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Short Communication

Enzymatic preparation of 1-*O*-hydroxycinnamoyl-β-D-glucoses and their application to the study of 1-*O*-hydroxycinnamoyl-β-D-glucose-dependent acyltransferase in anthocyanin-producing cultured cells of *Daucus carota* and *Glehnia littoralis*

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Abstract Four 1-*O*-hydroxycinnamoyl-β-D-glucoses (HCA-Glcs), sinapoyl-, feruloyl-, caffeoyl-, and 4-coumaroyl-glucoses, were synthesized using a recombinant protein of sinapate glucosyltransferase from *Gomphrena globosa* coupled with a recycling system of UDP-glucose by sucrose synthase from *Arabidopsis thaliana*. The substrate preference of HCA-Glc-dependent acyltransferase activity was examined in a protein extract prepared from anthocyanin-producing cultured cells of *Daucus carota* and *Glehnia littoralis*. The main anthocyanin molecule of the aglycon and the sugar moiety produced and accumulated in both cultured cells were exactly the same; the only difference was found in modification with sinapoyl moiety in *D. carota* and with feruloyl moiety in *G. littoralis*. The protein extracts from both *D. carota* and *G. littoralis* cultured cells showed higher activity with feruloyl-Glc than with sinapoyl-Glc. The major HCA-Glcs that accumulated in cultured cells of *D. carota* and *G. littoralis* were sinapoyl-Glc and feruloyl-Glc, respectively. These results suggested that the specificity of HCA moieties of major anthocyanin molecules in cultured cells of *D. carota* and *G. littoralis* might be dominated by produced and accumulated acyl donor molecules *in vivo* rather than by the substrate specificity of acyltransferase enzymes.

Key words: Anthocyanin, acyltransferase, *Daucus carota*, *Glehnia littoralis*, hydroxycinnamic acid.

Acylation of anthocyanins occurs in the final modification step of their biosynthesis, and is thought to stabilize the color of pigments in weakly acidic plant vacuoles (Goto and Kondo 1991). Acylation of anthocyanins also affects the absorbance properties of these molecules, and different patterns of acylation give rise to observable differences and variation in color (Honda et al. 2005). Recent understanding that acylation is related to bluing of anthocyanins can be applied to generate blue flowers using transgenic technology (Fukui et al. 2003; Katsumoto et al. 2007).

Two types of acyltransferases involved in the acylation of anthocyanins have been reported; one using hydroxycinnamic acid coenzyme A (CoA) and another using 1-*O*-hydroxycinnamoyl-β-D-glucose (HCA-Glc) as an acyl donor. The acyltransferase that modifies anthocyanins with HCA-CoA esters as donor substrates was identified more than 25 years ago (Kamsteeg et al. 1980; Teusch et al. 1987). An alternative pathway of ester formation by acyltransferases that accept 1-*O*-β-D-glucose esters as acyl donors instead of CoA thioester was reported later (Strack et al. 1988). In anthocyanin-producing cells of the cultured line of wild carrot, HCA-Glc, but not HCA-CoA, acted as the acyl donor for cyanidin 3-*O*-(2′′-*O*-xylosyl-6′′-*O*-glucosyl-galactoside) (cyanidin 3-(Xyl-Glc-Gal)) (Glassgen and Seitz 1992). More recent biochemical and molecular biological experiments have focused on acyltransferases requiring HCA-CoA of benzyl alcohol *O*-acetyltransferase, anthocyanin *O*-hydroxycinnamoyl

Abbreviations: AtSUS1, *Arabidopsis thaliana* sucrose synthase; CoA, coenzyme A; GgSGT, *Gomphrena globosa* sinapate glucosyltransferase; HCA-Glc, 1-*O*-hydroxycinnamoyl-β-D-glucose; LB, Luria–Bertani
This article can be found at http://www.jspcmb.jp/

transferase, anthranilate *N*-hydroxycinnamoyl/benzoyltransferase, and deacetylvindoline 4-*O*-acetyltransferase (Luo et al. 2007). Research has just begun on acyltransferases requiring HCA-Glc as an acyl donor, which is classified in the serine carboxypeptidase-like (SCPL) protein family (Fraser et al. 2007).

