Plant Biotechnology 21(5), 367–375 (2004)

Original Paper

Cloning and heterologous expression of cDNAs encoding flavonoid glucosyltransferases from *Dianthus caryophyllus*

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Received September 13, 2004; accepted October 5, 2004 (Edited by K. Yazaki)

Abstract Yellow petals of carnations contain chalcone 2′-*O*-glucoside. The glucosylation occurs after the *p*-coumaroyl CoA and malonyl-CoA condensation reaction by chalcone synthase (CHS), but the enzyme(s) transferring glucose from UDP-glucose to the 2′-OH position of chalcone has not been identified. The full-length cDNA clones for 18 *glucosyltransferase* (*GT*) genes were isolated from petal tissue of carnation (*Dianthus caryophyllus*) bearing various flower colors. The 18 GTs encoded in the cDNAs were enzymatically characterized in an *E. coli* expression system using chalcone, flavanone, flavone, flavonol and anthocyanidin as substrates. Three of the 18 were characterized as 3-GT possessing different substrate specificities for flavonoids and anthocyanidin and another two GTs catalyzed the transfer of glucose to the 2′-hydroxyl group of chalcone. In addition, these two enzymes glucosylated flavonol (3-OH and 7-OH), flavanone (7-OH), flavone (7-OH) and anthocyanidin (3-OH and 7-OH).

Key words: Carnation (*Dianthus caryophyllus*), chalcone 2′-*O*-glucoside, chalcone 2′-*O*-glucosyltransferase, flavonoid glucosyltransferase, heterologous expression, yellow flower color.

The flavonoid biosynthetic pathway in higher plants produces a wide array of compounds with biological significance. Glycosylation of flavonoids makes the molecules more stable and increases solubility in water for compartmentalization in the vacuole. Further, plant glucosyltransferases (GTs) are active in hormonal homeostasis and detoxification of xenobiotics (Lim et al. 2002). The small genome of *Arabidopsis thaliana* has been shown to contain about 120 individual GT sequences, of which only very few have been functionally characterized (Jackson et al. 2001; Lim et al. 2001).

Flavonoid GTs, which catalyze the transfer of the glucosyl moiety of UDP-glucose (UDP-Glc) to the hydroxyl group of flavonoid molecules, have been described in many plant species. The *Anthocyanindin 3-GT* gene was first isolated from *Zea mays* by transposon tagging with *Ac* (Fedroff et al. 1984) and subsequently from other species (Tanaka et al. 1996; Ford et al. 1998). Other flavonoid *GTs* have since been isolated from several plant species (Mato et al. 1998; Hirotani et al. 2000; Fukuchi-Mizutani et al. 2003).

The cyanic color of most flowers comes from anthocyanins, whereas betacyanins produce this color in the flowers of Caryophylladae excluding the genus *Dianthus* (Brouillard and Dangeles 1993). Betacyanins are red nitrogenous compounds derived from tyrosine and are distributed only in order Caryophyllales (Stafford 1994). Recently, Vogt et al. (Vogt et al. 1997; Vogt et al. 1999; Vogt 2002) indicated that betacyanin GT catalyzed the formation of anthocyanin from anthocyanidin in cultured cells of *Dorotheanthus bellidiformis*.

Cyclic malyl cyanidin or pelargonidin 3-*O*-glucoside-5-*O*-glucoside has been identified in the petals of carnation (*Dianthus caryophyllus*) (Bloor 1997; Nakayama et al. 2000). Moreover, the yellow flower color of carnations was found to be due to chalcone 2′-*O*-glucoside as a main flavonoid in vacuole (Forkmann

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Abbreviations: ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-β-hydroxylase; GT, glucosyltransferase; chalcone, chalcononaringenin (4,2′,4′,6′-tetrahydroxychalcone); HPLC, high performance liquid chromatography; K-Pi, potassium phosphate; PAL, phenylalanine ammonia-lyase; TLC, thin layer chromatography; UDP-Glc, Uridine 5′-diophosphoglucose.

and Dangelmayr 1980; Yoshida et al. 2004). This indicates that chalcone, which was synthesized from the condensation of *p*-coumaroyl-CoA and malonyl-CoAs by chalcone synthase (CHS), was converted to chalcone 2′-*O*-glucoside by UDP-Glc: chalcone glucosyltransferase (chalcone 2′-GT) (Figure 1). Chalcone 2′-*O*-glucoside can then be transported and accumulated into vacuoles.

In carnation, the flavonoid and anthocyanin biosynthetic pathway was genetically analyzed over a half century ago (Mehlquist 1940). Since then, biochemical and molecular biological techniques have allowed the identification of pathway enzymes and their corresponding genetic loci (Forkmann and Dangelmayr 1980; Itoh et al. 2002). Currently, most of the genes encoded the enzymes involved in flavonoid and anthocyanin synthesis have been characterized in other plant species. However, the biochemistry and genetics of chalcone 2′-*O*-glucoside synthesis are as yet unresolved.

In this work we used heterologous expression of cDNA encoding GT homologues from *D. caryophyllus* flower petals to identify chalcone 2′-GT clones. Substrate specificities of the recombinant GT enzymes *in vitro* and the expression of chalcone 2′-GT transcripts in flower petals bearing several colors were studied.

