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cDNA cloning of glycosyltransferases from Chinese wolfberry (*Lycium barbarum* L.) fruits and enzymatic synthesis of a catechin glucoside using a recombinant enzyme (UGT73A10)☆

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Abstract

To obtain glycosyltransferases that are useful for structural and functional modification of bioactive compounds by glycosylation, an array of cDNAs encoding plant secondary product glycosyltransferases (PSPGs) were cloned from Chinese wolfberry (*Lycium barbarum* L.) fruits. The cloning strategy was based on high sequence conservation of the C-terminal region of PSPGs. Nine of these cDNAs were heterologously expressed in *Escherichia coli*, resulting in the production of catalytically active proteins, thereby allowing for potential specific glycosylation of a wide range of natural products. We found that one of these PSPGs, UGT73A10, uniquely displayed regiospecific glucosyl transfer activity toward flavan-3-ols (e.g., (+)-catechin and epigallocatechin gallate), which occur only rarely in nature as glucosides. Thus, the biochemistry of this enzyme was characterized in detail. The enzyme was then used as a biocatalyst of the regiospecific glucosylation of (+)-catechin. UGT73A10 was highly specific for the glycosyl donor, UDP-glucose, and showed broad acceptor specificity with the highest preference for naringenin (k_{cat}/K_m value, 0.097 s^{-1} μM^{-1}). UGT73A10 was phylogenetically related to the flavonoid 7-*O*-glucosyltransferases and, in fact, glucosylated the 7-position of naringenin. UGT73A10-catalyzed glucosyl transfer to (+)-catechin was obtained in high yield (83%). Surprisingly, spectroscopic analyses showed that the transfer product was 4′-*O*-β-D-glucopyranoside, and not the 7-*O*-β-D-glucoside, of (+)-catechin. Stability studies showed that the transfer product was more stable than (+)-catechin under alkaline conditions and at elevated temperatures.
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Keywords: Plant secondary product glucosyltransferase (PSPG); Catechin; Enzymatic synthesis; UGT73A10; *Lycium barbarum* L.

1. Introduction

Functional properties, such as solubility, physicochemical stability, bioactivity, pharmacokinetics, and cellular localization of natural products, such as flavonoids, are greatly affected by glycosylation, which is, therefore, an important factor to be considered in industrial applications of these products. Glycosylation of plant secondary products, such as flavonoids, coumarins, terpenoids, and cyanohydrins, is generally catalyzed by plant secondary product glycosyltansferases (PSPGs, [1]), which are family-1 glycosyltransferases [2–7], catalyzing glycosyl transfer from nucleoside diphosphate-activated sugars (donor) to aglycon substrates (acceptors). It has been shown that, in addition to their physiological substrates, some PSPGs are able to transfer glycosyl groups to a wide variety of compounds

☆ The nucleotide sequences of LbGT cDNAs reported in this paper have been submitted to DDBJ database under the following accession numbers: AB360610 (UGT74P1), AB360611 (UGT73E5), AB360612 (UGT73A10), AB360613 (UGT75A2), AB360614 (UGT74N1), AB360615 (UGT71A12), AB360616 (UGT84A12), AB360617 (UGT74N2), AB360618 (UGT85A18), AB360619 (UGT74P2), AB360620 (UGT75L2), AB360621 (UGT75A3), AB360622 (UGT74B2), AB360623 (UGT85A20), AB360624 (UGT86A4), AB360625 (UGT73Q1), AB360626 (UGT73A12), AB360627 (UGT86A5), AB360628 (UGT85A12), AB360629 (UGT94E1), AB360630 (UGT71A14), AB360631 (UGT73E6), AB360632 (UGT72B10), and AB360633 (UGT94E2).

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in a regiospecific manner [8]. Thus, these enzymes efficiently catalyze specific modification of bioactive compounds [5].

The primary objective of the present study was to obtain cDNAs for an array of PSPGs that are able to allow for glycosylation of a wide variety of natural products of industrial importance. We chose *Lycium barbarum* L. (Chinese wolfberry) as the plant source of PSPG cDNAs, as the fruits (known as *Lycii fructus*), leaves (*Lycii folium*), and roots (*Lycii cortex radicis*) of this plant are used in Asian medicine and foods [9]. This plant species was expected to be a good source of PSPGs with different specificities, because a large number of glycosides (e.g. diterpenes, monoterpens, frostanols, tryptophan derivatives [10], flavonols [11], and ascorbic acid glycosides [12]) have been isolated from the *Lycium* species [13–18].

First, we isolated 24 PSPG cDNAs from a cDNA library of *L. barbarum* L. fruits by means of PCR using a strategy based on the conservation of the sequence of ∼45 amino acid residues (called the PSPG box) in the C-terminal domain [2]. Nine of these cDNAs successfully yielded catalytically active *L. barbarum* glycosyltransferases, termed LbGTs, which have the potential to biotransform a wide variety of compounds.

Second, we tested the LbGTs for their ability to glucosylate flavan-3-ols. Flavan-3-ols are plant flavonoids found mainly in tea (*Camellia sinensis*) leaves, which display bioactivities of nutritional and biomedical interest, such as strong antioxidant and antimicrobial activities. In tea leaves these flavonoids generally occur in the non-glycosylated form and are rather unstable and susceptible to oxidation in aqueous systems. Glycoconjugates of flavan-3-ols are expected to be more soluble [19,20] and stable in such systems and, therefore, are advantageous in pro-drug and dietary formulations. We found that one of the LbGTs, UGT73A10, was able to regiospecifically glucosylate flavan-3-ols, such as (+)-catechin (i.e., (2*R*, 3*S*)-3,5,7,3′,4′-pentahydroxyflavan) and epigallocatechin gallate (i.e., (2*S*, 3*S*)-3,5,7,3′,4′,5′-hexahydroxyflavan gallate] in high yields. The biochemistry of the UGT73A10 enzyme was characterized in detail; the structure of the glucoconjugate of (+)-catechin was determined, and its chemical stability was examined.

