Identification and characterization of *Camellia sinensis* glucosyltransferase, UGT73A17: A possible role in flavonol glucosylation

Shoji Ohgami1,†, Eiichiro Ono2,†, Hiromi Toyonaga2, Naoharu Watanabe3, Toshiyuki Ohnishi1,4,*

1Graduate School of Agriculture, Shizuoka University, Shizuoka, Shizuoka 422-8529, Japan; 2Research Institute, Suntory Global Innovation Center Ltd., Mishima, Osaka 618-8503, Japan; 3Graduate School of Engineering, Shizuoka University, Hamamatsu, Shizuoka 432-8561, Japan; †Graduate School of Green Science and Technology, Shizuoka University, Shizuoka, Shizuoka 422-8529, Japan
*E-mail: dtonish@ipc.shizuoka.ac.jp Tel & Fax: +81-54-238-3082

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**Abstract**  Tea plant (*Camellia sinensis*) biosynthesizes a wide variety of specialized metabolites, including phenolic compounds such as catechins. Flavanol, one of the major flavonoid subclasses, in *C. sinensis* is present in the O-glycoside form, such as quercetin 3-O-β-D-glucopyranoside, kaempferol 3-O-β-D-glucopyranoside, and rutin (quercetin 3-O-β-D-glucopyranosyl-6-O-α-rhamnopyranoside). These flavanol glycosides are highly accumulated, constituting up to 2–3% (w/w dry weight) of tea leaves; however, their biosynthetic machinery in *C. sinensis* remains elusive. Using high-throughput RNA sequencing from the fresh leaves of a cultivar (*C. sinensis* var sinensis cv Yabukita) and rapid amplification of cDNA ends (RACE) cloning with degenerate oligonucleotide primers, we identified a full-length cDNA of UDP-glycosyltransferase, designated as UGT73A17, and characterized the biochemical and molecular functions of UGT73A17. Recombinant UGT73A17 protein catalyzed 3'-O-glucosylation of quercetin, yielding quercetin 3-O-β-D-glucopyranoside in vitro. The preferential expression of UGT73A17 gene in the mature, relative to young leaves, stems and roots, is roughly consistent with the accumulation pattern of flavonol glycosides in *C. sinensis*, suggesting that UGT73A17, in part, participates in the biosynthesis of flavonol glycosides in *plant*.

**Key words:** *Camellia sinensis* (tea plant), flavonoid, quercetin, specialized metabolism, UDP-glycosyltransferase.
UDP-glucose: flavonol-3-O-glucosyltransferase in tea plant

moiety of anthocyanin 3-O-glucoside, resulting in formation of anthocyanidin rutinoside (Brugliera et al. 1994).

Despite the high accumulation of flavonol glycosides [up to 2–3% (w/w dry weight) in tea products] and their useful biological activities (Lin et al. 2003), the biosynthetic machinery of flavonol glycosides in *C. sinensis* remains elusive. Here we present the molecular and biochemical characterization of a novel *C. sinensis* UDP-glucosyltransferase, UGT73A17, that catalyzes 3-O-glucosylation for flavonols (Figure 1).

To assess the distribution of flavonol glycosides in *C. sinensis*, the composition and content of flavonol glycosides in the young and mature leaves, stems, and roots were investigated. The fresh leaves, stems, and roots of tea plants were harvested at the Center for Education and Research of Field Sciences, Shizuoka University (Shizuoka, Japan). By following the same procedure described in Katsumoto et al. (2007), flavonoid fractions were extracted from the young and mature leaves, stems, and roots. Flavonol aglycones were quantitatively measured in enzymatically hydrolyzed fractions by high-performance liquid chromatography (HPLC), whereas flavonol glycosides were directly measured without hydrolysis. Larger amounts of flavonols were observed in the mature leaves than in the young leaves, than in stems and roots (Figure 2). By hydrolysis of glycosides, quercetin was found to be the most abundant flavonol, rather than kaempferol and myricetin. Important to note, kaempferol was higher in the young leaves than in the mature leaves, whereas quercetin and myricetin were markedly increased in the mature leaves compared with those in the young leaves. Approximately twice the amounts of quercetin and its rutinoside (rutin) were found in the mature leaves of *C. sinensis* compared with those in the young leaves, suggesting that hydroxylation activity on the B-ring of flavonoids is activated along with leaf maturation. In contrast to rutin (a diglycoside), only small amount of quercetin monoglucoside was detected (data not shown), probably reflecting the metabolic coupling of 6″-O-rhamnosylation on the glucose moiety of flavonol monoglucoside, with the first 3-O-glycosylation of flavonols in vivo. These results show a spatiotemporal change of flavonol glycosides in *C. sinensis*. In the aspect of the biological function that the mature leaves highly accumulated flavonols compared with those in the young leaves, the mature leaves of *C. sinensis* may accumulate have phenolic compounds which protects the mesophyll tissue against UV-B radiation (280–315 nm) (Thompson et al. 1972).

