbiol. Biotechnol.* 32: 129–133.

Baker, M.E. and Blasco, R.E. (1992) Expansion of the mammalian 3β-hydroxysteroid dehydrogenase/plant dihydroflavonol reductase superfamily to include a bacterial cholesterol dehydrogenase, a bacterial UDP-galactose-4-epimerase, and open reading frames in vaccinia virus and fish lymphocytes disease virus. *FEBS Lett.* 301: 89–93.

Beld, M., Martin, C., Huits, H., Stuitje, R. and Gerats, A.G.M. (1989) Flavonoid synthesis in *Petunia hybrida: partial* characterization of dihydroflavonol 4-reductase genes. *Plant Mol. Biol.* 13: 491–502.

Bongue-Bartelman, M., O'Neill, S.D., Tong, Y. and Yoder, J.I. (1994) Characterization of the dihydroflavonol-reductase gene in tomato. *Gene* 138: 153–157.

Brugliera, F., Holton, T.A., Stevenson, T.W., Farcy, E., Lu, C.Y. and Cornish, E.C. (1994) Isolation and characterization of a cDNA clone corresponding to the *Rt* locus of *Petunia hybrida*. *Plant J.* 5: 81–92.

Do, C.B., Cormier, F. and Nicolas, Y. (1995) Isolation and characterization of a UDP-glucose:cyanidin 3-glucosyltransferase from grape cell suspension cultures (*Vitis vinifera* L.). *Plant Sci.* 112: 43–51.

Forkman, G. (1993) Genetics of flavonoids. *In* The Flavonoids: Advances in Research since 1986, Edited by Harborne, J.B. pp. 538–564. Chapman and Hall, London.

Forkman, G. and Ruhnau, B. (1987) Distinct substrate specificity of dihydroflavonol 4-reductase from flowers of *Petunia hybrida*. *Z. Naturforsch.* 42c: 1146–1148.

Furtek, D., Schiefelbein, J.W., Johnston, F. and Nelson, O.E., Jr. (1988) Sequence comparisons of three wild-type Bronze-1 alleles from *Zea mays*. *Plant Mol. Biol.* 11: 473–481.

Goto, T., Kondo, T., Tamura, H., Imagawa, H., Iino, A. and Takeda, K. (1982) Structure of gentiodelphin, an acylated anthocyanin isolated from *Gentiana makinoi*, that is stable in dilute aqueous solution. *Tetrahedr. Lett.* 23: 3695–3698.

Helariutta, Y., Elomaa, P., Kotilainen, M., Seppanen, P. and Teeri, T.H. (1993) Cloning of a cDNA for dihydroflavonol 4-reductase (DFR) and characterization of dfr expression in the corollas of *Gerbera hybrida* var. Regina (Compositae). *Plant Mol. Biol.* 22: 183–193.

Heller, W. and Forkman, G. (1993) Biosynthesis of flavonoids. *In* The Flavonoids: Advances in Research since 1986. Edited by Harborne, J.B. pp. 499–535. Chapman and Hall, London.

Holton, T.A., Brugliera, F., Lester, D.R., Tanaka, Y., Hyland, C.D., Menting, J.G.T., Lu, C.-Y., Farcy, E., Stevenson, T.W. and Cornish, E.C. (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature* 366: 276–279.

Holton, T.A. and Cornish, E.C. (1995) Genetics and Biochemistry of Anthocyanin Biosynthesis. *Plant Cell* 7: 1071–1083.

Holton, T.A. and Tanaka, Y. (1994) Blue roses—A pigment of our imagination? *Trends Biotechnol.* 12: 40–42.

Hosokawa, K., Fukushi, E., Kawabata, J., Fujii, C., Ito, T. and Yamamura, S. (1995) Three acylated cyanidin glucosides in pink flowers of gentiana. *Phytochemistry* 40: 941–944.

Hosokawa, K., Fukushi, E., Kawabata, J., Fuji, C., Ito, T. and Yamamura, S. (1996) Studies on flower pigments in *Gentiana* sp. III. Identification of anthocyanins in blue cultivar Albireo. *Ikushugakkaishi* 45 supp.1: 196.