2. Materials and methods

2.1. Plant materials and chemicals

L. barbarum L. is widespread throughout temperate regions, including China and Japan [9]. Mature and immature *L. barbarum* L. fruits were harvested from a species cultivated in Ningxia and Neimonggol, China, and were frozen at −80 °C until use. (*S*)-Naringenin, (+)-catechin hydrate, benzoic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, salicylic acid, salicyl alcohol, hydroquinone, caffeic acid, and *trans*-*p*-coumaric acid were purchased from Nacalai Tesque (Kyoto, Japan). Esculetin was purchased from Tokyo Kasei Industries (Tokyo, Japan). Kaempferol and quercetin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Apigenin and naringenin 7-*O*-glucoside were purchased from Funakoshi (Tokyo, Japan). Genistein, genistin, daidzein, and daidzin were products of Fujicco (Kobe, Japan). Capsaicin, UDP-glucose, UDP-galactose, and UDP-glucuronic acid were purchased from Sigma (St. Louis, MO, USA). Epigallocatechin gallate was a product of DSM Nutritional Products (Basel, Switzerland). Betanidin was prepared by treatment of betanin (red beet powder; Mitsubishi Kagaku Foods, Tokyo, Japan) with β-glucosidase (from almond; Sigma) followed by reversed-phase HPLC on a *J'*sphere ODS-M80 column (4.6 mm × 150 mm, YMC, Kyoto, Japan). Aureusidin was isolated and purified as described previously [21]. All other chemicals were of analytical grade.

2.2. cDNA library construction

Poly(A)$^+$ RNA was isolated from immature fruits of *L. barbarum* L. using the Straight A's^{TM} mRNA Isolation System (Novagen, Madison, WI, USA). cDNA was synthesized from 5 μg of poly(A)$^+$ RNA using the ZAP-cDNA^{TM} synthesis kit (Stratagene, La Jolla, CA, USA). The cDNA was ligated with EcoRI adapters and inserted into the Uni-ZAP XR vector (Stratagene). The resulting constructs were packaged using the Gigapack III Gold packaging extract (Stratagene). The resulting primary library contained 500,000 plaque-forming units.

2.3. Screening and sequencing of glucosyltransferase genes

Based on the highly conserved region of PSPGs (PSPG box), two degenerate PCR primers were designed: PSPG1, 5′-TT(C/T)ITIACICA(C/T)TG(C/T)GGITGGAA-3′; and, PSPG2, 5′-TG(C/T)GGITGGAA(C/T)TCI(A/G)(C/T)I(C/T)TIGA-3′. Total RNA was prepared from mature fruits of *L. barbarum* L. using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The reverse transcription-polymerase chain reaction (RT-PCR) was performed using the QIAGEN OneStep RT-PCR Kit (Qiagen) with PSPG1, oligo dT primer, and total RNA as the template. Thermal cycling conditions were as follows: the RT-PCR mixture was incubated at 50 °C for 30 min for reverse transcription and then at 95 °C for 15 min for the activation of DNA polymerase and inactivation of reverse transcriptase; 30 cycles of PCR (one cycle consisted of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min); and, a final incubation at 72 °C for 10 min. A portion of the RT-PCR product was used as the template for nested PCR using PSPG2 and oligo dT primers. Thermal cycling conditions used in the nested PCR were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and, finally, 72 °C for 10 min. The amplified fragments, which were about 500 bp in length, were cloned into TOPO-pCR2.1 (Invitrogen, Carlsbad, CA, USA). Randomly selected clone inserts were sequenced using a Dye-Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA) with a CEQ 2000 DNA analysis system (Beckman Coulter). This process gave 17 clones, all of which displayed significant similarity to PSPGs. The cloned fragments were DIG-labeled using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Basel, Switzerland), and then were used as probes to screen the cDNA library (500,000 plaques).

Hybridization was performed at 37 °C for 16 h in 5× SSC containing 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine, 2% (w/v) blocking reagent (Roche Molecular Biochemicals), and 30% (v/v) formamide. The filters were either washed twice in high-stringency conditions – 0.1 × SSC and 0.1% (w/v) SDS at 60 °C for 15 min or twice in low-stringency conditions – 2 × SSC and 1% (w/v) SDS at 50 °C for 15 min. A DIG-DNA Labeling and Detection Kit (Roche Molecular Biochemicals) was used to detect DIG-labeled DNA. The cDNAs of positive clones were cloned into the pBleuscript SK-phagemid following the *in vivo* excision protocol and then sequenced. The 5′-fragments of partial cDNAs were obtained using gene-specific primers (not shown) and a 5′RACE System for Rapid Amplification of cDNA Ends (Invitrogen).