To isolate candidate UGT genes involved in the glycosylation of flavonols in the leaves, we used transcriptome data derived from the sequencing of expressed sequence tag (EST) libraries. Total RNA was extracted from the young leaves of *C. sinensis* using a Plant RNeasy extraction kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. Construction of cDNA libraries for Roche-GS-FLX Titanium 454 sequencing (Roche Diagnostics, Tokyo, Japan), sequencing, trimming, and assembly of sequences were outsourced to Dragon Genomics Center (Mie, Japan) at TaKaRaBio Inc. In the resulting de novo transcript assembly from 454 sequencing, 187 independent ESTs assigned as UDP-glucosyltransferases by EST Viewer version 1.12 (TaKaRaBio) were obtained. Using TBLASTX search (Altschul et al. 1997), we
screened these assembled ESTs for structural similarity to previously characterized flavonoid UGTs, such as flavonoid 3-O-glycosyltransferases (F3GlcT) and found 8 candidate UGT genes. Among the candidates, contig 2948 was found to have an incomplete open reading frame, and rapid amplification of cDNA ends to obtain a full-length cDNA of contig 2948 was performed using RACE primers (GR-Cs85AlikeC1-RV: 5′-CGA TCT TGC CTT TCC TCA CAA CCC AGA TG-3′, and Cs85AlikeC1-nest-RV: 5′-CTC AAC CTC ATC AAT GGC TGA TTC TTT AC-3′) according to the procedure described previously (Ono et al. 2006).

The isolated full-length cDNA consists of a 1,422-bp open reading frame encoding a polypeptide of 473 amino acid residues (calculated molecular weight: 52.9 kDa) showing 78% structural similarity with a Fragaria vesca flavonoid: 3-O-glycosyltransferase (accession XM_004304666) and a Vitis vinifera flavonoid: 3-O-glucosyltransferase (accession XM_002282427). PSORT (http://psort.ims.u-tokyo.ac.jp/) analysis predicted that the isolated full-length cDNA harbored a conserved domain (W343-Q386; 44 amino acids), with UDP-glucuronosyltransferase. This contig 2948 was designated as UGT73A17 (accession AB847095.1) by the committee responsible for naming UDP-glucuronosyltransferases (Mackenzie et al. 1997).

We assessed the biochemical activity of UGT73A17 using the recombinant proteins in vitro, according to procedures described in previous reports (Noguchi et al. 2009; Ono et al. 2010). In order to express UGT73A17 protein heterologously in Escherichia coli, cDNA of full-length UGT73A17 was amplified with NdeI and BamHI restriction sites using the primer set (CAC-C-NdeI-CsUGT-C1-FW: 5′-CAC CCA TAT GGC TAA GCT TCA TTT CTT C-3′ and CsUGT-C1-BamHI-stop-RV: 5′-GGA TCC TTA TGA ACT CAT TTC TTG TAT CAG AG-3′), ligated to the NdeI and BamHI sites in a pET15b expression vector (Novagen, Tokyo, Japan), and the ligated plasmid was transformed into E. coli BL21 (DE3) (TOYOBO, Osaka, Japan) after sequence confirmation. The recombinant UGT73A17 proteins were partially purified with an N-terminal
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His-tag (Figure 3A) and tested with UDP-glucose as a sugar donor against quercetin as previously described in Noguchi et al. (2009). The expressed recombinant proteins in SDS-PAGE gels were also immunologically confirmed by western blotting analysis with His·Tag monoclonal antibody (mouse, Novagen) and ECL Anti-mouse IgG Horseradish Peroxidase-linked Whole antibody (sheep, GE Healthcare, Tokyo, Japan), respectively. (Figure 3). The enzymatic assays were performed using the purified protein, sugar acceptor, and UDP-sugar in 50 mM potassium phosphate buffer. The enzymatic reaction was stopped by addition of the same volume of methanol prior to LC-MS analysis. The apparent $K_m$ and $V_{max}$ values for glycosyl donors and the sugar acceptor (quercetin) 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 nonlinear regression analysis with Kaleidagraph (Synergy Software, PA, USA).

