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Comparative Functional Characterization of LuUGT74S1 from *Linum usitatissimum* L. and Its Closed Homologues from *Fragaria vesca* Subsp. *vesca* and *Camellia sinensis*

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ABSTRACT: The bioactive lignan secoisolariciresinol-diglucoside (SDG) accumulates in flaxseed (*Linum usitatissimum*), where LuUGT74S1 is involved in its formation by the sequential transfer of two glucose units to the aglycone secoisolariciresinol (SECO). Here, we investigated whether similar proteins catalyze the identical reaction in other SDG-producing plants. A phylogenetic analysis identified FvUGT74DH1 from *Fragaria vesca* subsp. *vesca* and CsUGT74DG1 from *Camellia sinensis* as LuUGT74S1-like. Recombinant LuUGT74S1 glucosylated (−)- and (+)-SECO to SDG, but none of the seven selected substrate-analogues. The LuUGT74S1-homologous proteins failed to glucosylate SECO but were active toward substrates structurally related to SECO. In contrast to CsUGT74DG1 and FvUGT74DH1, which were active in *Escherichia coli*, LuUGT74S1 was unable to convert SECO in intact transgenic *E. coli* cells, presumably due to the inhibitory Mg^{2+} concentration in the bacteria. LuUGT74S1 has become highly substrate-specific, probably due to selection pressure, while its homologues remained promiscuous. The SECO-glucosylating enzymes must have evolved through convergent evolution in different species.

KEYWORDS: *flax, lignan, UGTs, SDG, secoisolariciresinol, glucosylation, glycosyltranferases, Linum usitatissimum, Fragaria vesca, Camellia sinensis*

INTRODUCTION

Flax (*Linum usitatissimum* L.) is one of the earliest domesticated plants, which serves as a model for developmental studies and has a different evolutionary origin than *Arabidopsis thaliana*.[1] The seeds of the plant contain high levels of essential omega-3 fatty acids and bioactive phenolic compounds.[2−4] Lignans are a class of diphenolic nonsteroidal phytoestrogens that have various health-promoting effects, including preventing the development of breast and prostate tumors.[5−8] A variety of plant species, including *Forsythia* and *Podophyllum* species, accumulate lignans such as secoisolariciresinol (SECO) and its diglucoside (SDG), which are thus widely distributed.[9−11] Flaxseed is a particularly rich source of SECO (294 mg/100 g fresh weight) and SDG (26 mg/g dry weight), but strawberry fruit and tea also produce these pharmacologically important phytochemicals.[2,12−15] Pinoresinol-lariciresinol reductases (PLRs) catalyze the conversion of pinoresinol to (−) and (+) SECO and are thus crucial enzymes of lignan biosynthesis in many plant species, including flax. The coupling of two coniferyl alcohol molecules in the presence of dirigent proteins forms the precursor pinoresinol.[9,16,17]

Lignans are usually present in glycosylated form in oligomeric chains that are cross-linked via hydroxymethylglutaryl residues in plants.[18,19] Glycosylation is the final step in the biosynthesis of many natural compounds, including phytochemicals, increasing their structural complexity and diversity.[20] Binding to sugar molecules enhances water solubility and structural stability and reduces reactivity and toxicity.[21] Furthermore, the transport and storage within the cells is affected.[22] The reaction is catalyzed by glycosyltransferase enzymes (GTs), which are highly divergent and polyphyletic, forming a multigene family found in all living organisms.[23] GTs have been categorized into more than 100 families, with family 1 comprising the uridine diphosphate dependent glycosyltransferases (UGTs)[24,25] (http://www.cazy.org/), which use UDP-activated sugars, such as UDP-glucose, as donor substrates.[26,27] The UGT enzymes in plants are characterized by a conserved sequence of 44 amino acid residues, also called plant secondary product glycosyltransferase (PSPG) motif, which represents the donor-binding site and thus facilitates the finding of UGT genes in plant genomes.[28] Like the genomes of other higher plants, the flax genome contains more than 100 UGT genes, which are clustered into 14 phylogenetically distinct groups and show differential expression in various tissues.[2,29] Of five flax UGTs whose gene expression correlated with the formation of lignans and the gene expression of PLR, only LuUGT74S1 catalyzed the 2-fold glycosylation of SECO in vitro, first to monoglucoside (SMG) and then to SDG. LuUGT74S1 is the only enzyme identified to date that is able to glucosylate SECO 2-fold, resulting in the formation of SDG.

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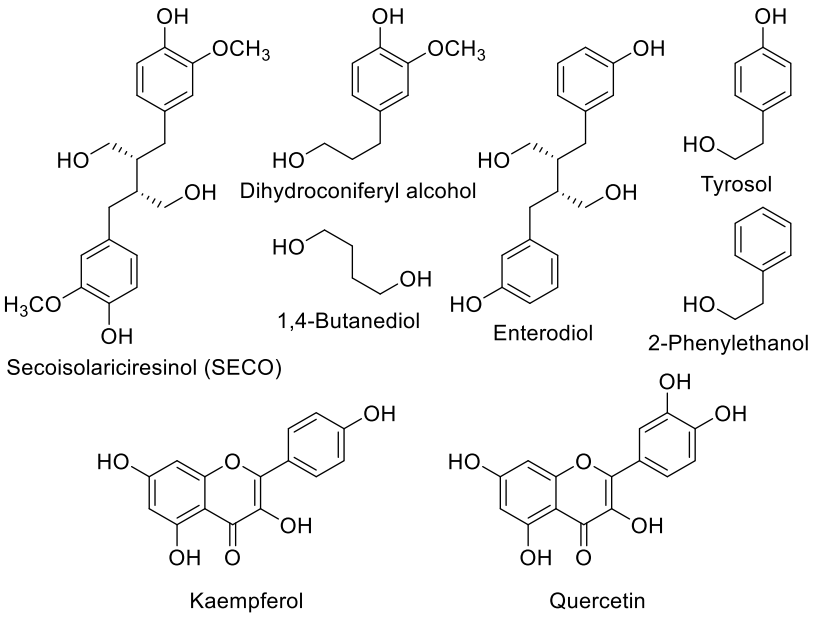