2.4. Heterologous expression of cDNA in Escherichia coli and purification of the expressed product

The coding sequences of *LbGT* cDNAs were amplified by PCR using primers (not shown) with appropriate restriction sites for ligation into the expression vector. The amplified fragments were cloned into a pCR-Blunt II-TOPO vector using a kit (Zero Blunt TOPO PCR Cloning Kit, Invitrogen) and sequenced to confirm the absence of PCR errors. The plasmids were digested with appropriate restriction enzymes, and the resulting DNA fragments were ligated into a pET-15b vector (Novagen) that had previously been digested with the appropriate restriction enzymes to obtain the plasmid pET-15b-LbGT, which encoded an N-terminal in-frame fusion of LbGT with a His_6 tag. *E. coli* BL21(DE3) cells were transformed with the resulting plasmids. After transformant cells were pre-cultured at 37 °C for 16 h in Luria–Bertani broth medium containing 50 μg/ml ampicillin, the culture (100 ml for UGT73A10, 6 ml for other LbGT) was inoculated into the same medium (4000 ml for UGT73A10, 300 ml for other LbGT). After cultivating the cells at 22 °C until the OD_{600} of the culture reached 0.6, isopropyl 1-β-D-thiogalactoside was added to the medium at a final concentration of 0.4 mM, followed by cultivation at 22 °C for 16 h. All subsequent operations were conducted at 0–4 °C. The cells were harvested by centrifugation (15 min, 5000 × *g*), washed with distilled water and resuspended in buffer A (20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl, 14 mM β-mercaptoethanol) containing 30 mM imidazole. The cell suspension was sonicated ten times for 1 min and the resulting cell debris was removed by centrifugation (15 min, 5000 × *g*). Polyethyleneimine was added at a final concentration of 0.12%, and the mixture was allowed to stand for 30 min. After centrifugation (15 min, 5000 × *g*), the supernatant was applied to a 1-ml HisTrap HP column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with buffer A containing 30 mM imidazole. The column was washed with buffer A containing 30 mM imidazole, and the enzyme was eluted with buffer A containing 200 mM imidazole. The column-bound fractions were concentrated and desalted using Amicon Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA, USA), followed by substitution with buffer B (20 mM potassium phosphate, pH 7.2, containing 14 mM β-mercaptoethanol). For UGT73A10, an extra purification procedure was carried out as follows. The enzyme solution was applied to a Mono Q HR 16/10 column (GE Healthcare Bio-Sciences) equilibrated with buffer B at a rate of 2 ml/min using an ÄKTAsystem (GE Healthcare Bio-Sciences). After washing the column with buffer B, the enzyme was eluted with a linear gradient of 0–420 mM NaCl in buffer B. The concentration of UGT73A10 was determined from the absorption coefficient of the purified enzyme, ε_{280} of 63910 M^{-1} cm^{-1}, which was calculated from the amino acid sequence. SDS-PAGE was carried out according to the method of Laemmli [22], and the proteins in the gels were visualized by Coomassie Brilliant Blue R250.

2.5. Enzyme assay

Method A (for UGT73A10): The standard reaction mixture (100 μl) consisted of 200 μM glucosyl acceptor, 216 μM UDP-glucose, 50 mM potassium phosphate (pH 7.2), and enzyme. The mixture without enzyme was pre-incubated at 30 °C for 10 min, and the reaction was started by addition of the enzyme. After incubation at 30 °C for 20 min, the reaction was stopped by the addition of 150 μl of 0.5% (v/v) trifluoroacetic acid. The reaction products were analyzed by reversed-phase high-performance liquid chromatography (HPLC) on a COSMOSIL $5C_{18}$-MS-II column (4.6 mm × 150 mm, Nacalai Tesque). The substrates and products were separated using a linear gradient of 4.5 to 90% (v/v) CH_3CN containing 0.5% (v/v) trifluoroacetic acid for 15 min, followed by isocratic elution with 90% CH_3CN containing 0.5% (v/v) trifluoroacetic acid for 5 min at a flow rate of 1 ml/min. The substrates and products were detected at 280 nm using an SPD-10A *VP* UV–vis detector (Shimadzu, Kyoto, Japan).

To determine the initial velocity of UGT73A10, the assays were carried out under steady-state conditions using the standard assay system (see above) with various substrate concentrations. The apparent K_m and V_{max} values for the glucosyl donor and acceptor substrates in the presence of a saturating concentration of the counter substrate were determined by fitting the initial velocity data to the Michaelis–Menten equation using non-linear regression analysis [23,24].

Method B (for other LbGTs): The standard reaction mixture (100 μl) was comprised of 100 μM glucosyl acceptor, 100 μM UDP-glucose, 50 mM potassium phosphate (pH 7.5), and enzyme. The mixture without the enzyme was pre-incubated at 30 °C for 10 min, and the reaction was started by addition of the enzyme. After incubation at 30 °C for 30 min, the reaction was stopped by addition of 100 μl of 0.5% (v/v) trifluoroacetic acid. The enzyme products were analyzed by HPLC on a Develosil column (C30-UG-5) (4.6 mm × 150 mm, NOMURA CHEMICAL, Aichi, Japan). The substrates and products were separated using a linear gradient of 4.5–45% (v/v) CH_3CN containing 0.1% (v/v) trifluoroacetic acid for 20 min at a flow rate of 1 ml/min. The substrates and products were detected using an L-7455 diode array detector (HITACHI, Tokyo, Japan). Relative activity was estimated based on the total amount of all transfer products.

2.6. Enzymatic synthesis and examination of stability of catechin glucoside

For enzymatic synthesis of catechin glucoside using UGT73A10, the reaction mixture (5.2 ml) contained 3.25 mg (+)-catechin hydrate, 16 mg UDP-glucose, 50 mM potassium phosphate (pH 7.5), 25%(v/v) glycerol, and 0.83 mg UGT73A10. The mixture was incubated at 30 °C for 66 h, and the reaction was stopped by addition of 0.52 ml of 5% (v/v) trifluoroacetic acid. The resulting mixture was applied to a Sephadex LH20 column (2.5 cm × 20 cm, GE Healthcare Biosciences) equilibrated with distilled water. The column was washed with distilled water, and the reaction product was eluted with 50% (v/v) ethanol. The product was lyophilized and subjected to spectroscopic analyses. NMR spectra (i.e., ^{1}H and ^{13}C NMR, double quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence spectroscopy (HSQC), rotating frame overhauser effect spectroscopy (ROESY), and heteronuclear multiple bond correlation spectroscopy (HMBC) spectra) in dimethylsulfoxide-d_6 were performed at 25 °C using a Bruker DMX-750 spectrometer (Bruker, Rheinstetten, Germany) operated at 750 MHz. ^{13}C NMR was performed using a Bruker DMX-500 spectrometer (Bruker) operated at 125 MHz. Positive mode time-of-flight mass spectrometric analyses were carried out using a z-spray ion source (Micromass, Manchester, UK).