Preparation of HCA-Glc molecules as acyl donor substrates is required for studying the enzymatic reaction of acylation of anthocyanin molecules. In previous works, HCA-Glc molecules were extracted and purified from plant materials, but such methods were inefficient and only a small amount could be obtained even from a large amount of plant materials. It is difficult to prepare various molecular species of HCA-Glcs, such as sinapoyl-, feruloyl-, caffeoyl-, and 4-coumaroyl-Glc, from plant materials. Instead of preparing these from plant materials, we established a method for the effective synthesis of HCA-Glcs by an enzymatic reaction using recombinant proteins of sinapate glucosyltransferase derived from globe amaranth (*Gomphrena globosa*) cDNA (GgSGT) coupled with a recycling system of UDP-glucose by sucrose synthase from *Arabidopsis thaliana* (AtSUS1) (Masada et al. 2007) to improve the synthetic efficiency (Figure 1). Using synthesized HCA-Glc molecules as acyl donors, we compared the substrate preference in protein extracts prepared from anthocyanin-producing cells of *Daucus carota* and *Glehnia littoralis*, which are taxonomically close species belonging to Umbelliferae. *In vivo*, *D. carota* cells synthesize and accumulate cyanidin 3-*O*-(2′′-xylosyl-6′′-sinapoyl-glucosyl-galactoside) (cyanidin 3-(Xyl-sinapoyl-Glc-Gal)) (Harborne et al. 1983), and *G. littoralis* cells synthesize cyanidin 3-(Xyl-feruloyl-Glc-Gal) (Miura et al. 1998) as the main anthocyanin molecule. The only difference between these species is the modification of the acyl moiety of sinapoyl or feruloyl. First, we studied whether the difference between these relates to distinct substrate preference of the acylation reaction in the protein extracts.

Globe amaranth plants bearing purple flowers were purchased from a flower shop, and the petals of freshly opening flowers were harvested and frozen in liquid nitrogen and stored at −80°C. Total RNA from the stored petals was prepared using a modified guanidinium thiocyanate-CsCl ultracentrifugation method (Chirgwin et al. 1979) followed by poly(A^+) RNA purification using Oligotex-dT30<*Super*> (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. The full-length cDNAs were synthesized using the GeneRacer^{TM} kit (Invitrogen, CA, USA). The nucleotide sequence of three reverse degenerate primers, which were designed based on the conserved regions of the amino acid sequences in various UDP-glucosyltransferases (UGTs) previously deposited in the database, as well as PCR conditions, cloning of amplified cDNA fragments

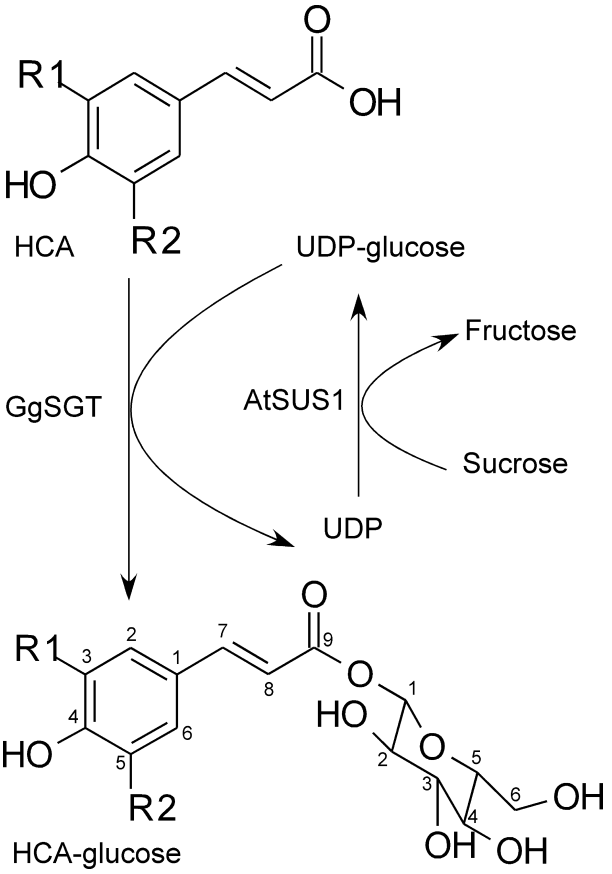

Figure 1. Schematic reaction pathway for the enzymatic synthesis of HCA-glucose established in this report. HCA, hydroxycinnamic acid as follows: sinapic acid, R1=OMe and R2=OMe; ferulic acid, R1=OMe and R2=H; caffeic acid, R1=OH and R2=H; 4-coumaric acid, R1=H and R2=H. GgSGT, *Gomphrena globosa* sinapate glucosyltransferase; AtSUS1, *Arabidopsis thaliana* sucrose synthase.

into pDONR221 (Invitrogen), isolation of full-length cDNA using 5′- and 3′-RACE, and determination of their nucleotide sequence have been described previously (Sasaki et al. 2005).