Figure 1. Chemical structures of the tested substrates.

According to a recent study, *LuUGT74S1* has evolved as a singleton in the flax genome under positive selection pressure and is closely related to two duplicate genes, *LuUGT74S4* and *LuUGT74S3*, whose encoded proteins, in contrast to LuUGT74S1, are unable to glucosylate SMG into SDG but produce minor amounts of SMG from SECO. Therefore, LuUGT74S1 plays an important role in controlling the glucosylation of SECO to SDG in flax, and its closely related genes contribute to provide the SMG precursor for LuUGT74S1.[30] Considering the economic and health benefits of these bioactive compounds, an analysis of similar UGT genes that can potentially glucosylate SECO should be performed. The aim of this study was to clone and characterize LuUGT74S1 in *Escherichia coli*, identify UGTs in strawberries and tea that are closely related to LuUGT74S1, and investigate their glycosylation activity toward SECO as well as other structurally similar substrates such as dihydroconiferyl alcohol, 2-phenylethanol, enterodiol, quercetin, tyrosol, and 1,4-butanediol. In addition, the use of the three enzymes in *E. coli* as whole-cell biocatalysts was to be tested.

MATERIALS AND METHODS

Chemicals. Secoisolariciresinol ((−)-SECO and (+)-SECO), dihydroconiferyl alcohol, 2-phenylethanol, enterodiol, quercetin, tyrosol, 1,4-butanediol, and other reagents were purchased in analytical grade from Sigma-Aldrich, Steinheim (Germany).

Search for Homologous Proteins of LuUGT74S1. A BLAST (Basic Local Alignment Search Tool) search (blast.ncbi.nlm.nih.gov/Blast.cgi) was performed with the full-length amino acid sequence of LuUGT74S1 (AGD95005.1) as the query sequence (Supporting Table S1). Evolutionary analyses were conducted in MEGA X.[31] The most similar protein sequences to LuUGT74S1 (>55% sequence identity) were extracted from GeneBank (ncbi.nlm.nih.gov/genbank). The proteins CsUGT74DG1 (KAF5954505.1) from *Camellia sinensis* and FvUGT74DH1 (XP_004290110.1) from *Fragaria vesca* subsp. *vesca* were selected for further analysis. Phylogenetic analysis was performed by Geneious Basic 5.6.7 (geneious.com/download/previous-versions/).

Cloning of LuUGT74S1, CsUGT74DG1, and FvUGT74DH1. The genes *LuUGT74S1* (JX011632.1), *CsUGT74DG1* (CM025498.1), and *FvUGT74DH1* (XM_004290062.2) were synthesized by Genewiz, Leipzig, Germany (www.genewiz.com) after codon optimization for *E. coli* (Supporting Information Figures S1−S3). The genes were ligated via *Eco*RI at the 5′ end and the NotI site at the 3′ end into the pGEX-4T-1 vector. For the transformation, 50 μL of NEB 10β competent cells (New England Biolabs, Frankfurt, Germany) were mixed with 1 μL (100 pmol) of dissolved gene in water and incubated for 20 min on ice according to the manufacturer's instruction. The cells were heat shocked for 45 s at 42 °C, kept on ice for 2 more min, and incubated for 2 h at 37 °C after the addition of 950 μL of SOC medium. After the incubation, the samples were centrifuged for 45 s and 200 μL was spread on agar plates and incubated overnight at 37 °C. Positive colonies were confirmed using colony PCR and stored in glycerol at −80 °C.

Protein Production. Protein expression was performed using *E. coli* BL21(DE3) pLysS cells transformed with pGEX-4T-1-*LuUGT74S1*, pGEX-4T-1-*CsUGT74DG1*, and pGEX-4T-1-*FvUGT74DH1* (Supporting Information Figure S4). After preculturing overnight at 37 °C and 150 rpm in Luria-Bertani medium containing 100 mg/mL ampicillin and 34 mg/mL chloramphenicol, 10 mL of the preculture was added to 1 L of the main culture containing the corresponding antibiotics and incubated at 37 °C and 120 rpm until OD_{600} reached 1 in a chicane flask. Gene expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cultures were incubated overnight at 18 °C and 150 rpm. Cells were harvested by centrifugation and stored at −80 °C. Recombinant fusion proteins with an N-terminal GST-tag were purified with Novagen GST Bind Resin following the manufacturer's instructions. Briefly, after resuspension, the cells were disrupted by sonication. After centrifugation, the crude protein extract was incubated overnight at 4 °C with the resin to bind the GST fusion protein and eluted with GST elution buffer containing reduced glutathione. The quality of the purified proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein concentration was determined with Roti-Nanoquant (Carl Roth, Karlsruhe, Germany) in 96-well microtiter plates according to the manufacturer's instructions. Absorption was measured at 450 and 590 nm using a CLARIO star plate reader (BMG Labtech, Germany).