Materials and methods

Chemicals and plant materials

Kaempferol (3,5,7,4′-tetrahydroxyflavone), kaempferol 7-*O*-glucoside, chalcone (4,2′,4′,6′-tetrahydroxychalcone) 2′-*O*-glucoside and chalcone 2′,4′-*O*-diglucoside were generous gifts from. M. Yamaguchi and T. Iwashina. Apigenin (5,7,4′-trihydroxyflavone) 7-*O*-glucoside, naringenin (5,7,4′-trihydroxyflavanone) 7-*O*-glucoside and kaempferol 3-*O*-glucoside were purchased from Funakosi Co., Ltd. (Tokyo, Japan), and UDP-D-[U-^{14}C] glucose was purchased from PerkinElmer, Inc. (NEN Life Science Products, Inc., Boston, USA). Other non-radioisotope glycosyl donor reagents (UDP-Glc, ADP-glucose, TDP-glucose and UDP-galactose), naringenin and apigenin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Quercetin (3,5,7,3′,4′-pentahydroxyflavone), quercetin 3-*O*-glucoside, quercetin 7-*O*-glucoside, pelargonidin (3,5,7,4′-tetrahydroxyflavilium), pelargonidin 3-*O*-glucoside, cyanidin (3,5,7,3′,4′-pentahydroxyflavilium), cyanidin 3-*O*-glucoside and cyanidin 3,5-*O*-diglucoside were extracted and purified in our laboratory. Betanin was a gift from San-Ei Gen F.F.I., Inc. (Toyonaka, Japan). Chalcone was synthesized from naringenin according to the method of Moustafa and Wong (1967). Betanidin was isolated from betanin (betanidin 5-*O*-glucoside), after treatment with β-glucosidase (Almond) according to the method of Heurer et al. (1996).

Carnation (*D. caryophyllus*) cultivars ‘Symphony Rose’ bearing red flowers, ‘A66’ bearing yellow flowers and other cultivars bearing petals of several colors were prepared from inbred lines collected by Japan Tobacco Inc. The petals of four developmental stages (see Figure 3), leaves, stems and roots were separated, weighed, frozen immediately in liquid nitrogen and stored at −80°C.

Preparation of poly (A)$^+$ RNAs and cloning of GT

Total RNA was prepared using a modified guanidinium thiocyanate-CsCl ultracentrifugation method (Chirgwin et al. 1979). Poly (A)$^+$RNA was prepared from total RNA using Oligotex-dT30 〈Super〉 (TaKaRa Bio Inc., Ohtsu, Japan) according to the supplier’s manual. First strand cDNA was synthesized using poly(A)$^+$ RNA extracted from red or yellow petals of closed flower buds (see Figure 3, stage 2) of cultivars ‘Symphony Rose’ and ‘A66’, respectively, using First Strand cDNA Synthesis Kit (TaKaRa). Synthesis reactions were diluted with 40 μl H_2O and used as templates for PCR. Reverse primers A (5′-TGCWTWWTWASTGATGCWTTYTT-RTGGT-3′) and B (5′-GTBRTWNTSAAYWSYTTYK-AKGARYTKGA-3′), and forward primers C (5′-TN-GARTTCCANCCRCARTGHGTNACRAA-3′) and D (5′-TCCAYCCRCARTGHGWMACRAAACAWCC-3′), were designed based on conserved amino acid sequences of GT enzymes previously reported, they were *Arabidopsis thaliana* putative anthocyanin 5GT (AC005106), *Dorotheanthus bellidiformis* betanidin 5GT (Y18871), *Lycopersicon esculentum* GT (twi1, X85138), *Nicotiana tabacum* phenylpropanoid GT (togt1, AF346431), *Perilla frutescens* flavonoid 3GT and anthocyanin 5GT (AB002818 and AB013596), *Petunia hybrida* anthocyanidin 3GT and anthocyanin 5GT (AB027454 and AB027455), *Scutellaria baicalensis* flavonoid 7GT (AB031274), *Solanum melongena* flavonoid 3GT (X77369), and *Verbena hybrida* anthocyanin 5GT (AB013598). The LA-PCR mixture (10 μl) was prepared according to the supplier’s recommendations containing 1 μl of the diluted carnation cDNA and 4 pmol of degenerate primers of combination of A or B and C or D. The mixture was incubated for 1 min at 94°C, and 0.5 unit of TaKaRa LA-*Taq* DNA polymerase was added. PCR was carried out for 35 amplification cycles (30 s at 92°C; 45 s at 55°C; 1 min at 72°C). The amplified cDNA fragments were cloned into the pBluscript SK plasmid and sequenced using a Thermo Sequenase Cycle Sequencing Kit (USB Co., Cleveland, USA) with DNA Sequencer model 4000L (Aloka Co., Ltd, Tokyo, Japan). Specific primers corresponding to the nucleotide sequences of the individual cDNA fragments of *GT* candidates isolated above were synthesized and used to isolate the 5′ and 3′ regions of *GT* cDNAs by GeneRacer Kit (Invitrogen Co., Groningen, The Netherlands). Finally the full-length carnation *GT* cDNAs were

amplified by PCR using a 5′ primer corresponding to the nucleotide sequence containing ATG for the putative 1st methionine and a 3′ primer containing the stop codon using cDNAs as templates.

Northern analysis

Poly(A)$^+$ RNA from the petals of carnation cultivar 'Symphony Rose' were separated by electrophoresis in a 1.5% formaldehyde-denaturing agarose gel, blotted and fixed on nylon membranes then hybridized with the ^{32}P-labeled cDNA of *DicGT1* at 65°C overnight. The membranes were washed twice with 2×SSC, 0.5% SDS solution at room temperature for 15 min, then twice with 0.1×SSC, 0.1% SDS at room temperature for 10 min, and finally twice with 0.1×SSC, 0.1% SDS at 65°C for 30 min. The filters were then exposed to X-ray film at −80°C.