The deduced amino acid sequences encoded in the eight candidates of UGT cDNAs isolated here were aligned using the multiple alignment program CLUSTAL W (Thompson et al. 1994) on the server at GenomeNet (http://www.genome.jp/). Based on this alignment, phylogenetic analysis was performed by the neighbor-joining method (Saitou and Nei 1987) to prepare a phylogenetic tree (Figure 2). A deduced amino acid sequence of cDNA designated as GgSGT was shown to share high identity with UGTs of *A. thaliana* and *Brassica napus*, which catalyze the formation of HCA-Glcs. The group of phenylpropanoid 1GT including the sequence of *GgSGT* cDNA was separated clearly from the branches of the sequence of flavonoid UGTs and phenylpropanoid 4GTs. Analysis of the molecular phylogenetic tree led us to hypothesize that the protein of GgSGT catalyzes the formation of HCA-Glcs. To obtain the GgSGT recombinant protein with high efficiency, the

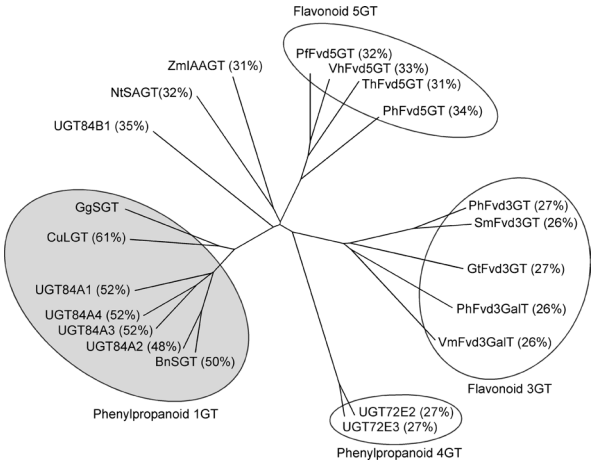

Figure 2. A molecular phylogenic tree of plant UGTs based on the amino acid sequences. The GTs used here and their accession numbers (in parentheses) are as follows: GgSGT, *Gomphrena globosa* sinapate glucosyltransferase (AB362221); SmFvd3GT, *Solanum melongena* flavonoid 3-*O*-GT (X77360; Toguri); PhFvd3GalT, *Petunia hybrida* 3-*O*-galactosyltransferase (AB027454; Miller et al. 1999); VmFvd3GalT, *Vigna mungo* 3-*O*-GT (AB009370; Mato et al. 1998); GtFvd3GT, *Gentiana triflora* 3-*O*-GT (D85186; Tanaka et al. 1996); PhFvd3GT, *Petunia hybrida* 3-*O*-GT (AF165148; Yamazaki et al. 2002); PhFvd5GT, *Petunia hybrida* 5-*O*-GT (AB027455; Yamazaki et al. 2002); ThFvd3GT, *Torenia hybrida* 3-*O*-GT (AB076698; Mizutani); VhFvd3GT, *Verbena hybrida* 3-*O*-GT (AB013598; Yamazaki et al. 1999); PfFvd5GT, *Perilla frutescens* 5-*O*-GT (AB013596; Yamazaki et al. 1999); CuLGT, *Citrus unshiu* limonoid GT (AB033758; Kita et al. 2000); BnSGT, *Brassica napus* sinapate 1GT (AF287143; Milkowski et al. 2000); UGT84A1, UGT84A3, UGT84A4, AtHCAGT, *Arabidopsis thaliana* hydroxycinnamate GT-1, -2, and -3 (Z97339; Lim et al. 2001); UGT84A2A, *A .thaliana* sinapate 1GT (B019232; Lim et al. 2001); UGT72E2, *A. thaliana* sinapoyl alcohol 4-*O*-GT (AB018119; Lim et al. 2001); UGT72E3, *A. thaliana* sinapate 4-*O*-GT (AF077407; Lim et al. 2001); UGT84B1, *A. thaliana* indole-3-acetic acid GT (AC002391; Jackson et al. 2001); ZmIAAGT, *Zea mays* indole-3-acetic acid GT (L34847; Szerszen et al. 1994); and NtSAGT, *Nicotiana tabacum* salicylic acid GT (AF190634; Lee and Raskin 1999). The analytical methods used for multiple sequence alignment, phylogenetic analysis, and drawing the phylogenetic tree are described in Materials and Methods. The percentages in parentheses in the figure represent the amino acid sequence similarities between GgSGT and each of the other GTs.