In Vitro Enzyme Assays and Identification of Products by Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis. The recombinant proteins isolated from the *E. coli* cultures expressing LuUGT74S1, CsUGT74DG1, and FvUGT74DH1 were reacted with different aglycone substrates, including SECO, dihydroconiferyl alcohol, 2-phenylethanol, enterodiol, kaempferol, quercetin, tyrosol, and 1,4-butanediol (Sigma-Aldrich, Taufkirchen, Germany) in the

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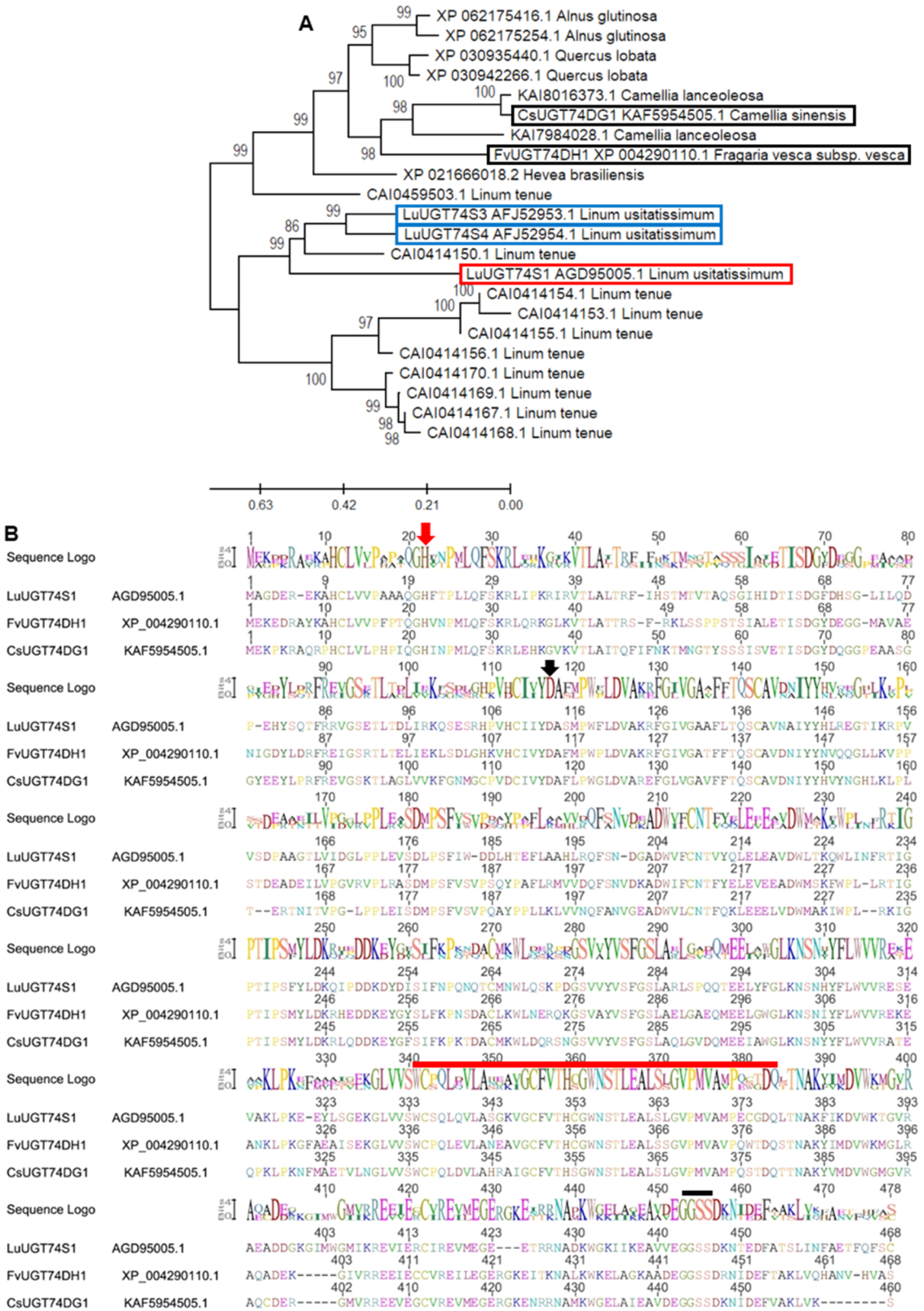

Figure 2. Candidate enzymes are similar to LuUGT74S1. (A) Phylogenetic tree showing enzymes with >55% sequence identity according to BLAST search. Genbank accession numbers and the species are shown. The bar indicates substitution per site. The evolutionary history was inferred by using the maximum likelihood method and JTT matrix-based model.[36] The tree with the highest log likelihood (−9290.33) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X.[31] (B) Amino acid sequence alignment of LuUGT74S1 and its closed homologues from *F. vesca* subsp. vesca (FvUGT74DH1) and *C. sinensis* (CsUGT74DG1). The red and black arrows indicate the catalytically active His and activating Asp, respectively, and the red and black bars indicate the PSPG box and GSS motif, respectively.