RT-PCR analyses

Total RNA used for the RT-PCR analysis was prepared according to the same method described above. The primer pairs for amplification of cDNA fragments for semi-quantitative PCR were as follows; phenylalanine ammonia-lyase (*PAL*) (Yoshimoto et al. 2000), 5′-CTTCTACAAGGGTACTCCGGTATTCGT-3′ and 5′-ACTGAAGCTCAGAACAGTAAGATGCC-3′; *CHS* (AF267173), 5′-GGAGAAATTTCGACGCATGTGCGAT-3′ and 5′-CAATCGAGCCCTTCACCTGTTGTG-3′; chalcone isomerase (*CHI*) (Itoh et al. 2002), 5′-GGATGGAGATTGGAGGCAGATTC-3′ and 5′-CATCTTGTGGAATCGACTCGTGCT-3′; flavanone 3-β-hydroxylase (F3H) (X72592), 5′-GAACGTCCGAAAGTGGCGTATAATG-3′ and 5′-TTAGCAAGCCTCTTATGACGGGCGA-3′; dihydroflavonol 4-reductase (*DFR*) (Itoh et al. 2002), 5′-CATTTGTAACAAAAAAAGATGGTTTC-3′ and 5′-GGAAAACACAAATCTGATAACTAAGCAGA-3′; anthocyanidin synthase (*ANS*) (U82432), 5′-TGAGGATCCAGAGGTTCGAGCCAAG-3′ and 5′-CGGAAGACTTATGTTGAATATGT-3′; *D. caryophyllus* UDP-Glc: flavonoid glucosyltransferase (designated "*DicGT1*" as follows), 5′-AGCTCCGGGGATCCACACAATGTCAGCAAATTCTAACT ACATGAACA-3′ and 5′-ACTAGAGCGGCCGCAGTTTAGTTAGAAGTGACGATCATGTCG-3′; *DicGT2*, 5′-AGCTCCGGGGATCCAATTCCTAGCCATTCCTCCAAAATTAC-3′ and 5′-ACTAGAGCGGCCGCATAAATGATGATGATTTATTGAAGAACATC-3′; *DicGT3*, 5′-AGCTCCGGGGATCCATCTTCTATTAACAATCAGAAACGATA-3′ and 5′-ACTAGAGCGGCCGCTACTATTATGAACTTGTGATTAGTTCA-3′; *DicGT4*, 5′-ATGGATATCATGAGAAAATCATACATATACACAAACAATA-3′ and 5′-GACGGAGCTCTTTTATCAAACTATATTTTTGTACTAGGA-3′; *DicGT5*, 5′-GATCGATATCATTCATATTTCCGCCAACATCAATT GAGA-3′ and 5′-ATTGCTGCAGCTATGGGTAATACAACACTTTGATTCTAAAG-3′; ubiquitin (UBQ), 5′-ATATTGCAGTTCTAGCATTCCCTTTCGGCGGA-3′ and 5′-CGTATTTCGCTCTATCCGCCTTGTACATGTGAG-3′. The LA-PCR mixture (10 μl) was prepared according to the supplier's recommendations using 1 μl of the cDNA mixture and 2 pmol of each primer. The mixture was incubated for 1 min at 92°C, and then 0.5 units TaKaRa LA-*Taq* DNA polymerase were added. The PCR was performed for 20 or 32 amplification cycles (30 s at 92°C; 1 min at 55°C; 1 min at 72°C). Reaction mixtures were separated by electrophoresis in a 1.5% agarose gel, followed by ethidium bromide staining.

Protein extraction from recombinant E. colis and GT assay

The *GT* full-length cDNAs were introduced into the pBluescript SK plasmid in the orientation that would allow expression to be controlled by the *T7* promoter. Transformed *E. coli* expressing the recombinant GT protein and vector without insert as a control were cultured in 5 ml of liquid 2 YT medium containing ampicillin (50 μg/ml) at 37°C overnight, and then 6 h at 30°C. Cells were collected by centrifugation (10,000 *g*, 30 s) and suspended in 500 μl of 50 mM K-Pi buffer (pH 7.0) followed by centrifugation as above. The pellet was suspended in 300 μl of the same buffer as above containing 14 mM 2-mercaptoethanol, and the bacteria were lysed with an ultrasonic disruptor (TOMY UD-201, Tokyo, Japan). Cell debris was removed by centrifugation (12,000 *g*) for 20 min at 4°C.

The 50 μl reaction mixture for the assay of enzyme activity consisted of 20 μl crude *E. coli* extract, 25 nmol of several flavonoid and 25 nmol UDP-D-glucose (925Bq UDP-D-[^{14}C]-glucose) in 50 mM K-Pi (pH 7.5). The mixture was incubated for 15 min at 30°C. The reaction was stopped by addition of 20 μl of 10% HCOOH, and the reaction products were extracted with 200 μl of ethyl acetate. Aliquots of 100 μl of the organic phase were transferred to scintillation vials and monitored for radioactivity in a toluene-based scintillation fluid.