DNA fragment corresponding to the open reading frame of *GgSGT* cDNA was amplified by PCR and introduced into the expression vector pDEST17 (Invitrogen) using LR clonase (Invitrogen) and transformed into *Escherichia coli* KRX. Our preliminary experiments using the crude extract of *E. coli* harboring *GgSGT* cDNA in pDEST17 prepared above showed UGT activity of various HCA molecules as acceptors, which we expected to be useful for studying HCA-Glc synthesis *in vitro*.

We prepared GgSGT recombinant protein from *E. coli* cultured at 30°C in Luria–Bertani (LB) medium containing 50 g ml^{-1} ampicillin, 0.4% glucose, and 0.1% rhamnose for 12 h, which was harvested by centrifugation and stored at −80°C until use. A protein extract from the recombinant *E. coli* was prepared by sonication in 0.1 M potassium phosphate, pH 7.5, containing 7 mM 2-mercaptoethanol, and was centrifuged to remove cell debris. The resultant supernatant was then used as a crude extract for the enzymatic reaction. We also prepared AtSUS1 recombinant protein, which catalyzes the formation of UDP-glucose (UDP-Glc) from sucrose and UDP (Figure 1) as the UDP-Glc recycling system for the efficient synthesis of HCA-Glcs as reported previously (Masada et al. 2007). A slight modification was that a HisTrap HP column (GE Healthcare, Buckinghamshire, England) was used to affinity purify the recombinant protein. The purified AtSUS1 protein concentrated by ultrafiltration was used in the enzyme reaction.

The reaction mixture contained 20 ml of GgSGT protein extract with 91.8 mg protein in total, 1 ml of AtSUS1 purified protein (4.2 mg) in the buffer of 0.1 M potassium phosphate (pH 7.5), 7 mM 2-mercaptoethanol, 0.2 mmol of UDP-Glc, 0.42–0.63 mmol of hydroxycinnamic acid (sinapic, ferulic, caffeic, and 4-coumaric acid), and 4.5 mmol of sucrose in a final volume of 30 ml. The reaction mixture was incubated at 30°C for 12 h and centrifuged at 5,800×*g* for 15 min. The supernatant was passed through an ion exchange resin of DOWEX 1X8-100 Cl^- (Sigma-Aldrich, MO, USA), and the flow-through was combined with fractions washed with water and then applied to Sep-Pak Plus C18 Cartridges (Waters Corporation, MA, USA). Each HCA-Glc preparation was eluted with 10% EtOH, sinapoyl-Glc, and 4-coumaroyl-Glc and purified further by recrystallization. The yields were then quantified by their weight. Feruloyl-Glc and caffeoyl-Glc were purified by reverse phase HPLC (ELITE LaChrom L-2130 Pump, L-2420 UV-VIS Detector, D-2500 Chromato-Integrator, Hitachi High-Technologies Co., Tokyo, Japan) equipped with an Xterra Prep MS C_{18} column (i.d. 10×100 mm; Waters Corporation) with detection at 330 nm. The substrates and products were separated by linear gradient elution (1.5 ml min^{-1}) for 4 min with a 30%–80% gradient of solvent B (90% methanol) in solvent A (1.0% formic acid in water). The yield of these two HCA-Glcs was quantified from the HPLC areas using those of the corresponding HCA as the standard.

The molecular structure of these four HCA-Glcs was identified from the electrospray ionization mass spectra (ESI MS) (AccuTOF MS, JMS-T100LC, JEOL, Tokyo, Japan) and ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMBC, and HMQC nuclear magnetic resonance (NMR) (JEOL ECA/ECX400, JEOL). Four glucose conjugates showed molecular MS for $[M-H]^-$ at *m*/*z* 385, 355, 342, and 325, and $[M+Na]^+$ at *m*/*z* 409, 379, 365, and 349, confirming that these were sinapoyl-, feruloyl-, caffeoyl-, and 4-coumaroyl-Glc, respectively. ^{1}H NMR analysis of sinapoyl-Glc was recorded at 400 MHz in deuterated methanol as follows: C-2, 6; δ 6.29 (2H, s), C-7; δ 7.71 (1H, d, J=15.9 Hz), C-8; δ 6.42 (1H, d, J=16.0 Hz),