To compare the stability of the glucoconjugate of (+)-catechin under acidic and alkaline conditions with that of (+)-catechin, the flavonoids (100 μM) were treated either with 0.1 M HCl or NaOH for 1, 2, 4, 8 and 19 h at 25 °C. After the treatment with either acid or base, the mixture was neutralized by addition of two volumes of 1 M potassium phosphate (pH 7.2). To compare the thermal stability of the glucoside of (+)-catechin with that of (+)-catechin, the flavonoids (100 μM) were exposed to temperatures of 4, 60, 80 and 100 °C for 1, 2, and 4 h at pH 7.0. The mixtures were chilled on ice and two volumes of 0.1% (v/v) trifluoroacetic acid were added. The remaining (+)-catechin and catechin glucosides were quantified using HPLC as described in Section 2.5.

3. Results and discussion

3.1. cDNA cloning and phylogenetics of LbGTs

Seventeen PSPG cDNA fragments (~500 bp in length) were obtained from an RNA sample extracted from mature fruits of *L. barbarum* L. by RT-PCR using primer sets designed on the basis of the amino acid sequence of the PSPG box. Four full-length and twenty partial PSPG cDNAs were isolated by plaque hybridization of the cDNA library (~500,000 plaques in size) using the DIG-labeled partial cDNA fragments as probes under low- and high-stringency conditions. The 5′-portions of cDNAs, if missing, were clarified by means of 5′-rapid amplification of cDNA ends using gene-specific primers. A total of 24 full-length cDNAs of different PSPGs were obtained and collectively were referred to as *LbGT*s. According to the glycosyltransferase nomenclature guidelines [25], the systematic names of the LbGTs are as follows: UGT71A12, UGT71A14, UGT72B10, UGT73A10, UGT73A12, UGT73E5, UGT73E6, UGT73Q1, UGT74B2, UGT74N1, UGT74N2, UGT74P1, UGT74P2, UGT75A2, UGT75A3, UGT75L2, UGT84A12, UGT85A12, UGT85A18, UGT85A20, UGT86A4, UGT86A5, UGT94E1, and UGT94E2 (see also Fig. 1).

A phylogenetic tree was constructed for the LbGTs along with other PSPGs by means of the neighbor-joining method (Fig. 1). Currently, PSPG function and specificity cannot be fully predicted based on sequence information alone. However, the phylogenetic tree of functionally characterized PSPGs showed several clusters [2,6,7,26,27] (see Fig. 1), which were, at least in part, characterized by the specificity of their flavonoid glycosyltransferase activities. This observation provides useful information regarding the choice of PSPGs as biocatalysts for synthetic purposes. For example, Clusters I, II, and III (Fig. 1) are characterized by flavonoid 3-*O*-glycosyltransferase (e.g., UGT78 PSPGs), flavonoid 5-*O*-glycosyltransferase (e.g., UGT75 PSPGs), and flavonoid 7-*O*-glycosyltransferase (UGT73 and UGT89 PSPGs) activity, respectively. However, Cluster III also contains glycosyltransferases that are capable of glycosylating the 4′-position of flavonoids (e.g., [28]) or that are unrelated to flavonoid biosynthesis (e.g., TOGT1 and 2 from *Nicotiana tabacum* [29], abscisic acid glucosyltransferase from *Vigna angularis* [30], and UGT73C5 from *Arabidopsis thaliana* [31]). Cluster IV contains PSPGs that catalyze glycosyl transfer to sugar moieties of flavonoid glycosides [2–7]. As expected from the wide variety of glycosides in *Lycium* species (see Section 1), PSPGs that were obtained from *L. barbarum* L. fruits were diversely distributed throughout the phylogenetic tree, suggesting that the LbGTs obtained were diverse with regards to specificity. Thus, the LbGTs have the potential to specifically glycosylate a wide range of natural products, including flavonoids (see below).

3.2. Heterologous expression and glucosyltransferase activity of LbGTs

All of the *LbGT* cDNAs obtained in this study were expressed under the control of the T7 *lac* promoter in *E. coli* BL21(DE3) cells as His_6 tagged fusion proteins. Crude extracts of the transformant cells were subjected to chromatography using a nickel-affinity column. When the column-bound fractions were incubated with various glucosyl acceptors and UDP-glucose, we found that nine LbGTs, i.e., UGT71A14, UGT72B10, UGT73A10, UGT73A12, UGT73Q1, UGT74N2, UGT75L2, UGT84A12, and UGT86A4, exhibited glucosyltransferase activity toward flavonoids and coumarins (Supplementary Fig. 1). Among these LbGTs, UGT73A10 uniquely displayed a weak, but significant, level of glucosyltransferase activity toward flavan-3-ols (i.e., (+)-catechin and epigallocatechin gallate). This specificity of UGT73A10 is noteworthy; although the glucosides of these flavonoids rarely occur in nature, glycosylation of these flavonoids increases their industrial significance (see Section 1). Thus, the specificity and molecular properties of UGT73A10 were examined in detail (see Section 3.3). The kinetic properties and specificities of some of the other LbGTs