Ethyl acetate extractions from the reaction were concentrated from 200 μl to 10 μl followed by addition of authentic flavonoids as internal standards and applied to a thin layer chromatography (TLC) plate (Avicel SF, Funakoshi Co., Ltd., Tokyo, Japan). Compounds were separated using solvent systems of 15% CH_3COOH or *n*-BuOH–CH_3COOH–H_2O (4 : 1 : 5, v/v (BAW)). Radioactivity on the chromatograms was determined with a bioimage analyzer (BAS 1500, Fuji Photofilm), and *Rf* values of reaction products and authentic flavonoids determined with UV illumination (280 nm or 360 nm).

Reaction mixtures with anthocyanin or betanidin as substrate were terminated by adding 50 μl CH_3Cl/CH_3OH (2 : 1(v/v) plus 5% HCOOH). The mixture was

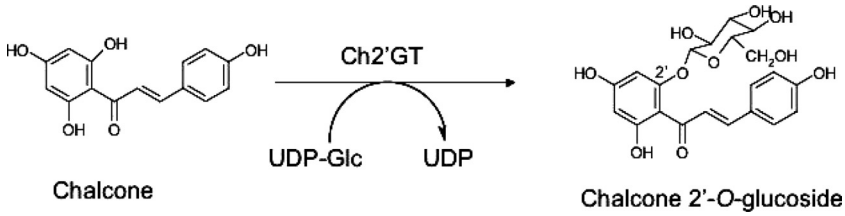

Figure 1. Reaction scheme illustrating the glucosylation of chalcone by chalcone 2′-GT.

then centrifuged at 3,000 *g* for 3 min to remove insoluble materials, such as denatured proteins. Reaction products were identified by co-migration with anthocyanin and betacyanin on TLC. Radioactive detection on TLC of anthocyanin and betacyanin were carried out as above.

Identification of non-radiolabeled reaction products was carried out by HPLC (Beckman System Gold, Beckman Instruments, Inc., Fullerton, USA) equipped with a reverse-phase column (4.6 mm i.d.×250 mm, Wakosil-II 5C18 AR, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Linear gradient elution (1 ml/min) was used for HPLC with solvent A (1.5% H_3PO_4 in H_2O) and solvent B (80% MeOH) from 20 to 80% solvent B in solvent A over 20 min with a monitoring absorbance at 520,370,360 or 280 nm. Reactions using non-radiolabeled glucosyl donors (UDP-Glc, ADP-glucose, TDP-glucose and UDP-galactose) were terminated by addition of 10 μl of a stop solution (10% HCOOH) and the mixture was then centrifuged at 3,000 *g* for 3 min. The supernatant (50 μl) was freeze-dried and re-dissolved in 10 μl HPLC solvent (A : B=80 : 20). The peaks were identified by retention time and co-chromatography with authentic compounds.

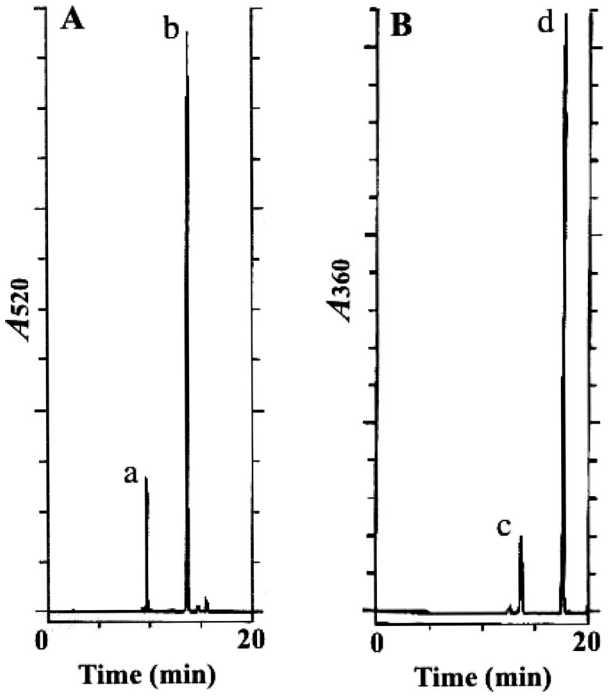

Figure 2. Analysis of flavonoid 3-*O*-glucosyltransferase activities of DicGT1 by HPLC. (panel A) HPLC profile of reaction mixture of the enzymatic glucosyl transfer containing cold UDP-Glc, cyanidin and recombinant DicGT1; (panel B) HPLC elution profile of a reaction mixture containing cold UDP-Glc, quercetin and recombinant DicGT1. Identified peaks were as follows; a, cyanidin 3-*O*-glucoside; b, cyanidin; c, quercetin 3-*O*-glucoside; d, quercetin.

Results and discussion

cDNA cloning of GTs from carnation flower petals

Of the twenty-three cDNA fragments identified as homologous to flavonoid *GT* nucleotide sequences, 18 could be extended by 5′- and 3′-RACE and introduced into the pBluescript SK vector under control of the *T7* promoter for examination of flavonoid GT and anthocyanin GT activity. For flavonoid GT activity, chalcone, quercetin or naringenin and UDP-[^{14}C]Glc were incubated with crude extracts, the reaction products were extracted in ethyl acetate and the radioactivity in the organic phase was measured. Flavonoid GT activity was detected in five of the *E. coli* extracts harboring cDNAs, designated as DicGT1 through DicGT5 (accession numbers were AB191245 through AB191249). As a simple screening assay for anthocyanidin 3-GT activity, cyanidin and UDP-Glc were mixed with crude extract and incubated at room temperature for 1 h. Anthocyanidin 3-*O*-glucoside is much more stable in neutral conditions (pH 7.5) than anthocyanidin. Thus, if an extract has anthocyanidin 3-GT activity, cyanidin in the reaction mixture would be converted into cyanidin 3-*O*-glucoside, giving a stable red color, whereas cyanidin is quickly decomposed to indicate brown color. Only the crude extract for DicGT1 was positive for anthocyanidin 3-GT activity in the screening assay. DicGT2 through DicGT5 had glucosyl transfer activity to the 3-OH position of flavonols (kaempferol or quercetin). In order to confirm cyanidin 3-GT activity, the reaction mixture of DicGT1 with cyanidin was analyzed by HPLC, showing that the reaction product was undoubtedly cyanidin 3-*O*-glucoside (Figure 2A).