Huits, H.S.M., Gerats, A.G.M., Kreike, M.M., Mol, J.N.M. and Koes, R.E. (1994) Genetic control of dihydroflavonol 4-reductase gene expression in *Petunia hybrida*. *Plant J.* 6: 295–310.

Hughes, J. and Hughes, M.A. (1994) Multiple secondary plant product UDP-glucose glucosyltransferase genes expressed in cassava (*Manihot esculenta* Crantz) cotyledons. *DNA Seq.* 5: 41–49.

Kikuchi, Y., Kiyokawa, S., Shimada, Y., Shimada, R., Ohbayashi, M. and Okinaka, Y. (1993) Novel plant gene. International patent publication number WO 93/18155.

Kristiansen, K.N. and Rohde, W. (1991) Structure of *Hordeum vulgare* gene encoding dihydroflavonol 4-reductase and molecular analysis of ant18 mutants blocked in flavonoid synthesis. *Mol. Gen. Genet.* 230: 49–59.

Larson, R. and Bussard, J.B. (1986) Microsomal flavonoid 3'-monooxygenase from maize seedlings. *Plant Physiol.* 80: 483–486.

Martin, C. and Gerats, T. (1992) The control of flower coloration. *In* Molecular Biology of Flowering, Edited by Jordan, B. pp. 219–255. CIB international, Wallingford.

Martin, C., Prescott, A., Machay, S., Bartlett, J. and Vrijklandt, E. (1991) The control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. *Plant J.* 1: 37–49.

Ralston, E.J., English, J.J. and Dooner, H.K. (1988) Sequence of three bronze alleles of maize and correlation with the genetic fine structure. *Genetics* 119: 185–197.

Sambrook, J., Fritsch, E.F. and Maniatis, T.(1989) Molecular Cloning: a Laboratory Manual. 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY.

Schwarz-Sommer, Z., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclerq, L., Mattes, M., Berndtgen, R., Peterson, P.A., Saedler, H. (1987) Influence of transportable elements on the structure and function of the A1 gene of *Zea mays*. *EMBO J.* 6: 287–294.

Shirley, B., Hanley, S. and Goodman, H.M. (1992) Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis *transparent testa* mutations. *Plant Cell* 4: 333–347.

Sparvoli, F., Martin, C., Scienza, A., Gavazzi, G. and Tonelli, C. (1994) Cloning and molecular analysis of structural genes involved in flavonoid and stibene biosynthesis in grape (*Vitis vinifera* L.). *Plant Mol. Biol.* 24: 743–755.

Stotz, G. and Forkmann, G. (1982) Hydroxylation of the B-ring of flavonoids in the 3'- and 5'-position with enzyme extracts from flowers of *Verbena hybrida*. *Z. Naturforsch.* 37c: 19–23.

Sun, Y. and Hrazdina, G. (1991) Isolation and characterization of a UDPGlucose: Flavonol *O*-3-Glucosyltransferase from illuminated red cabbage (*Brassica oleracea* cv. Red Danish) seedlings. *Plant Physiol.* 95: 570–576.

Tanaka, Y., Fukui, Y., Fukuchi-Mizutani, M., Holton, T.A., Higgins, E. and Kusumi, T. (1995) Molecular cloning and characterization of *Rosa hybrida* dihydroflavonol 4-reductase gene. *Plant Cell Physiol.* 36: 1023–1031.

Tanaka, T., Kita, H., Murakami, T. and Narita, K. (1977) Purification and amino acid sequence of mating factor from *saccharomyces cerevisiae*. *J. Biochem.* 82: 1681–1687.

Toguri, T., Umemoto, N., Kobayashi, O. and Ohtani, T. (1993) Activation of anthocyanin synthesis genes by white light in eggplant hypocotyl tissues, and identification of an inducible P-450 cDNA. *Plant Mol. Biol.* 23: 933–946.

Wise, R.P., Rohde, W. and Salamini, F. (1990) Nucleotide sequence of the Bronze-1 homologous gene from *Hordeum vulgare*. *Plant Mol. Biol.* 14: 277–279.

Yoshida, K., Kondo, T. and Goto, T. (1992) Intramolecular stacking conformation of gentiodelphin, a diacylated anthocyanin from *Gentiana makinoi*. *Tetrahedron* 48: 4313–4326.

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