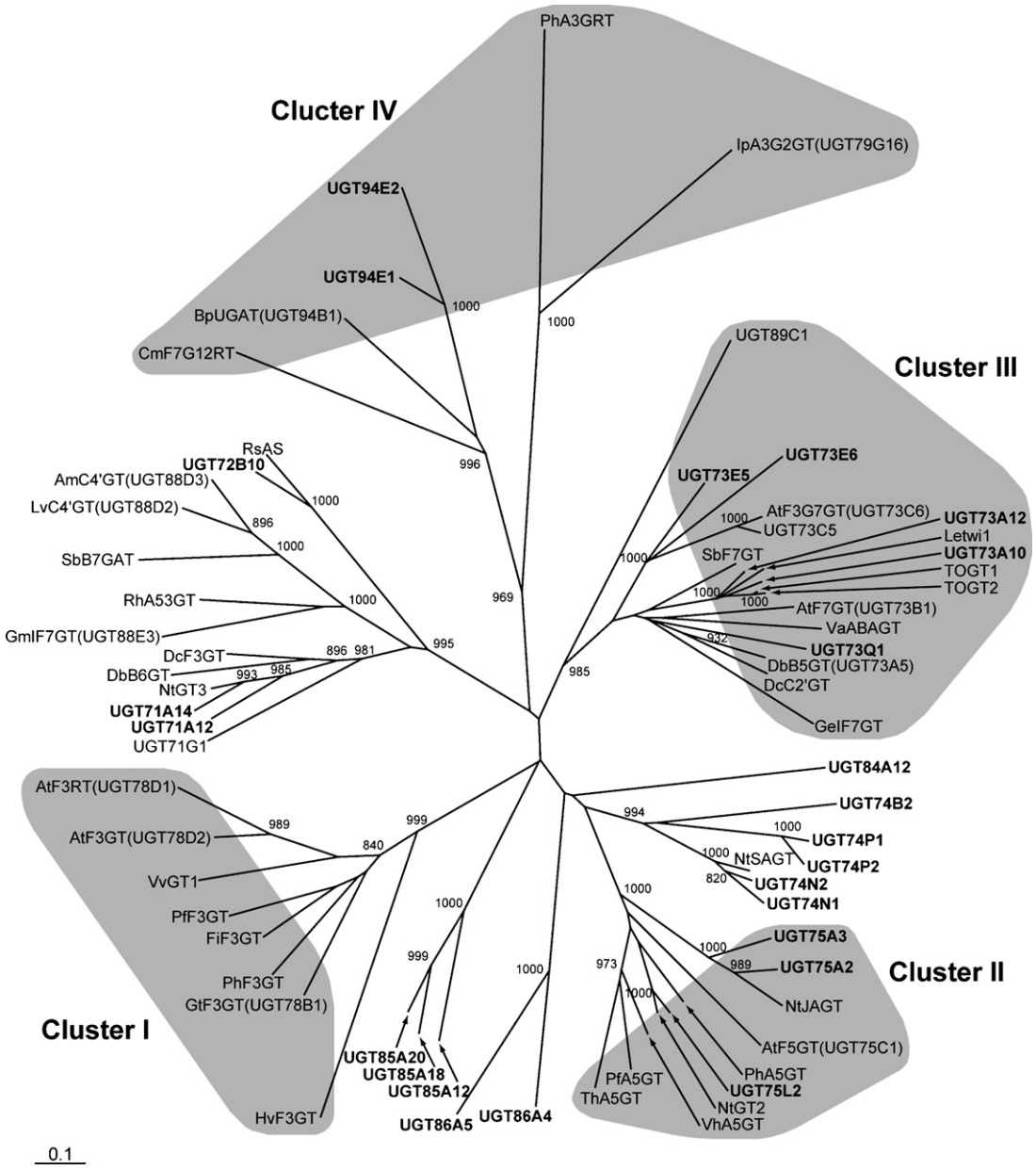

Fig. 1. Phylogenetic trees of PSPGs. LbGTs are shown in boldface letters. The sequences used for the alignment were primarily those of PSPGs that have been functionally characterized. The tree was constructed from a ClustalW program multiple alignment using the neighbor-joining method of TreeView [37]. Bar = 0.1 amino acid substitution/site. Numbers indicate bootstrap values >800. Known clusters (Cluster I, II, III, and IV) of PSPGs [1,2,7,26] are shown with gray background. The names and DDBJ/GenBankTM/EBI accession numbers of PSPGs used for the alignment are as follows: AtF3G7GT, *Arabidopsis thaliana* UDP-glucose: flavonol-3-*O*-glycoside 7-*O*-glucosyltransferase (UGT73C6; accession number Q9ZQ95); AtF7GT, *A. thaliana* flavonoid 7-*O*-glucosyltransferase (UGT73B1; accession number AAL90934); AtF5GT, *A. thaliana* flavonoid 5-*O*-glucosyltransferase (UGT75C1; accession number AAM91686); AtF3GT, *A. thaliana* flavonoid 3-*O*-glucosyltransferase (UGT78D2; accession number AAM91139); AtF3RT, *A. thaliana* flavonoid 3-*O*-rhamnosyltransferase (UGT78D1; accession number AAM91139); AmC4′GT, *Antirrhinum majus* UDP-glucose:chalcone 4′-*O*-glucosyltransferase (UGT88D3; accession number BAE48239); BpUGAT, *Bellis perennis* UDP-glucuronic acid:anthocyanidin-3-*O*-glucoside 2″-*O*-glucuronosyltransferase (UGT94B1; accession number AB190262); CmF7G12RT, *Citrus maxima* UDP-rhamnose:flavonoid-7-*O*-glycoside 1,2-*O*-rhamnosyltransferase (accession number AAL06646); DbB5GT, betanidin 5-*O*-glucosyltransferase from *Dorotheanthus bellidiformis* (UGT73A5; accession number CAB56231); DbB6GT, betanidin 6-*O*-glucosyltransferase from *D. bellidiformis* (accession number AAL57240); DcC2′GT, *Dianthus caryophyllus* UDP-glucose:chalcononaringenin 2′-*O*-glucosyltransferase (accession number BAD52006); DcF3GT, *D. caryophyllus* UDP-glucose:flavonol 3-*O*-glucosyltransferase (accession number BAD52004); FiF3GT, *Forsythia intermedia* UDP-glucose:flavonoid 3-*O*-glucosyltransferase (accession number AAD21086); GeIF7GT, *Glycyrrhiza echinata* UDPglucose: isoflavonoid 7-*O*-glucosyltransferase (accession number BAC78438); GmIF7GT, *Glycine max* UDP-glucose:isoflavone 7-*O*-glucosyltransferase (UGT88E3; accession number AB292164); GtF3GT, *Gentiana triflora* flavonoid 3-*O*-glucosyltransferase (UGT78B1; accession number BAA12737); HvF3GT, *Hordeum vulgare* UDP-glucose:flavonoid 3-*O*-glucosyltransferase (accession number CAA33729); IpA3G2GT, *Ipomoea purpurea* UDP-glucose:anthocyanidin-3-glucoside 2″-*O*-glucosyltransferase (UGT79G16; accession number BAD95882); Letwi1, *Lycopersicon esculentum* probable glucosyltransferase *twi1* (accession number X85138); LvC4′GT, *Linaria vulgaris* UDP-glucose:chalcone 4′-*O*-glucosyltransferase (accession number BAE48240); NtGT2, *Nicotiana tabacum* glucosyltransferase-2 (accession number BAB88935); NtGT3, *N. tabacum* glucosyltransferase-3 (accession number BAB88934); NtSAGT, *N. tabacum* UDP-glucose:salicylic acid glucosyltransferase (accession number AAF61647); NtJAGT, *N. tabacum* jasmonate-induced glucosyltransferase (accession number T02238); PfF3GT, *Perilla frutescens* UDP-glucose:flavonoid 3-*O*-glucosyltransferase