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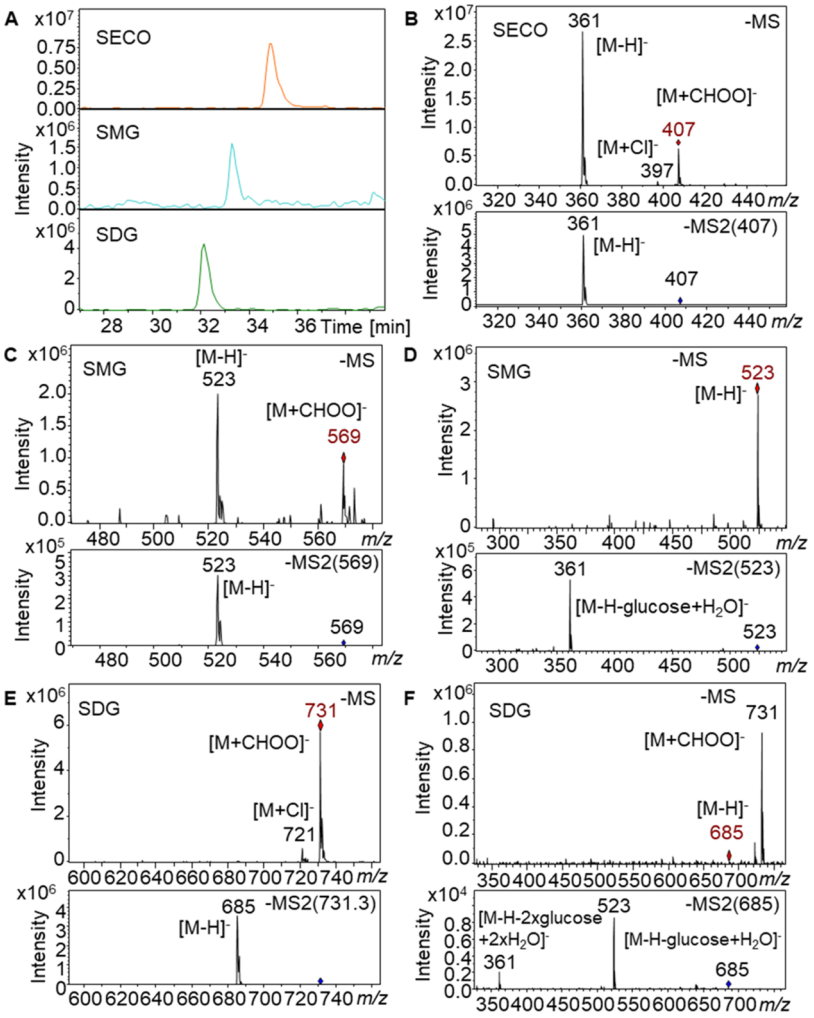

Figure 3. LC-MS analysis of the in vitro enzymatic activity of LuUGT74S1. (A) Chromatographic separation of substrate SECO and products SMG and SDG. (B) Mass spectrum (MS) and product ion spectra (MS2) of m/z 407 acquired in negative mode (−) of SECO. (C) MS and MS2 of m/z 569 of SMG. (D) MS and MS2 of m/z 523 of SMG. (E) MS and MS2 of m/z 731 of SDG. (F) MS and MS2 of m/z 685 of SDG.

presence of UDP-glucose (Figure 1). For the initial substrate screening, the UGT reaction was performed in a final volume of 100 μL of 100 mM sodium phosphate buffer (pH 7.5) containing 5 μg of recombinant protein, 1 mM UDP-glucose, and 600 μM substrate dissolved in dimethylsulfoxide (DMSO). The reaction was incubated at 30 °C with constant shaking at 400 rpm overnight. The enzymatic reaction was stopped by heat inactivation for 10 min at 95 °C. After centrifugation, the glycosides in the supernatant were identified by LC-MS. The LC System 1100 (quaternary pump and diode array detector) was from Agilent (Bruker Daltonics, Bremen, Germany). The reaction mixtures were separated by a LUNA C18 100 Å 150 × 2 mm^2 column (Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.2 mL/min.

The binary gradient system consisted of solvent A, water with 0.1% formic acid, and solvent B, 100% methanol with 0.1% formic acid, with the following gradient program: 0−6 min: 0−100% B; 6−14 min: 100% B; 14−14.1 min: 100−0% B; 14.1−25 min: 0% B. A Bruker esquire 3000 plus mass spectrometer with an ESI interface monitored the mass spectra. The ionization voltage of the capillary was 4000 V, and the end plate was set to −500 V. The substances detected at 280 nm, which indicate phenolic compounds, were validated with authentic reference materials (Supporting Information Table S2).

