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Identification of the glucosyltransferase that mediates direct flavone *C*-glucosylation in *Gentiana triflora*

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ABSTRACT

The major flavonoids accumulated in leaves of Japanese gentian (*Gentiana triflora*) were determined as isoorientin (luteolin 6-*C*-glucoside) and isoorientin 4′-glucoside. A cDNA (*GtUF6CGT1*) was isolated that encoded the UDP-glucose-dependent glucosyltransferase that is involved in *C*-glycosylflavone biosynthesis. The recombinant GtUF6CGT1 protein could transfer a glucose group to the C6 position of a flavone skeleton through C-linkage, using UDP-glucose as the glucosyl donor. These *C*-glycosylflavones also accumulated in petals. A good correlation was observed between *GtUF6CGT1* expression and *C*-glycosylflavone accumulation in leaves and petals. GtUF6CGT1 is the first reported *C*-glucosyltransferase that mediates direct *C*-glucosylation of the flavone skeleton.

1. Introduction

Flavonoids are a major class of plant secondary metabolites that are commonly accumulated in the vacuole as their glycosides, with some exceptions [1]. Flavone, a major class of flavonoids, is commonly conjugated with a sugar group through an *O*-glycosidic linkage. Some flavones exist as the *C*-glycosylated form and are often modified with *O*- and *C*-glycosylations. *C*-glycosylflavones are an important subgroup of flavonoids present in many plants [1]. *C*-glycosylflavones are involved in UV protection, defense against pathogens and inhibition of caterpillar growth [2]. *C*-glycosylflavones are also co-pigments for flower coloration, antioxidants and anti-diabetic compounds [3]. Flavonoid *O*-glycosides are commonly generated by the glycosylation of the flavone skeleton, mediated by uridine diphosphate (UDP)-sugar-dependent glycosyltransferase (UGT) [4]. The biosynthetic pathway of *C*-glycosylflavone was determined recently [4]. In 2009, Brazier-Hicks et al. first identified *C*-glucosyltransferases (CGTs) involved in the biosynthesis of *C*-glycosylflavone in rice (*Oryza sativa*). *C*-glycosylflavone is synthesized via glucosylation of the open-chain form of 2-hydroxyflavanone produced by the hydroxylation of flavanone mediated by a cytochrome P450 family protein, followed by enzymatic dehydration (Fig. 1, *Route 1*) [5,6]. Thus, in cereals, *C*-glycosylflavones are generated by *C*-glycosylation before the flavone skeleton is complete. The same pathway exists in buckwheat (*Fagopyrum esculentum*) [7].

Japanese-cultivated gentians, *Gentiana triflora*, *Gentiana scabra* and their hybrids, are popular ornamental flowers in Japan. They typically show blue to violet flower color, but some pink- or white-colored cultivars have been bred [8–10]. These cyanic flower colors arise from polyacylated anthocyanins and the structures of those compounds were determined as gentiodelphin [11,12]. Besides anthocyanins, Japanese-cultivated gentians accumulate unpigmented flavonoids, including flavone derivatives. Previously, we isolated the *flavone synthase II* (*FNSII*) gene, which is involved in flavone biosynthesis in gentian petals [13]. Recently, we characterized the transcription factors involved in the activation and repression of flavonoid biosynthetic genes in Japanese gentians [14,15].

Abbreviations: ABEE, *p*-aminobenzoic acid ethylester; CGT, *C*-glucosyltransferase; DTT, dithiothreitol; ESI-MS, electrospray ionization-mass spectrometry; EST, expressed sequence tag; FNSII, flavone synthase II; GT, glycosyltrasnferase; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PDA, photodiode array; qRT-PCR, quantitative real-time reverse transcription-PCR; RACE, rapid amplification of cDNA ends; TFA, trifluoroacetic acid; UDP, uridine diphosphate; UF6CGT, UDP-glucose:flavone 6-*C*-glucosyltrasnfease

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However, the molecular structures of these flavonoids have not been determined and no enzymatic genes involved in flavone modification have been isolated.

In this report, we attempted to determine the structures of flavones accumulated in Japanese gentian and to identify a cDNA encoding the UDP-glucose-dependent glucosyltransferase. The results showed that *C*-glycosylflavones accumulate in the leaves and petals of Japanese gentian. A cDNA, *GtUF6CGT1*, encoding a protein involved in the formation of flavone 6-*C*-glucosides was identified. In vitro assays using flavonoids as substrates demonstrated that the recombinant GtUF6CGT1 protein could transfer a glucose moiety to the 6 position of the flavone skeleton with a *C*-linkage. GtUF6CGT1 could catalyze direct *C*-glucosylation to the flavone skeleton with high stereo selectivity, which differs from previously reported CGTs.

2. Materials and methods

2.1. Plant materials and chemicals

Japanese-cultivated gentian (*G. triflora*) cv. Maciry was grown in the field of the Iwate Agricultural Research Center. The petals at developmental stages 1–4, as described in our previous report [16], young and mature leaves, and stems were collected and immediately frozen in liquid nitrogen and stored at −80 °C. 2-Hydroxynaringenin and norathyriol were purchased from ChemFaces (Wuhan, China). Maclurin was purchased from Sigma (St. Louis, MO, USA). Other flavonoids were purchased from Funakoshi (Tokyo, Japan).