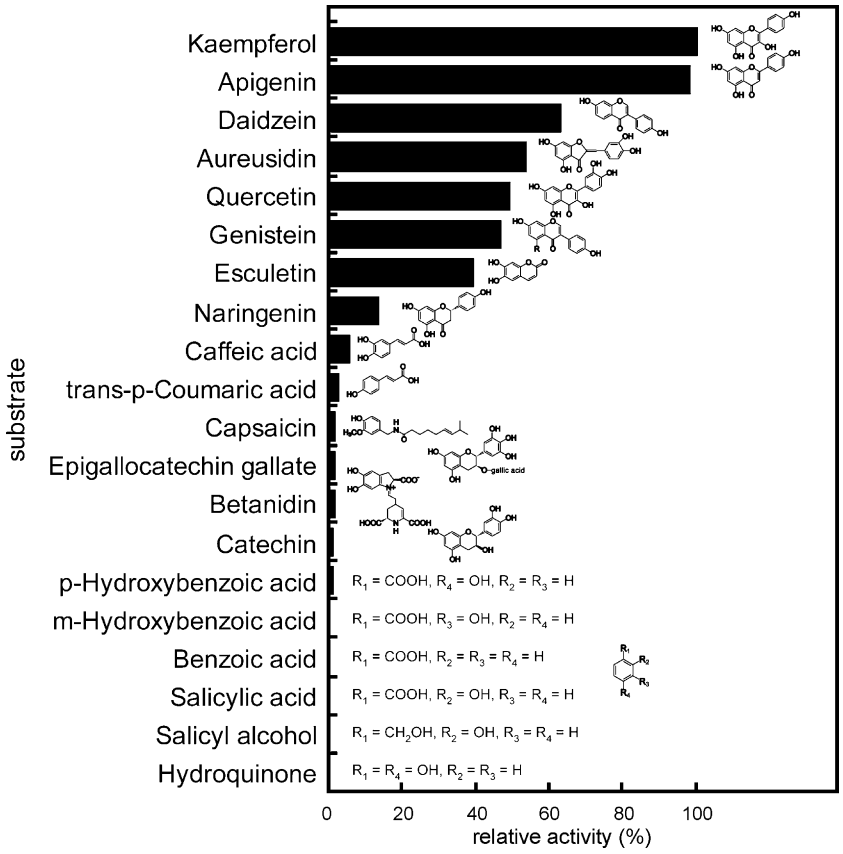

Fig. 2. Substrate specificity of UGT73A10. Relative activities of UGT73A10 toward flavonoids and related compounds (final concentration, 200 μM) are shown. Glucosyl transfer activity was assayed at a final UDP-glucose concentration of 216 μM as described in Section 2.5. Activity toward kaempferol was set equal to 100%. Structures of the aglycons are shown.

will be reported elsewhere. It should be mentioned that LbGTs other than those listed above were unable to glucosylate these phenolics, probably because they were neither soluble nor correctly folded into their catalytically active forms; alternatively, it is possible that they could utilize neither UDP-glucose as the glycosyl donor nor the phenolics examined here as the glycosyl acceptor.

3.3. Biochemical characterization of UGT73A10

UGT73A10 was stably and abundantly expressed in *E. coli* cells. The enzyme showed appreciable activity toward the following phenolics: kaempferol (relative activity, 100%), apigenin (98%), daidzein (63%), aureusidin (53%), quercetin (49%), genistein (47%), esculetin (39%), naringenin (13%), caffeic acid (5.6%), trans-*p*-coumaric acid (2.7%), capsaicin (1.5%), betanidin (1.4%), epigallocatechin gallate (1.4%), catechin (1.1%), *p*-hydroxybenzoic acid (0.9%), and *m*-hydroxybenzoic acid (0.4%). None of the following aromatic compounds served as glucosyl acceptors: benzoic acid, salicylic acid, salicyl alcohol, and hydroquinone (Fig. 2). HPLC analysis showed that the reactions of UGT73A10 with esculetin, naringenin, capsaicin, betanidin, and catechin yielded a single transfer product, whereas reaction with other substrates yielded two or more transfer products. All of these transfer products were hydrolyzed by β-glucosidase (from sweet almond) to produce aglycons, indicating that these are β-glucosides. The transfer product obtained from naringenin, as well as one of the transfer products obtained from apigenin, quercetin, daidzein and genistein, were co-eluted with their 7-*O*-glucosides in analytical HPLC (data not shown).