In LC-MS analysis, two newly formed peaks in the reaction of quercetin with UGT73A17 were found at the retention time of 7.8 min and 8.8 min with $m/z$ 463. The dominant peak at 7.8 min was consistent with formation of quercetin 3-O-β-D-glucopyranoside (Figure 3B). Although we could not determine the chemical structure of the minor peak at the retention time of 8.8 min, it has been reported that Arabidopsis UGT73B1 (At4g34138) catalyzes 7-O-glucosylation of quercetin (Kim et al. 2006). UGT73A17 may be a bifunctional UGT forming quercetin 3-O-β-D-glucopyranoside and quercetin 7-O-β-D-glucopyranoside. The maximum velocity ($V_{max}$) and estimated apparent $K_m$ values of UGT73A17 with quercetin were determined to be 143.7 ± 9.7 µmol min$^{-1}$ mg$^{-1}$ protein and 9.9 ± 2.3 (µM), respectively. Two further flavonoids (quercetin and cyanidin) and three aromatic alcohols were tested as potential substrates. In contrast to its high activity toward quercetin (100%), UGT73A17 showed negligible activity toward 2-phenylethanol (6.7%), eugenol (2.9%) and benzyl alcohol (2.7%) (Figure 3C). Trace activity toward cyanidin was observed in spite of the structural similarity to quercetin. To evaluate the sugar donor specificity of UGT73A17, we tested four UDP-sugars (UDP-glucose, UDP-xylose, UDP-galactose, and UDP-glucuronic acid), with quercetin as a sugar acceptor. The relative activity of UGT73A17 toward UDP sugars was estimated as 100%
for UDP-glucose, 17.8% for UDP-galactose, 16.3% for UDP-xylene, and 1.1% for UDP-glucuronic acid (Figure 3D). Taken together, these data showed that UGT73A17 preferentially catalyzes 3-O-β-glucosylation of quercetin, with narrow substrate specificity in vitro.

The spatial gene expression of UGT73A17 was investigated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), with each organ of C. sinensis (young leaf, mature leaf, stem, and root) according to the procedure described by Noguchi et al. (2008), with the following specific primer sets: qRT-CsUGT_C1-FW: 5′-TCA GCC ATT GAT GAG GTT GA and qRT-CsUGT_C1-RV: 5′-ATG AAT TGT TGC CCT GAA GC for UGT73A17, and Cs18SrRNA-FW: 5′-CAC GGG GAG GTA GTG ACA AT-3′ and Cs18SrRNA-RV: 5′-CCT CCA ATG GAT CCT CGT TA-3′ for 18S rRNA of C. sinensis (accession number; AB120309) as an internal standard gene. Real-time PCR was performed using a 7500 Real-Time PCR system (Life Technologies) and a Power SYBR Green PCR kit (Qiagen). Relative transcription levels were analyzed by the DD cycle threshold method (Life Technologies) after normalization to expression of an internal standard (18S rDNA).

UGT73A17 gene was found to be expressed at high abundance in the mature leaves (Figure 4). Along with leaf maturation, its expression increased, which is in accordance with the accumulation profile of quercetin glucoside (Figure 2). These results suggest that UGT73A17 partly participates in flavonol glycosylation in the leaves of C. sinensis.

This is the first report of C. sinensis UGT catalyzing glucosylation of flavonols. Owing to the reporting of various substrates as sugar acceptors for UGT73-family enzymes (Fukuchi-Mizutani et al. 2003), it is difficult to identify the bona fide substrates of enzymes of this family on the basis of phylogenetic relationships and in vitro characterization. However, our findings on the preferential expression of UGT73A17 in mature leaves, where rutin highly accumulates, and the catalysis of the recombinant protein for quercetin suggest that flavonols are potent substrates of this protein in vivo.

Given that rutin is the most abundant form of flavonol in leaves of C. sinensis (Figure 2), the second UGTs catalyzing 6″-O-rhamnosylation for glucose moiety of quercetin 3-O-glucoside should be active for efficient conversion of quercetin 3-O-glucoside to rutin (Figure 1). Among the 8 contigs showing structural similarity to UGT described above, we found contig2937 to be a candidate base on the homology to Petunia hybrida UGT79A1, which catalyzes 6″-O-rhamnosylation of anthocyanin 3-O-β-glucoside (Bruglieri et al. 1994; Kroon et al. 1994). Future functional characterization of contig2937 and UGT73A17 in association with flavonol synthase in vivo will clarify their biological impacts on tea flavonoid metabolism.

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References


Figure 4. Relative transcript abundance of UGT73A17 in various organs (young leaves, mature leaves, stems, and roots) in C. sinensis. Expression level of UGT73A17 in young leaf is set to 1.0. Values of relative expression level were determined in three biological replicate measurements, error bars indicate standard deviation.