2.2. Purification of flavonoids extracted from gentian leaves

Freeze-dried leaves (20 g, dry weight) were soaked in 80% methanol. The filtered extract was evaporated and dissolved in pure water. Flavonoids were absorbed to a Diaion HP20 column (Mitsubishi Chemical, Tokyo, Japan) equilibrated with pure water. Flavonoids were eluted with 50% methanol after washing with 20% methanol. Flavonoids were separated using high-performance liquid chromatography (HPLC; L-6250 Intelligent Pump, L-4250 UV–VIS detector, D-2500 Chromato-Integrator, Hitachi High-Technologies, Tokyo, Japan) equipped with a reverse-phase column (Inertsil ODS, 10 i.d. × 250 mm, GL Science, Tokyo, Japan). The first separation used a linear gradient elution (2 ml min^{-1}) of 5–60% methanol in 0.1% aqueous trifluoroacetic acid (TFA) for 40 min. Fractions were analyzed by an HPLC-PDA (HPLC-photodiode array detector; AS-4010 Auto sampler, L-6320 Intelligent pump, L-2455 Diode Array Detector, D-2000 Elite chromatography DATA station, Hitachi High-Technologies) at 1-min intervals and the fractions containing flavonoids were combined and subjected to the second purification step. The second cycle of HPLC separation used a linear gradient elution (2 ml min^{-1}) of 5–45% acetonitrile in 0.1% aqueous TFA for 40 min. The fraction containing compound 1 was subjected to flash chromatography (YFLC AI-580, Yamazen Corp., Osaka, Japan) equipped with a doubled reverse-phase column (Hi-Flash column, ODS-SM, 26 i.d. × 100 mm, Yamazen) using a linear gradient elution (10 ml min^{-1}) of 10–80% methanol in water for 40 min. Purified flavonoids were analyzed by electrospray ionization-mass spectrometry (ESI-MS, AccuTOF MS, JMS-T100LC, JEOL Ltd., Tokyo, Japan) and nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, ^{1}H{^{13}C} HMQC, ^{1}H{^{13}C} HMBC, ^{1}H-^{1}H COSY, and NOESY) in CD_3SOCD_3 on a JNM-ECA-500 spectrometer (JEOL). For ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), chemical shifts were referenced to the residual solvent (CD_3SOCD_3) signals at δ_H 2.50 and δ_C 39.5. The assignments of NMR signals are shown in Table S1. Purified compound 1 was hydrolyzed by treatment with 4 N HCl at 80 °C. The hydrolysate was labeled with *p*-aminobenzoic acid ethylester (ABEE) following the manufacturer's protocol (ABEE labeling kit, J-oil mills, Tokyo, Japan). Labeled sugars were separated by HPLC on a reverse-phase column (COSMOSIL5C18-MS-II, 4.6 mm i.d. × 50 mm) using isocratic elution (1.5 ml min^{-1}) of 0.2 M potassium borate buffer (pH 8.9), including 7% acetonitrile, for 5 min. The sugars were identified by comparing their retention times with standard hexoses (glucose and galactose).

2.3. C-glucosylation assay using the crude protein prepared from gentian young leaf

Frozen leaves (0.5 g) were ground into powder in liquid nitrogen using a mortar and pestle and then placed into 5 ml of extraction buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM dithiothreitol (DTT)). Cell debris was removed by centrifugation at 15 000×*g* for 5 min. Proteins were precipitated with 80% ammonium sulfate and re-suspended in extraction buffer. Crude protein was desalted using a G-25 spin column (GE Healthcare, Piscataway, NJ, USA). The reaction mixture comprised 100 mM Tris–Cl buffer, pH 8.5, 1 mM DTT, 0.2 mM of substrate and 40–60 μg of crude protein. A CBB protein assay kit (BioRad Laboratories, Hercules, CA, USA) determined the protein concentrations, using bovine serum albumin as the standard.

2.4. Cloning CGT candidate cDNAs and the production of recombinant proteins

UGT homologous cDNAs were searched for using our in-house expressed sequence tag (EST) database [14]. Six cDNA sequences containing putative ATG start codons were selected and PCR primers were designed to include the first ATG (Supple. Table S2). Total RNA was extracted from young leaves using an RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) following the manufacturer's manual. First strand cDNA was synthesized by rapid amplification of

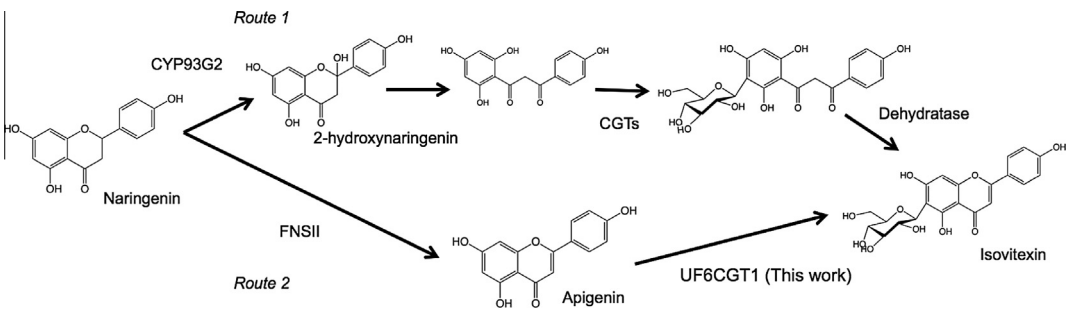

Fig. 1. The possible biosynthetic pathways for *C*-glycosylflavone. The pathways of *C*-glycosylflavone synthesis have been proposed as: *Route 1*, glycosylation occurs at the open-chain form of 2-hydroxyflavanone in rice and maize. *Route 2* illustrates the other possible pathway for *C*-glycosylflavone in Japanese gentian.