(accession number BAA19659); PfA5GT, *P. frutescens* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (accession number BAA36421); PhF3GT, *Petunia hybrida* UDP-glucose:anthocyanin 3-*O*glucosyltransferase (accession number BAA89008); PhA5GT, *P. hybrida* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (accession number BAA89009); PhA3GRT, *P. hybrida* UDP-rhamnose:anthocyanidin 3-*O*-glycoside rhamnosyltransferase (accession number CAA50376); RhA53GT, *Rosa hybrida* UDP-glucose:anthocyanidin 5,3-*O*-glucosyltransferase (accession number BAD99560); RsAS, *Rauvolfia serpentine* hydroquinone glucosyltransferase (arbutin synthase; accession number CAC35167); SbB7GAT, *Scutellaria baicalensis* UDP-glucuronate:baicalein 7-*O*-glucuronosyltransferase (accession number BAC98300); SbF7GT, *S. baicalensis* UDP-glucose:flavonoid 7-*O*-glucosyltransferase (accession number BAA83484); ThA5GT, *Torenia hybrida* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (accession number BAC54093); TOGT1, *N. tabacum* phenylpropanoid:glucosyltransferase 1 (accession number, AAK28303); TOGT2, *N. tabacum* phenylpropanoid:glucosyltransferase 2 (accession number, AAK28304); UGT71G1, *Medicago truncatula* triterpene UDP-glucosyltransferase (accession number AAW56092); UGT73C5, *A. thaliana* deoxynivalenol glucosyltransferase (accession number NP_181218); UGT89C1, *A. thaliana* flavonol 7-*O*-rhamnosyltransferase (accession number AAP31923); VaABAGT, *Vigna angularis* abscisic acid glucosyltransferase (accession number BAB83692); VhA5GT, *Verbena hybrida* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (accession number BAA36423); VvGT1, *Vitis vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase-1 (accession number AAB81682).

(a)

Position	^{1}H δ (ppm)	^{1}H J (Hz)	^{13}C δ (ppm)
(+)-Catechin			
2	4.6	d; 7.1	80.6
3	3.9	dd; 7.0, 6.1	66.1
4a	2.6	dd; 16, 4.5	27.7
4b	2.4	dd; 16, 7.9	
5			156.2
6	5.9	d; 2.3	95.1
7			156.5
8	5.7	brs	93.7
9			155.2
10			99.0
1'			134.5
2'	6.8	brs	116.4
3'			146.4
4'			144.8
5'	7.1	d; 8.2	114.6
6'	6.7	brd; 8.3	118.3
Glucose			
1"	4.6	d; 7.3	102.4
2"	3.3	dd; 9, 7	73.3
3"	3.3	t; 9	75.8
4"	3.2	t; 9	69.8
5"	3.3	m	77.2
6" a	3.7	brd; 12, 1	60.7
6" b	3.5	dd; 12, 5	

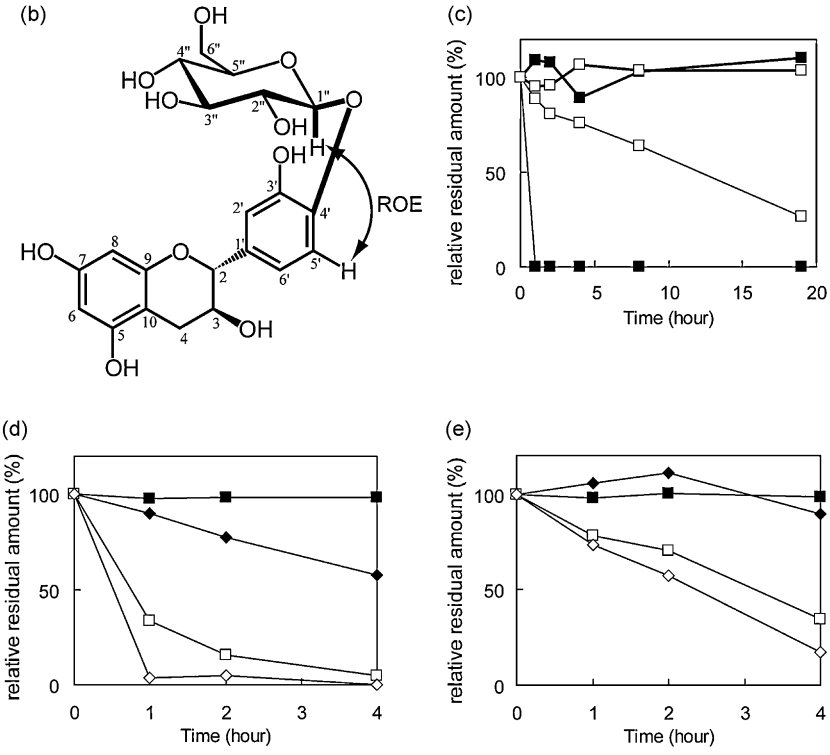

Fig. 3. NMR assignments, structure, and stability of the product of UGT73A10-catalyzed glucosyl transfer to (+)-catechin. (a) Assignment of NMR spectra of the reaction product. (b) Structure of the reaction product was confirmed by ROESY and HMBC analyses. Rotating frame overhauser enhancement detected on ROESY is indicated by a bent double-headed arrow and the HMBC cross peak is indicated by thick lines. (c) Comparison of the stabilities of (+)-catechin (closed square) and its glucoside (open square) under acidic (in 0.1 M HCl, thick lines) and alkaline (in 0.1 M NaOH, thin lines) conditions. (d and e) Thermal stability of (+)-catechin (d) and its glucoside (e) at 4 °C (closed square), 60 °C (closed rhombus), 80 °C (open square), and 100 °C (open rhombus).