OMe; δ 3.89 (6H, s), Glc C-1; δ 5.57 (1H, d, J=8.0), Glc C-2–5; δ 3.35–3.49 (4H, m), Glc C-6; δ 3.84 (1H, dd, J=1.8), and δ 3.69 (1H, dd, J=4.6). The correlation between the Glc C-1 proton signal at δ 5.57 and the C-9 carbonyl signal at δ 167.3 observed in the HMBC spectrum showed that the sinapic acid moiety is linked to the position through its carbonyl carbon. The configuration of the anomeric center was confirmed to be β from the ^{1}H NMR coupling constant (J=8.0 Hz) of the anomeric signal, and the spectra were assigned according to published information (Lim et al. 2001). Consequently, the enzymatic reaction product was identified as 1-*O*-sinapoyl-β-D-Glc. Other products produced by the enzymatic reaction using ferulic, caffeic, or 4-coumaric acid instead of sinapic acid as the acyl donor were the conjugates expected of the same structure as sinapoyl-Glc. The yields of sinapoyl-, feruloyl-, caffeoyl-, and 4-coumaroyl-Glc synthesized using recombinant GgSGT coupled with AtSUS1 were 7.0, 5.7, 2.7, and 2.3 mg, respectively.

The structure of the major pigment in the anthocyanin-producing cells of a suspension culture of *D. carota* (Itoh and Ozeki 2002) was confirmed by ESI MS and NMR analysis (data not shown) as cyanidin 3-(Xyl-sinapoyl-Glc-Gal), similar to that reported previously (Harborne et al. 1983). Cyanidin 3-(Xyl-Glc-Gal) for the acceptor substrate of HCA acylation was prepared from purified cyanidin 3-(Xyl-sinapoyl-Glc-Gal) from anthocyanin-producing carrot cells by alkaline hydrolysis as follows. The pigment of anthocyanin-producing carrot cells was extracted with 70% EtOH and purified twice by mass paper chromatography. The purified pigment was hydrolyzed with 10% NaOH in the presence of N_2 for 5 min and then neutralized by the addition of HCl. Hydroxycinnamic acid was removed using diethyl ether, and the deacylated anthocyanin was purified using reverse phase HPLC equipped with a Xterra Prep MS C_{18} column (i.d. 10×250 mm; Waters Corporation) with detection at 530 nm separating by linear gradient elution (6.6 ml min^{-}1) for 20 min with a 17%–30% gradient of solvent B (90% methanol) in solvent A (1.0% formic acid in water). The purified pigment showed the molecular MS $[M]^+$ at *m*/*z* 743.20, corresponding to the value for cyanidin 3-(Xyl-Glc-Gal) obtained by AccuTOF MS (JEOL).

Cells (0.5 g) were homogenized with a mortar and pestle in liquid nitrogen, and the frozen powder was added gently to 4 ml of 0.1 M potassium phosphate buffer, pH 7.5. The homogenate was centrifuged using QIAshredder (QIAGEN, Hilden, Germany) at 12,000×*g* for 5 min to homogenize and remove cell debris. An aliquot of the extract (750 μl) was added to 321 μl of 4.1 M $(NH_4)SO_4$ at a final concentration of 30% saturation, and the mixture was incubated on ice for 5 min and then centrifuged. An aliquot of the supernatant (357 μl) was added to 893 μl of 4.1 M $(NH_4)SO_4$ at a final concentration of 80%, and the mixture was incubated on ice for 5 min and then centrifuged. The precipitate was dissolved in 50 μl of 0.1 M potassium phosphate buffer, pH 7.5, and desalted with a MicroSpin G-25 column (GE Healthcare) preequilibrated with 0.1 M sodium citrate buffer, pH 6.0. The amount of protein in the preparations was quantified using a Coomassie Plus Protein Assay Kit (Pierce Biotechnology, IL, USA) with bovine serum albumin as the standard. The reaction mixture of HCA-Glc-dependent acyltransferase activity in a final volume of 30 μl comprised 11–14 ng of the protein extract, 23 pmol of cyanidin 3-(Xyl-Glc-Gal) as an acyl acceptor, and 30 pmol of sinapoyl-Glc as an acyl donor. The reaction mixture was incubated at 30°C for 60 min, and the reaction was terminated by the addition of 1.5 μl of 20% phosphoric acid. The mixture was centrifuged at 20,000×*g* for 5 min, and the supernatant was subjected to HPLC analysis using a Chromolith Performance RP-18e 100-4 column (i.d. 4.6 m×100 mm; Merck KGaA, Darmstadt, Germany) with detection at 530 nm. The substrates and products were separated by linear gradient elution (2.5 ml min^{-1}) for 10 min using a 17%–30% gradient of solvent B (90% methanol) in solvent A (1.5% phosphoric acid in water).