It has been reported that anthocyanidin 3-GT has flavonol 3-GT activity in several plant species (Tanaka et al. 1996; Ford et al. 1998). To determine if clone DicGT1 has additional activity, the substrate specificity of DicGT1 was tested for anthocyanidins (pelargonidin and cyanidin), flavonols (kaempferol and quercetin) and

betanidin. Cyanidin proved to be the best substrate for crude extracts from DicGT1 but activity was also observed with quercetin as substrate (Figure 2B). ADP-glucose, TDP-glucose and UDP-galactose could not serve as glycosyl donors (data not shown). It was reported that *Dorotheanthus bellidiformis* betanidin 6-GT has anthocyanidin 3-GT activity (Vogt et al. 1997; Vogt 2002). Both *D. caryophyllus*, which exclusively produces red pigmentation from anthocyanins and *D. bellidiformis*, which only produces betacyanins for its red pigmentation, are in order Caryophyllales. However, there was no evidence of glucosyltransferase activity of DicGT1 extracts on betanidin (data not shown).

From the results of northern blot analysis (Figure 3), the *DicGT1* gene was expressed strongly in stages 2 to 4 during the development of petals, in which anthocyanin synthesis occurred. This expression profile was similar to those of *DFR* and *ANS* genes in petals of cyanic carnation (Mato et al. 2000). *DicGT1* transcripts could not be detected in roots, stems or leaves (data not shown).

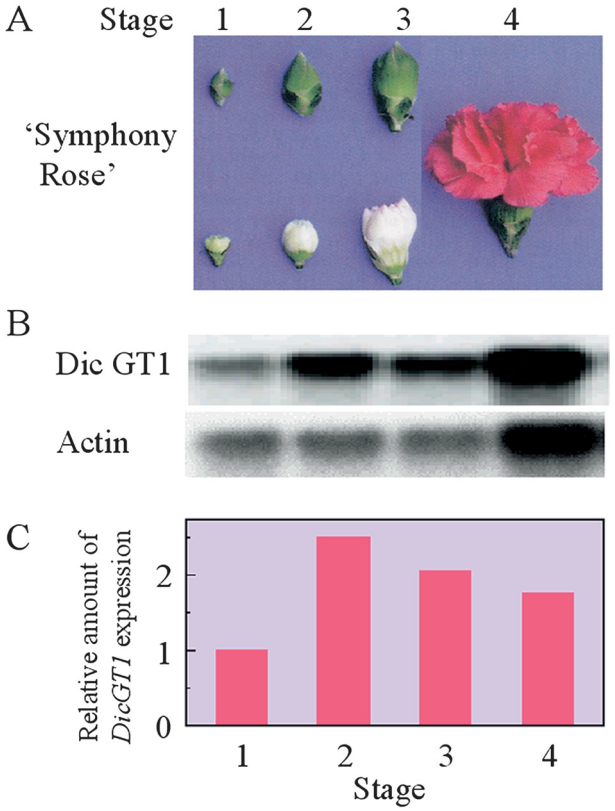

Figure 3. Northern blot analysis on the expressed transcripts of *DicGT1* genes. A, flower developmental stages of carnation, "Symphony Rose". Sepals of the upper lane were peeled to show the development of petals in the buds (lower lane). B, the poly(A)$^+$ RNA was prepared from the petals of flowers at each stages shown in A and *DicGT1* transcripts were detected. Actin probes were used as a loading control (bottom panel). C, the signal intensity of the bands on the X-ray film were measured and the relative amount of the expression of *DicGT1* gene was calculated in comparison with that of actin gene using Image Quant (Amersham Biosciences Corp., Piscataway, USA).

Chalcone 2′-O-glucosyltransferase activity of DicGT4 and DicGT5

When the individual extracts above were incubated with chalcone and UDP-Glc, we found that both DicGT4 and DicGT5, but not DicGT1, 2 or 3, catalyzed transfer of the glucose residue from UDP-Glc to chalcone. The co-chromatography and retention time of the reaction products were consistent with chalcone 2′-*O*-glucoside (Figure 4), indicating that DicGT4 transferred a glucose to the 2′-hydroxyl group of chalcone. The chromatogram of DicGT5 activity for chalcone as a substrate was similar to that of DicGT4, but the 2′-GT activity of DicGT5 was lower than that of DicGT4 (data not shown). Despite the considerable overlap in GT activity, DicGT4 and DicGT5 share about 30% amino acid identity (data not shown).