These results, along with the fact that UGT73A10 is a member of Cluster III (see above), suggested that this enzyme might be a flavonoid 7-*O*-glucosyltransferase with broad acceptor specificity. HPLC analysis also showed that the reaction products of UGT73A10-catalyzed glucosyl transfer to esculetin, betanidin, and capsaicin co-eluted with esculetin 6-*O*-glucoside (esculin), betanidin 5-*O*-glucoside (betanin), and capsaicin 4-*O*-glucoside, respectively [32–34]. The k_{cat} and K_m values of several acceptor substrates determined at pH 7.2 and 30 °C are shown in Table 1. Among the substrates examined, naringenin was the best substrate, judging from kinetic parameters, with a k_{cat}/K_m value of 0.097 s^{-1} μM^{-1}. The glycosyl-donor specificity of UGT73A10 was examined using UDP-glucose, UDP-glucuronic acid, UDP-galactose, ADP-glucose, GDP-glucose, and TDP-glucose with esculetin as the glycosyl acceptor. Only UDP-glucose could be utilized, indicating enzyme specificity for UDP-glucose. The calculated K_m for UDP-glucose at pH 7.2 and 30 °C, using esculetin as the glucosyl acceptor, was 150 ± 30 μM (Table 1).

Table 1
Kinetic parameters of UGT73A10[a]

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Esculetin	2.7 ± 0.1	150 ± 20	0.0172
Naringenin	0.89 ± 0.10	9.3 ± 2.9	0.097
Capsaicin	0.038 ± 0.004	110 ± 27	0.00036
Betanidin	0.0032 ± 0.0001	64 ± 8	0.00005
Catechin	0.014 ± 0.001	49 ± 6	0.00029
UDP-Glucose[b]		150 ± 30	

[a] Kinetic parameters (means ± standard errors) were determined by non-linear regression analysis [23] as described in Section 2.5.
[b] Esculetin was used as the glucosyl acceptor.

UGT73A10 was active over a pH range of 7.0–9.0 with maximal activity at pH 8.0 (at 30 °C). The enzyme was stable at pH 5.0–10.0 (at 20 °C for 60 min) and below 40 °C (at pH 7.2 for 20 min). Enzyme activity was completely inhibited by 0.1 mM Hg^{2+} and partially inhibited by 0.1 mM Fe^{2+} (residual activity, 43%). Other 0.1 mM metal ions had negligible effects on catalytic activity (Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+}, and Zn^{2+}): 1 mM EDTA, 1 mM uridine, 1 mM UMP, 1 mM UDP, 1 mM UTP, 1 mM glucose, 1 mM diethylpyrocarbonate, and 1 mM phenylmethanesulfonyl fluoride.

3.4. Glucosylation of catechin catalyzed by UGT73A10 and structural and stability studies of the reaction product

The UGT73A10-catalyzed reaction of (+)-catechin and UDP-glucose at 30 °C for 66 h yielded a single transfer product of (+)-catechin in an overall conversion yield of 83% [based

on (+)-catechin; see Section 2.6 for details]. Positive mode time-of-flight mass spectrometric analysis of the product furnished a molecular ion at *m/z* 475 $[M+Na]^+$, suggesting the presence of one hexose moiety in the product. In the ^1H NMR spectra, the anomeric hydrogen signal (H-1″ in Fig. 3b) was observed at δ 4.64 (1H, d, $J = 7.3$ Hz); the hexose moiety was confirmed to be β-D-glucopyranoside based on the coupling constants of the hydrogen signals (Fig. 3a). A cross peak of ROESY was observed between anomeric hydrogen (H-1″ in Fig. 3b) and the 5′-H of catechin (H-5′ in Fig. 3b; δ 7.1). In addition, in the ^1H $\{^{13}C\}$-HMBC analysis, a cross peak was observed between anomeric hydrogen (H-1″ in Fig. 3b) and the 4′-carbon atoms (C-4′ in Fig. 3b; δ 144.7). Thus, the transfer product was identified as 4′-*O*-β-D-glucopyranoside of (+)-catechin (Fig. 3b) [35]. This result was unexpected because UGT73A10 showed flavonoid 7-*O*-glucosyltransferase activity (see above) toward some flavonoids, but catechin 4′-*O*-β-D-glucopyranoside was the sole transfer product.

The stability of 4′-*O*-β-D-glucoside of (+)-catechin was then examined and compared with that of catechin. The stability of 4′-*O*-β-D-glucoside of (+)-catechin in 0.1 M HCl at 25 °C was similar to that of (+)-catechin (Fig. 3c). In contrast, the stability of the 4′-*O*-β-D-glucoside in 0.1 M NaOH at 25 °C was significantly higher than that of (+)-catechin. Similarly, thermal stability of 4′-*O*-β-D-glucoside was higher than that of (+)-catechin (Fig. 3d,e). These results show that 4′-*O*-glucosylation of (+)-catechin by UGT73A10 enhances its stability, most likely due to modification of the reactive catechol moiety in the (+)-catechin molecule.

4. Conclusion

The array of PSPG cDNAs (LbGTs) isolated from fruits of *L. barbarum* L. in this study is expected to serve as a good source of biocatalysts for specific glycosylation of a wide variety of compounds. Indeed, from among these LbGTs, we identified UGT73A10, which effectively catalyzed the regiospecific glucosylation of (+)-catechin. The spectroscopic analyses showed that the glucosyl transfer product was 4′-*O*-D-glucopyranoside of (+)-catechin. This reaction product was more stable than aglycon under acidic conditions and at elevated temperatures. LbGT-catalyzed glucosylation may be coupled with an *in situ* UDP-glucose recycling system [36] for efficient production of glycosides of useful compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2008.02.001.

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