The incubation of both cyanidin 3-(Xyl-Glc-Gal) and sinapoyl-Glc with the protein preparations from the cultured cells of *D. carota* for 60 min led to the appearance of an additional peak in HPLC (Figure 3B, arrow) as the cyanidin 3-(Xyl-Glc-Gal) peak became smaller. The retention time of this peak in HPLC coincided with that of authentic cyanidin 3-(Xyl-sinapoyl-Glc-Gal), and the molecular MS of the peak purified by HPLC showed $[M]^+$ at *m*/*z* 949, corresponding to the authentic substance.

Using a similar method, the HCA-Glc-dependent acyltransferase activity of *D. carota* cultured cells was examined for other HCA-Glc molecules using feruloyl-Glc, caffeoyl-Glc, or 4-coumaroyl-Glc instead of sinapoyl-Glc as acyl donors. Each new peak emerging in the HPLC profiles after the reaction was subjected to ESI MS analysis. The products showing the molecular MS $[M]^+$ at *m*/*z* 919 and 889 were confirmed as cyanidin 3-(Xyl-feruloyl-Glc-Gal) and cyanidin 3-(Xyl-4-coumaroyl-Glc-Gal), respectively. The UV/VIS spectrum suggested that the new product using caffeoyl-Glc as an acyl donor was cyanidin 3-(Xyl-caffeoyl-Glc-Gal). However, the molecular mass was not able to be determined because of low yield. The apparent substrate preference of the protein extract of cultured *D. carota* cells showed clearly the highest activity with feruloyl-Glc (Table 1). Compared with feruloyl-Glc, the relative activity with sinapoyl-Glc and 4-coumaroyl-Glc was 39% and 44%, respectively, which corresponded with values published previously (Glässgen and Seitz 1992). Caffeoyl-Glc has not been prepared previously, but we succeeded in

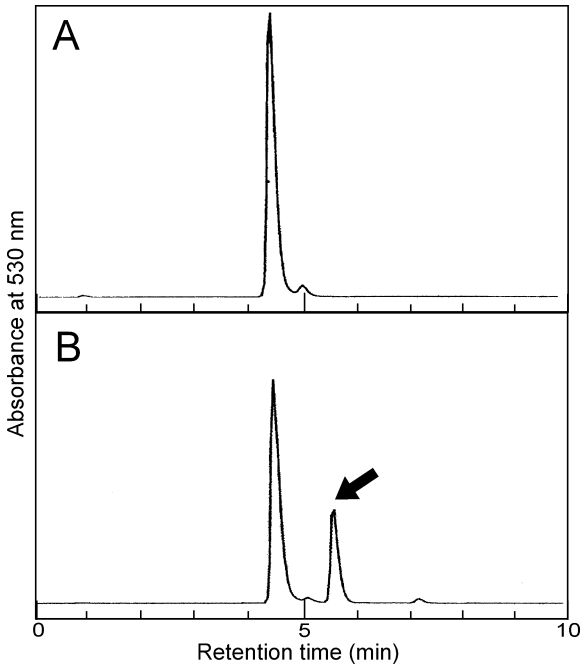

Figure 3. Reaction assay of HCA-Glc-dependent acyltransferase activity in the protein extracts prepared from a suspension of cultured cells of *Daucus carota* using sinapoyl-Glc as an acyl donor. The reaction mixture of HCA-Glc-dependent acyltransferase activity in a final volume of 30 μl, comprising 11–14 ng of the protein extract and 23 pmol of cyanidin 3-(Xyl-Glc-Gal) as an acyl acceptor in the absence (A) or presence (B) of sinapoyl-Glc (30 pmol) as an acyl donor. The reaction mixture was incubated at 30°C for 60 min, and the reaction was terminated by the addition of 1.5 μl of 20% phosphoric acid. The reaction mixture was centrifuged at 20,000×*g* for 5 min, and the supernatant was subjected to HPLC analysis. The peak at about 4.5 min of the HPLC profiles of A and B corresponds to the substrate, cyanidin 3-(Xyl-Glc-Gal), and the peak shown with an arrow corresponds to the product, cyanidin 3-(Xyl-sinapoyl-Glc-Gal), produced by HCA-Glc-dependent acyltransferase activity.