The reaction products of DicGT4 with chalcone as substrate contained naringenin 7-*O*-glucoside (peak b in Figure 4B), presumably because naringenin is a by-product of a spontaneous chemical reaction in the mixture (Moustafa and Wong 1967) and was glucosylated by DicGT4 activity. Furthermore, the DicGT4 and DicGT5 enzymes were able to glucosylate various hydroxyl positions of flavonoids (Figures 5 and 6). TLC analysis of the reaction products for kaempferol,

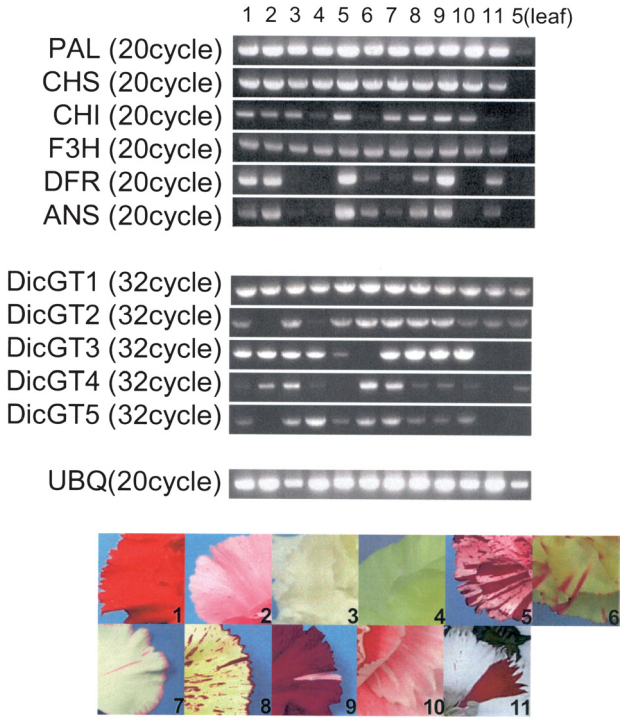

Figure 7. RT-PCR analysis to detect the expression of anthocyanin biosynthesis genes and flower phenotypes. cDNA was extracted from petals at all four developmental stages and from leaves of cultivar no. 5. Specific primers corresponding to the *PAL, CHS, CHI, F3H, DFR, ANS* and *DicGTs* were used for RT-PCR.

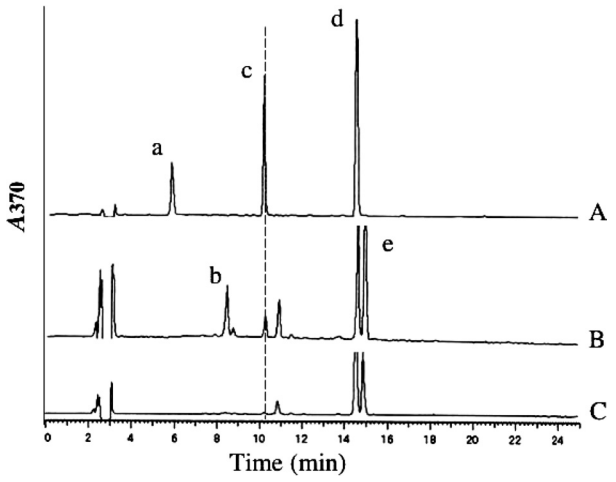

Figure 4. Analysis of chalcone 2′-*O*-glucosyltransferase activities of DicGT4 by HPLC. (chromatogram A) HPLC elution profile of authentic samples of chalcone 2′,4′-*O*-diglucoside, chalcone 2′-*O*-glucoside and chalcone; (chromatogram B) HPLC profile of reaction mixture of the enzymatic glucosyl transfer containing UDP-Glc, chalcone and recombinant DicGT4; (chromatogram C) HPLC profile of a vector-only control reaction mixture that contained UDP-Glc, chalcone and the extract prepared from *E. coli* harboring pBluescript with no insert. a, chalcone 2′,4′-*O*-diglucoside; b, naringenin 7-*O*-glucoside; c, chalcone 2′-*O*-glucoside; d, chalcone; e, naringenin, respectively.

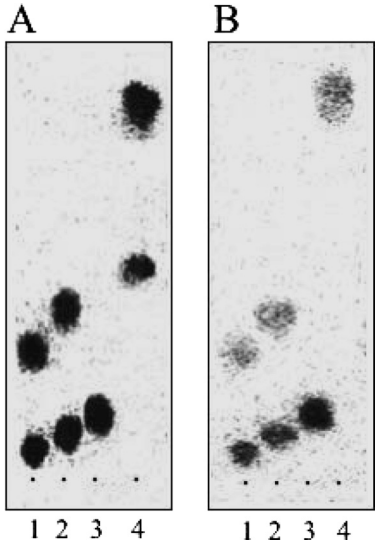

Figure 5. TLC profiles of reaction mixture of the enzymatic glucosyl transfer containing UDP-[^{14}C]Glc, flavonoids and recombinant DicGT4 (panel A) and DicGT5 (panel B). 1. quercetin, 2. kaempferol, 3. apigenin, 4. naringenin as substrates. Developmental solvent of 15% CH_3COOH was used for analysis of reaction mixture. In panel A, 1st lane upper spot, quercetin 3-*O*-glucoside; lower spot, quercetin 7-*O*-glucoside; 2nd lane upper spot, kaempferol 3-*O*-glucoside; lower spot, kaempferol 7-*O*-glucoside; 3rd lane spot, apigenin 7-*O*-glucoside; 4th lane upper spot, naringenin 7-*O*-glucoside; lower spot, unknown naringenin glucoside(s).