Screening of UGTs In Vivo-Whole-Cell Biotransformation. For the in vivo screening of the enzymatic activity of the three UGTs toward the substrates SECO, dihydroconiferyl alcohol, 2-phenylethanol, enterodiol, kaempferol, quercetin, tyrosol, and 1,4-butanediol in a small-scale biotransformation process, a 50 mL overnight culture of each *E. coli* W: pGEX-4T1-UGT in M9 minimal media containing 1% sucrose with 50 mg/L ampicillin was prepared.[32] After measuring the OD_{600}, 25 mL of the bacterial culture was centrifuged at 5000*g*, at room temperature (RT), for 15 min, and the pellet was resuspended in M9 minimal media containing 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to OD_{600} of 1.5 mL of the bacterial suspension was transferred to a 25 mL deep well microtiter plate (HJ-BIOANALYTIK GmbH, Erkelenz, Germany) and incubated for 3 h at 18 °C and 300 rpm. The biotransformation was started by adding the substrate at a final concentration of 1 mg/mL. After 1 day of incubation with shaking at RT, 0.5% sucrose was added to the culture. On the second day, the culture supernatant was centrifuged at 12,000*g* for 2 min, diluted 1:10, and analyzed for glucoside formation by LC-UV-MS (Supporting Information Table S2).

Biochemical Characterization and Analysis of the Enzyme Kinetics of LuUGT74S1. To determine the optimal reaction parameters of LuUGT74S1, such as pH, temperature, enzyme concentration, and effect of metal cofactors on enzyme activity, different conditions were tested. Investigated were pH values from 6.0 to 9.0, temperatures from 25 to 50 °C, enzyme amounts from 10 to 100 μg and three concentrations (5, 10, and 15 mM) of four metal cofactors (NaCl, $MgCl_2$, $MnCl_2$, and $CaCl_2$), and ethylenediaminetetraacetic acid (EDTA) in 100 μL reaction mixture. To determine the initial rate of the recombinant LuUGT74S1 enzyme, a time course study (5, 10, 15, 30, 45, 60 min) was performed at 30 °C, pH 8, using the optimum enzyme concentration and a fixed excess of substrate

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(280 μM SECO; 1.67 mM UDP-glucose) as described by.[33] Linearity was maintained in assays up to 30 min at 30 °C. The initial rate of reaction was measured at 10 min, at which time no more than 10% of SECO was converted to SDG. Assays were then run under optimal conditions for 30 min to determine kinetic parameters using different substrate concentrations (70−1400 μM SECO with UDP-glucose fixed at 1.67 mM). Lineweaver−Burk plots were used to determine the apparent v_{max} and K_m for the acceptor substrate in the presence of 80 μg of the enzyme. The v_{max} was divided by the enzyme concentration to obtain k_{cat}.

Data Analysis. All reactions were performed in triplicate, and the data are presented as means ± standard deviations. To test the statistical significance of metabolite production levels by LuUGT74S1, CsUGT74DG1, and FvUGT74DH1, a one-tailed Student's t test was performed.

RESULTS

CsUGT74DG1 and FvUGT74DH1 Are Homologues of LuUGT74S1. Since LuUGT74S1 is the only biocatalyst known so far to catalyze the glucosylation of SECO, we used BLAST (blast.ncbi.nlm.nih.gov) to search for similar enzymes in different plant species. In addition to several proteins from other *Linum* species, similar amino acid sequences were found in *Alnus glutinosa*, *Quercus lobata*, *Hevea brasiliensis*, as well as *Camellia* and *Fragaria* species, when the threshold was set to >55% sequence identity (Figure 2; Supporting Information Table S2). LuUGT74S3 and LuUGT74S4 are paralogs of LuUGT74S1 but show only weak glycosylation activity and produce small amounts of SMG.[34] We selected CsUGT74DG1 and FvUGT74DH1 for further analysis because SDG and SMG have already been detected in tea and strawberry fruits.[15,35]

Both proteins carry the catalytically active His22, the activating Asp114/117, the PSPG box, which represents the donor-binding site, and the GSS motif, which is characteristic of monosaccharide-forming UGTs (Figure 2). The proteins from tea plant and woodland strawberries show a sequence similarity to LuUGT74S1 of 55 and 56%, respectively. LuUGT74S1 was expressed in yeast and in *E. coli* and characterized together with CsUGT74DG1 and FvUGT74DH1, which were produced in *E. coli*. SDS-PAGE analysis confirmed that the molecular weights of the expressed proteins without the GST-Tag were 52.7, 51.5, and 52.6 kDa for LuUGT74S1, CsUGT74DG1, and FvUGT74DH1, respectively, in agreement with their predicted sequences (Supporting Information Figure S4).

Enzyme Activity of LuUGT74S1 Determined by LC-MS Analysis. The enzymatic activity of the proteins LuUGT74S1 expressed in *E. coli* was determined by LC-MS with various possible substrates such as SECO and substances sharing structural elements with SECO, such as dihydroconiferyl alcohol, 2-phenylethanol, enterodiol, tyrosol, and 1,4-butanediol (Figure 1). In addition, the universal substrates kaempferol and quercetin were used, as they are glucosylated by about 30% of the UGTs in *Arabidopsis*.[37] Of these possible substrates, LuUGT74S1 was only able to glucosylate SECO, yielding two products (Figure 3). These were identified as SMG and SDG based on the mass spectra and retention times of the authentic reference material. Thus, LuUGT74S1 catalyzes the sequential glucosylation of SECO. The two products exhibited a pseudomolecular ion at a mass-to-charge ratio (m/z) of 523 $[M - H]^-$ and 731 $[M + HCOO]^-$ for SMG and SDG, respectively, consistent with their molecular weights of 524 and 686 Da, respectively (Figure 3). In addition, the LuUGT74S1 protein was used to test the activity against enantiomerically pure (−)-SECO and (+)-SECO. LuUGT74S1 does not stereoselectively catalyze the transformation of SECO, as both enantiomers were glucosylated with similar efficiency (Figure 4).