Table 1. HCA-Glc-dependent acyltransferase activity in protein extracts from anthocyanin-producing suspensions of cultured cells of *Daucus carota* and *Glehnia littoralis*.

acyl donor	pkat/mg protein (relative activity (%))	
	Daucus carota	*Glenhnia littoralis*
sinapoyl-glucose	149.9 (44.2)	55.3 (38.1)
feruloyl-glucose	339.1 (100.0)	145.0 (100.0)
caffeoyl-glucose	5.8 (1.7)	ND
4-coumaroyl-glucose	114.6 (39.4)	83.4 (57.5)

The reaction mixture of HCA-Glc-dependent acyltransferase activity in a final volume of 30 μl, comprising 11–14 ng of the protein extract, 23 pmol of cyanidin 3-(Xyl-Glc-Gal) as an acyl acceptor, and 30 pmol of sinapoyl-Glc, feruloyl-Glc, caffeoyl-Glc, or 4-coumaroyl-Glc as an acyl donor. The reaction mixture was incubated at 30°C for 60 min, and the reaction was terminated by the addition of 1.5 μl of 20% phosphoric acid. The mixture was then centrifuged at 20,000×*g* for 5 min, and the supernatant was subjected to HPLC analysis. The products were quantified using the HPLC areas with cyanidin as the standard with detection at 530 nm to calculate the moles of reaction products. We confirmed the linearity of the reaction over the range of reaction time and amount of protein extracts used here in our preliminary experiments. The relative activity of the individual *D. carota* and *G. littoralis* protein extracts for each acyl donor was presented relative to feruloyl-Glc as 100%. ND, not detectable.

preparing it by enzymatic synthesis; the transfer activity with caffeoyl moiety was extremely low in the protein extract from cultured *D. carota* cells (Table 1). Although the major anthocyanin molecule produced *in vivo* and accumulated in cultured cells of *D. carota* was cyanidin 3-(Xyl-sinapoyl-Glc-Gal), *in vitro* activity in the protein extract was highest for feruloyl-Glc.

To consider the discrepancy between the HCA-modifying property of anthocyanin molecules of carrot cells *in vivo* and *in vitro*, we analyzed the *in vitro* HCA-Glc-dependent acyltransferase activity of anthocyanin-producing cultured cells of *G. littoralis*, because *G. littoralis* is a taxonomically close species to *D. carota* and cyanidin 3-(Xyl-feruloyl-Glc-Gal) is the main pigment produced and accumulated in cultured *G. littoralis* cells (Miura et al. 1998). The only difference in the main pigment between *D. carota* and *G. littoralis* was their acyl moiety—sinapoyl or feruloyl. The protein extract was prepared similarly in cultured *G. littoralis* cells to that in cultured *D. carota* cells by introducing into the acyltransferase assay each HCA-Glc as an acyl donor with cyanidin 3-(Xyl-Glc-Gal) as an acyl acceptor. The protein extract from cultured *G. littoralis* cells *in vitro* clearly showed the highest activity with feruloyl-Glc. The activity was 38% for sinapoyl-Glc and 58% for 4-coumaroyl-Glc compared with the activity of feruloyl-Glc; these values were similar to those obtained using *D. carota* cells (Table 1). Here, the question was raised that if the *in vitro* HCA-Glc-dependent acyltransferase activity in cultured cells of the taxonomically close species *D. carota* and *G. littoralis* had similar preference for the substrate feruloyl-Glc, then why was the acyl moiety of the major anthocyanin molecules produced and accumulated *in vivo* completely different from the sinapoyl and feruloyl moiety, respectively. One possibility is the difference in the production and accumulation of the acyl donor molecules as substrates in the metabolic pools between these two species.

In order to determine the amount of *in vivo* acyl donors accumulated in cultured cells of *D. carota* and *G. littoralis*, 10 g (fresh weight) of cultured cells were extracted with 200 ml 80% MeOH for 14 h at 4°C, and then evaporated and dissolved in water. To avoid loss of HCA-Glc molecules during further purification steps such as column chromatography, we used authentic HCA-Glc molecules synthesized here to establish analytical conditions for HPLC analysis using a Develosil Combi RP-5 C_{30} column (Nomura Chemical Co., Ltd, Aichi, Japan) to separate and quantify them. No purification step was used for the concentrated extracts prepared above. The major HCA-Glc molecules accumulated in the cells of *D. carota* and *G. littoralis* were sinapoyl-Glc (314 pmol g^{-1} fresh weight) and