quercetin, apigenin and naringenin gave radioactive products, indicating that DicGT4 and DicGT5 could transfer [^{14}C]glucose to the 7-OH position of these compounds as well as to 3-OH of kaempferol and quercetin. The strength of the radioactivity signal was almost the same between kaempferol-3-[^{14}C]glucose and kaempferol-7-[^{14}C]glucose, and between quercetin-3-[^{14}C]glucose and quercetin-7-[^{14}C]glucose in the DicGT4 reaction (lanes 1 and 2 of Figure 5A), indicating that DicGT4 transfers glucose equally to the 3- and 7-OH positions, whereas for DigGT5 signal strength was greater for 7-OH (Figure 5B). DicGT4 produced an unknown reaction product with quercetin as a substrate (peak a in Figure 6A). The unknown is possibly a quercetin diglucoside, as estimated by the HPLC retention time. However, the product corresponding to the HPLC peak was not detected by TLC (Figure 4), probably because this compound is more soluble in water and may not be extracted in ethyl acetate after the enzyme reaction.

In the case of cyanidin as a substrate in the DicGT4 reaction, in addition to cyanidin 3-*O*-glucoside (Figure 6B, peak e), an unknown cyanidin glucoside (Figure 6B, peak f) was observed, indicating that the DicGT4 could transfer glucose to other positions than just the 3 position of hydroxyl residue. This alternative glucosylation may be at the 7-OH position based on HPLC retention time and TLC *Rf* values. When cyanidin 3-*O*-glucoside was a substrate for DicGT4, an unidentified peak was observed (peak h in Figure 6C), which did not correspond to

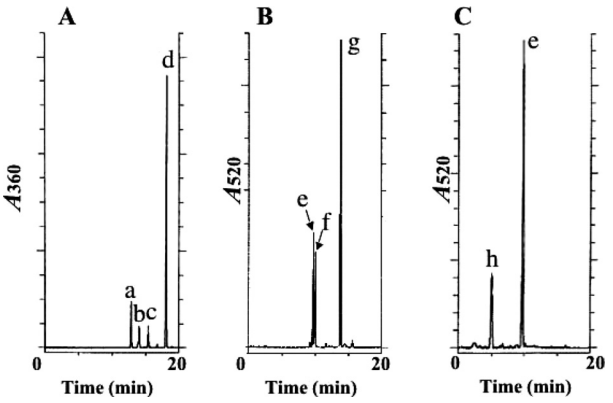

Figure 6. Analysis of flavonoid glucosyltransferase activities of DicGT4 by HPLC. (panel A) HPLC profile of a reaction mixture containing UDP-Glc, quercetin and recombinant DicGT4; (panel B) HPLC elution profile of reaction mixture containing UDP-Glc, cyanidin and recombinant DicGT4; (panel C) HPLC profile of reaction mixture containing UDP-Glc, cyanidin 3-*O*-glucoside and recombinant DicGT4. In panel A, peaks a, unknown quercetin glucoside(s); b, quercetin 3-*O*-glucoside; c, quercetin 7-*O*-glucoside; d, quercetin, respectively. Peaks of panel B and C are e, cyanidin 3-*O*-glucoside; f, unknown cyanidin glucoside; g, cyanidin; h, unknown cyanidin glucoside(s).

cyanidin 3,5-*O*-diglucoside. The unknown peaks, peak f in Figure 6B and peak h in Figure 6C, could not be identified, because they did not correspond to elution times for authentic samples. As a future research project, these unknown compounds will be produced *in vitro* on a

large scale followed by purification, and their structure will be determined using NMR analysis. Whereas, when quercetin and cyanidin were substrates for DicGT5, only two mono-glucoside reaction products were detected, but the other products, e.g., di-glucoside reaction products, could be under the detectable level (data not shown).

Our results presented here show that DicGT4 and DicGT5 proteins produced in *E. coli* exhibited broad substrate specificity, similar to tobacco GT genes isolated from elicitor-treated leaves of *Nicotiana tabacum* L. cv. Samsun NN (Fraissinet-Tachet et al. 1998). Vogt et al. (2002) showed that GT enzymatic activity prepared from cultured cells of *D. bellidiformis* catalyzed various flavonoid glucosides. For example, the betanidin 5GT could transfer glucose residues to the 4′- or 7-hydroxyl group of quercetin. However, DicGT4 and DicGT5 could not transfer glucose to betanidin (data not shown).

RT-PCR analysis of GT gene expression

In preliminary experiments, gene expression of *DicGT2* through *DicGT5* were quite low relative to *DicGT1* and below levels detectable by northern blotting. RT-PCR analyses were performed to reveal expression levels in the petals of carnation cultivars bearing various flower colors (Figure 7). Consequently, expression of the genes encoding anthocyanidin biosynthesis enzymes was detectable after 20 cycles of PCR (Figure 7). Expression of *DicGT2* through *DicGT5* could not be detected after 20 cycles (data not shown), but could after 32 cycles. It was found that both *DicGT4* and *DicGT5* were expressed in cultivars of carnation with yellow color petals (lanes 3, 4, 6, 7 and 8 in Figure 7), but they were also expressed in pink or red color petals (lanes 1, 5, 9 and 10), indicating that both genes were expressed in the petals lacking chalcone 2′-*O*-glucoside. Similarly, *DicGT1*, the putative *anthocyanidin 3-GT* gene, also expressed in all cultivars (Figure 7). *DicGT1 DicGT2* and *DicGT4* was also expressed in leaf (Figure 7, lane 12, cultivar 5 (leaf)).