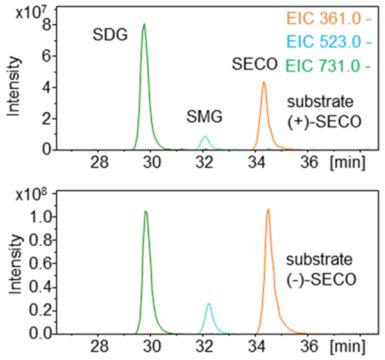

Figure 4. LuUGT74S1 catalyzes the glucosylation of (+) and (−) SECO. (+)-SECO (above) and (−)-SECO (below) were used as substrates for LuUGT74S1 in independent experiments. SMG and SDG were produced by LuUGT74S1 from both enantiomers.

Enzyme Activity of CsUGT74DG1 and FvUGT74DH1 Determined by LC-MS Analysis. The enzymatic activity of the proteins CsUGT74DG1 and FvUGT74DH1 expressed in *E. coli* was determined by LC-MS with SECO, substances with structural similarity to SECO, as well as kaempferol and quercetin. Neither protein was able to catalyze the glucosylation of SECO, kaempferol, and enterodiol (Table 1). In contrast, CsUGT74DG1 glucosylated 2-phenylethanol, quercetin, and tyrosol (Figure 5). In the case of 2-phenylethanol, the pseudomolecular ion of the glucoside was observed at m/z 329 $[M + HCOO]^-$.

Table 1. Substrates and Enzymes Used in This Study and Their Catalytic Activities: +: Active, −: Inactive

	FvUGT74D-H1		CsUGT74D-G1		LuUGT74S1	
substrate	in vitro	in vivo	in vitro	in vivo	in vitro	in vivo
SECO	−	−	−	−	+	−
2-phenylethanol	−	−	+	+	−	−
dihydroconiferyl alcohol	+	+	−	−	−	−
enterodiol	−	−	−	−	−	−
kaempferol	−	−	−	−	−	−
quercetin	+	+	+	+	−	−
tyrosol	+	+	+	+	−	−
1,4-butanediol	+	+	−	−	−	−

CsUGT74DG1 also produced quercetin 3-glucoside and 4′-glucoside similar to FvUGT74DH1 (Figure 6), which was confirmed by the pseudomolecular ions of the products of m/z 463 $[M - H]^-$. The formation of quercetin diglucoside could not be detected. Both enzymes transferred glucose to tyrosol, as the products formed by the two proteins produced ions of m/z 345 $[M + CHOO]^-$. In the case of tyrosol, glycosylation took place on the primary hydroxyl of tyrosol to form salidroside 2-(4-hydroxyphenyl)ethyl-β-D-glucopyranoside ($[M - H]^-$, m/z 299). Furthermore, in contrast to CsUGT74DG1, FvUGT74DH1 glucosylates 1,4-butanediol

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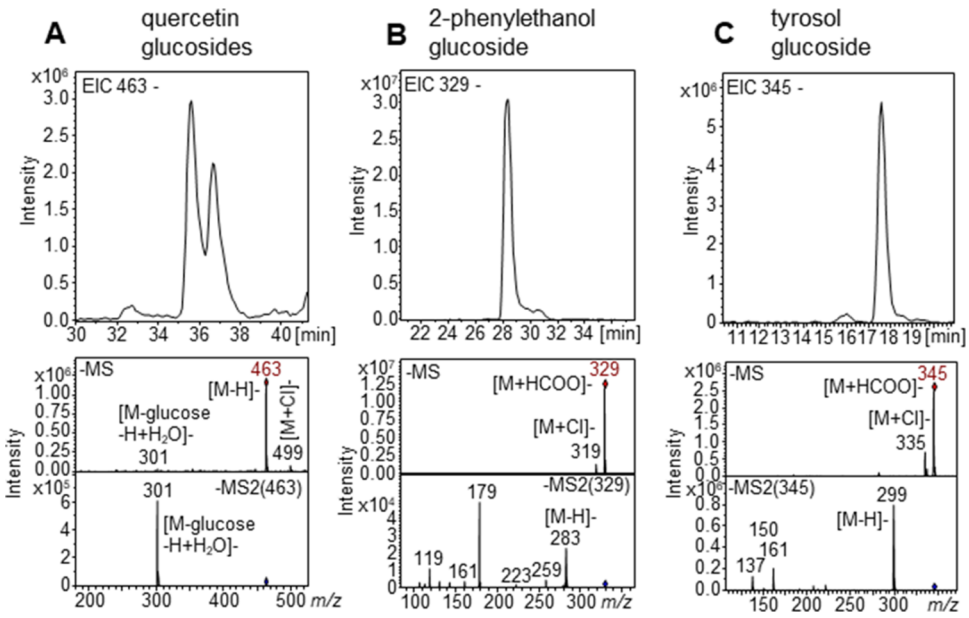

Figure 5. Glucosides produced by CsUGT74DG1. (A) Quercetin, (B) 2-phenylethanol, and (C) tyrosol were used as substrates. Glucoside formation was verified by LC-UV-MS (negative mode). Ion traces (extracted ion chromatogram EIC) of the indicated m/z ratio, mass spectra (MS), and product ion spectra (MS2) of the indicated m/z ratio are shown.