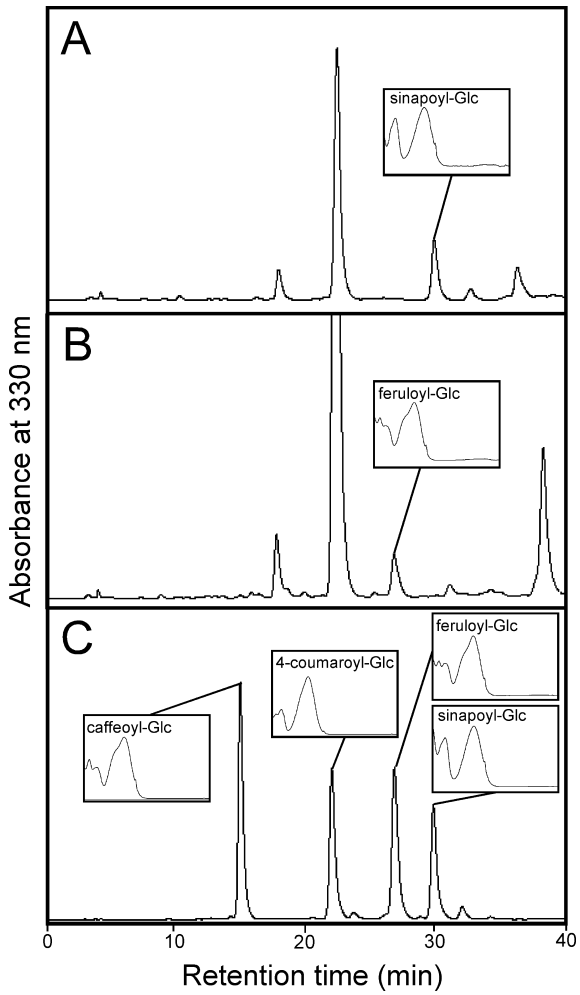

Figure 4. HPLC analysis of the extracts from cultured cells of *Daucus carota* (A), *Glehnia littoralis* (B), and authentic HCA-Glcs enzymatically synthesized here as the standard (C). The extracts that passed the filter were evaporated and resuspended in water and subjected to HPLC analysis using a Develosil Combi RP-5 C_{30} column (i.d. 4.6×250 mm; Nomura Chemical Co., Ltd, Aichi, Japan) with detection at 330 nm. HCA-Glcs were separated by linear gradient elution (1 ml min^{-1}) for 40 min with a 7%–14% gradient of solvent B (100% acetonitrile) in solvent A (1.5% phosphate in water). The peak fractions at 29.71 min in A and 26.81 min in B were introduced into ESI MS, and their molecular masses were confirmed to correspond to sinapoyl-Glc and feruloyl-Glc in C, respectively (data not shown).

feruloyl-Glc (59 pmol g^{-1} fresh weight), respectively (Figure 4). The retention times of the peaks of 22.22 min and 22.25 min in A and B (Figure 4), respectively, indicated that the molecules were not 4-coumaroyl-Glc. This was proved by cochromatography with authentic 4-coumaroyl-Glc (data not shown). These results strongly suggested that the specificity of HCA moieties of major anthocyanin molecules of *D. carota* and *G. littoralis* cultured cells might be dominated by the production and accumulation of different acyl donor molecules rather than by the substrate specificity of acyltransferase enzymes.

Our results should provide important clues for the modification and alteration of anthocyanin molecules by transgenic strategies to generate new flower colors. Recent approaches to manage these modifications and alterations concerning flower color have focused on the introduction of foreign genes for acyltransferases having different substrate specificities from the host plants (Katsumoto et al. 2007). In order to introduce foreign acyltransferases into transgenic plants, other gene(s) for the enzyme(s) involved in the supplement of acyl donor molecules should be introduced into the plants together with the acyltransferase genes.

We showed a method for the effective preparation of HCA-Glcs by enzymatic reaction. We plan to use them as substrates to analyze HCA-Glc-dependent acyltransferase activities by isolating their encoding cDNAs from cultured *D. carota* and *G. littoralis* cells. These cDNAs will give us detailed information on the characteristics and properties of each *D. carota* and *G. littoralis* acyltransferase. These acyl donor substrates will help in the characterization of enzyme activities of SCPL acyltransferase family members for anthocyanin and other metabolites.

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