Considering the broad expression profiles, the low expression level of these genes in comparison with *DicGT1* and the broad substrate specificity of the enzymes encoded by these genes, we could not determine that *DicGT4* and/or *DicGT5* were chalcone 2′-GT genes acting *in vivo* in yellow petals of carnation. However, this is the first report to isolate and identify GT cDNAs encoding chalcone 2′-GT activity.

Carnation cultivars bearing yellow flowers have *CHI* and *DFR* genes disrupted by the transposable element *dTdic1s* (Itoh et al. 2002). The insertion event of *dTdic1* likely occurred due to the breeding process within the past several centuries, because all carnation lines bearing yellow flowers have identical insertion sites of *dTdic1* in the C*HI* and *DFR* genes (Itoh et al. 2002). It is likely then that one mutant carnation harboring both disrupted *CHI* and *DFR* genes bearing yellow flowers emerged in the way of breeding has spread worldwide by breeders. Before the insertion events in the genes, carnation may not have produced chalcone derivatives as a main pigment in petals. When the insertion event occurred, the CHI activity was almost curtailed, resulting in the accumulation of chalcone in cells of petals. If carnation did not have chalcone GT activity, the accumulated chalcone might be autochemically converted to equal amounts of (2*S*)-naringenin and (2*R*)-naringenin in cells under acid or neutral pH conditions (Moustafa and Wong 1967). Most plant enzymes involved in the flavonoid and anthocyanin pathways can catalyze (2*S*)-naringenin as a substrate, but not (2*R*)-naringenin, so that most (2*R*)-naringenin would not be catalyzed. (2*R*)-Naringenin is highly hydrophobic and permeates cell and vacuole membranes, thus its accumulation might disrupt the membrane structure and cause the death of petal cells. The presence of flavonoid GTs in carnation with broad substrate specificity for several species of flavonoid molecules would allow the transfer of glucose to the 2′-OH position of chalcone. Glucosylation to a hydrophilic molecule would facilitate detoxification by allowing compartmentation into vacuoles. The tissue would thus be rescued by preventing the formation and accumulation of (2*R*)-naringenin and to counteract chalcone catalyzed to chalcone 2′-*O*-glucoside by chalcone 2′-GT activity. Although the original yellow carnation is no longer in existence, its petals may have been pale yellow, because chalcone 2′-GT activity may not have been high enough for all of the accumulated chalcone to be converted to chalcone 2′-*O*-glucoside. Thus, some (2*R*)-naringenin could be formed autochemically. Early *Dianthus* breeders may have selected for a deep yellow petal color, resulting in the propagation of elevated chalcone 2′-GT activity from the original flavonoid GT by selection pressure. However, the more efficient glucosylation could be either because of increased expression of an inefficient enzyme or because of selection for enzymes with higher activity to chalcone. In either case, the enzyme may still show substrate specificity for flavonoids other than chalcone, so that the recombinant proteins encoded by *DicGT4* and *DicGT5* have glucosylation activity *in vitro* not only with chalcone, but also naringenin, apigenin, kaempferol, quercetin and cyanidin. It is thought that that *DicGT4* and *DicGT5* are evolving to become *chalcone 2′-GT* genes, but have not become a specific chalcone 2′-GT with no or little substrate specificity for other flavonoids or anthocyanidins. Furthermore, the expression of the genes encoding GT may not have been yet limited only to petals but may also occur in leaves, apart from the *anthocyanindin 3-GT* gene. Here, we identified chalcone 2′-GT activity in *DicGT4* and *DicGT5*. It is supposed that carnation may have over hundreds *GT* genes because

Arabidopsis, with a small genome size, has 120 *GT* genes (Jackson et al. 2001; Lim et al. 2001). Other DicGT genes in the carnation genome, which were not isolated in this experiment, possibly encode enzyme(s) having much higher activity and more defined substrate specificity for chalcone 2′-GT and showing more specific expression in petals than *DicGT4* and *DicGT5*.

To confirm whether the enzymes encoded in *DicGT4* and/or *DicGT5* catalyze the synthesis of chalcone 2′-*O*-glucside from chalcone *in vivo*, these cDNAs could be introduced into plant expression vectors and transferred into the other plant species in which *CHI* transcription is blocked by RNAi, for example, giving rise to yellow flower colors synthesizing and accumulating chalcone 2′-*O*-glucoside. We are now preparing these constructs to generate plants bearing yellow flowers which can not be produced by conventional breeding.

Acknowledgements

We greatly appreciate the skillful floriculturists, Mr. Masahiko Ayusawa, Mr. Hirohito Suzuki and Mr. Akira Nasuno for kindly providing us with many carnation plants. Chalcone glucoside, some flavonoids and betanin as authentic samples were kindly provided by Prof. Masa-atsu Yamaguchi (Minami-Kyushu University, Miyazaki, Japan) and Prof. Tsukasa Iwashina (Tsukuba Botanical Garden, National Science Museum, Tukuba, Japan) and San-Ei Gen F. F. I., Inc. This work was partially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, the National Institute of Floricultural Science (NIFS), the National Agricultural Research Organization (NARO), Society for Technoinnovation of Agriculture Forestry and Fisheries (STAFF, Technical Development Program for Making Agribusiness in the Form of Utilizing the Concentrated Know-how from Private Sector), and the Japan Food Chemical Research Foundation.

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