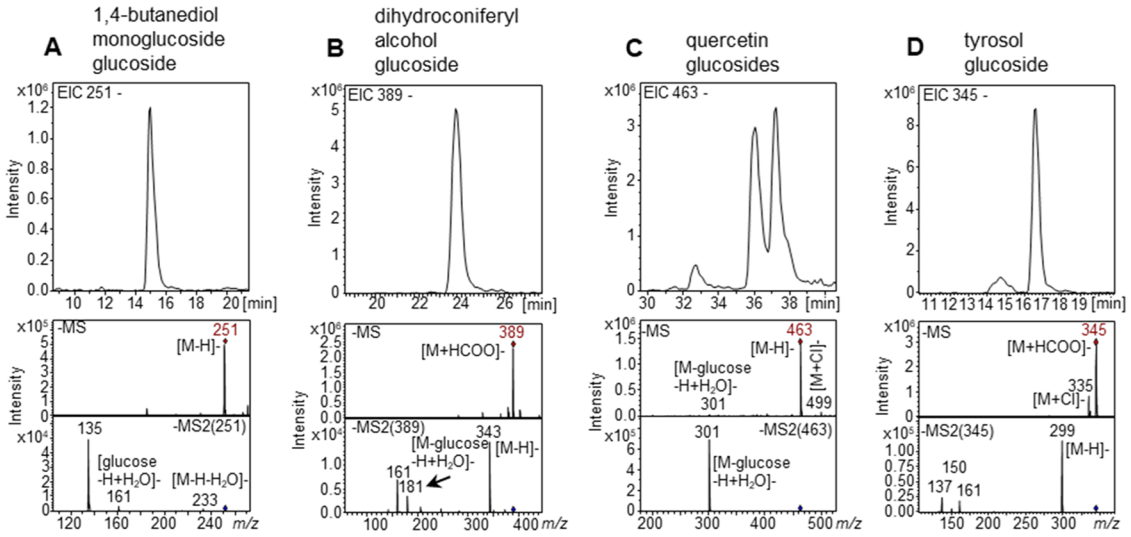

Figure 6. Glucosides produced by FvUGT74DH1. (A) 1,4-Butanediol, (B) dihydroconiferyl alcohol, (C) quercetin, and (D) tyrosol, were used as substrates. Glucoside formation was verified by LC-UV-MS (negative mode). Ion traces (extracted ion chromatogram EIC) of the indicated m/z ratio, mass spectra (MS), and product ion spectra (MS2) of the indicated m/z ratio are shown.

and dihydroconiferyl alcohol, as evidenced by m/z 251 $[M - H]^-$ and m/z 389 $[M + CHOO]^-$, respectively (Figure 6). The three enzymes can be clearly distinguished by their substrate tolerance. While LuUGT74S1 appears to be a highly selective catalytically active protein, the polypeptides from *F. vesca* and *C. sinensis* are promiscuous with regard to their substrates but show a differing preference (Table 1).

Testing of UGTs In Vivo. Considering the pharmacological importance of SDG, we wanted to investigate whether LuUGT74S1 could also be used to produce glucosides in a whole-cell biocatalytic approach based on *E. coli* cells expressing the recombinant plant UGT. Such a system has already been successfully applied for the biotechnological production of various natural product glucosides.[32,38] In addition to LuUGT74S1, the catalytic activity of CsUGT74DG1 and FvUGT74DH1 was also studied via a whole-cell biotransformation approach. The experiments were carried out using 25 mL well microplates to which 5 mL of bacterial solution was added. This allowed *E. coli* to be cultivated without inhibiting bacterial growth by oxygen deprivation. Expression of recombinant enzymes in *E. coli* cells harboring the *UGT* genes was induced with isopropyl-ß-D-1-thiogalactopyranoside (IPTG), and SECO, dihydroconiferyl alcohol, 2-phenylethanol, enterodiol, kaempferol, quercetin, tyrosol, and 1,4-butanediol were added to initiate the biotransformation experiment. The glucoside products were identified in the supernatants by LC-MS analysis (Supporting Information Table S2). Of the three different plant UGTs used as whole-cell biocatalysts in *E. coli*, LuUGT74S1 showed no activity against any of the substrates tested, including SECO,

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for which activity was detected in the previous in vitro experiment (Supporting Figure S5; Table 1). In contrast, CsUGT74DG1 showed activity toward 2-phenylethanol, quercetin, and tyrosol and FvUGT74DH1 transformed dihydroconiferyl alcohol, quercetin, 1,4-butanediol, and tyrosol (Table 1). This result thus confirms the activities of CsUGT74DG1 and FvUGT74DH1 in the in vitro enzyme assays. None of the enzymes was able to glucosylate SECO in the whole-cell test. Although different substrate concentrations, incubation temperatures, media, and incubation times were used, no SECO-glucosides were detected in the whole-cell approach, even with LuUGT74S1-expressing *E. coli* cells (Supporting Information Figure S5).

Biochemical Characterization of UGT74S1. To improve the LuUGT74S1 reaction with SECO, different pH ranges, temperatures, and SECO and enzyme concentrations were tested (Supporting Information Figure S6). The optimal pH for the enzyme was 7.5, and the enzyme activity increased linearly with the protein concentration until a saturation point of 75 μg was reached. The optimal temperature for LuUGT74S1 activity was 30 °C, and at an SECO concentration of 280 μM, sequential diglucosylation became incomplete, as SMG was detectable. The activity of LuUGT72AY1 was inhibited by divalent ions such as Mg^{2+}, Mn^{2+}, and Ca^{2+}, whereas Na^{+} had no effect on the enzymatic activity (Supporting Information Figure S7).

In *E. coli*, calcium ion concentrations are in the submillimolar range, while magnesium ion concentrations are between 1 and 100 mM, calculated for the total amount of free and bound forms.[39−44] Therefore, additional experiments were performed to determine whether these cofactors were responsible for the lack of SDG production in the whole-cell biotransformation assay. Different concentrations of $MgCl_2$ in the range of 0−11 mM were tested. The enzyme inhibition increased proportionally with the Mg^{2+} ion concentration in the medium (Figure 7; Supporting Information Figure S8).

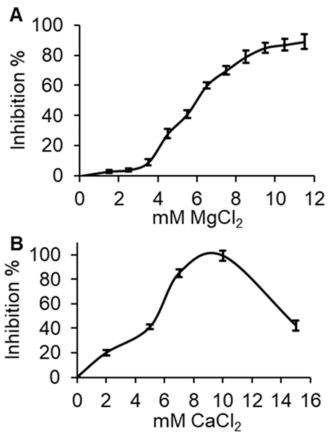

Figure 7. Inhibition of LuUGT74S1 activity with an increasing concentration of divalent metal ions. (A) $MgCl_2$. (B) $CaCl_2$.

The inhibitory effect of Mg^{2+} ions was reduced by the use of EDTA, from which we conclude that Mg^{2+} is not required for the enzymatic activity of the enzyme (Supporting Information Figure S9). We also investigated the effect of increasing Ca^{2+} concentrations on the activity of LuUGT74S1. The inhibitory effect of Ca^{2+} ions peaked at 10 mM and decreased thereafter (Figure 7). Under the optimized conditions, a catalytic efficiency (k_{cat}) of LuUGT74S1 for SDG production of 0.8 ± 0.1 s^{-1}, a K_m value versus SECO for SDG production of 74 ± 1 μM, and k_{cat}/K_m (catalytic efficiency) of 11 mM^{-1}/s were determined.

DISCUSSION

Using the conserved PSPG signature motif, 137 *UGT* genes have been identified in the draft genome sequence of *L. usitatissimum* and have been classified into 14 different evolutionary groups.[29] *LuUGT74S1* was grouped in phylogenetic group L and is an exception as it only contains intron 3 instead of intron 4, which is shared by the other members of group L. Among all of the genes expressed, *LuUGT74S1* showed the highest expression in seed coat at the torpedo stage, indicating its putative in planta function as SECO glycosyltransferase. Finally, flax LuUGT74S1 was identified as the enzyme that sequentially glucosylates SECO to its monoglucoside SMG and diglucoside SDG when expressed in yeast.[30,33,34,45] Since the enzyme could also be functionally expressed in *E. coli* in our study, we assume that post-translational modifications are not essential for the catalytic activity.

LuUGT74S1 is a Unique SECO Glucosyltransferase Probably Created by Convergent Evolution. Due to the bioactivity of SECO-glucosides and the associated interest in their production, we sought sequence-like proteins to study their catalytic activities after heterologous expression. Although CsUGT74DG1 and FvUGT74DH1 from *C. sinensis* and *F. vesca* subsp. *vesca*, respectively, were the most similar proteins from a plant other than Linum species, they were unable to glycosylate SECO. The proteins showed 55 and 56% sequence identity to LuUGT74S1, respectively, and yielded high protein levels when expressed in *E. coli* (Supporting Information Figure S4). However, CsUGT74DG1 showed activity toward substrates that are structurally similar to SECO, such as 2-phenylethanol and tyrosol, which conversely are not converted by LuUGT74S1. On the other hand, FvUGT74DH1 glucosylated dihydroconiferyl alcohol, 1,4-butanediol, and tyrosol, which also have structural similarities with SECO. Neither CsUGT74DG1 nor FvUGT74DH1 is able to glycosylate SECO; therefore, no further biochemical characterization or optimization was performed with these two enzymes. Since SECO-glucosides have been detected in both the tea plant and strawberries,[15] other proteins less closely related to LuUGT74S1 must be responsible for the formation of the lignan glucosides in these plants. Consequently, this indicates a convergent evolution of the lignan UGTs similar to the UGTs involved in the formation of cyanogenic glucosides.[46] The uniqueness and specificity of the catalytic activity of LuUGT74S1 are further supported by the fact that homozygous flax lines LuUGT74S1-nonsense mutants were unable to produce SDG.[34] This proves that LuUGT74S1 is the only enzyme in flax that catalyzes the formation of SDG from SECO. The optimal enzyme conditions determined for LuUGT74S1 produced in *E. coli*, including pH, temperature, cofactors, and substrate and enzyme concentrations, are largely identical to the previously published data for the enzyme produced in yeast.[33] While at pH 8.0 and 30 °C, a k_{cat} for SDG production and K_m toward SECO of 0.9 s^{-1} and 79 μM, respectively were determined for the enzyme from yeast, at pH 7.5 and 30 °C, we determined a k_{cat} of 0.8 s^{-1} and K_m of 74 μM for the protein expressed in *E. coli*. In addition, we were able to prove for the first time that the enzyme does not exhibit

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enantioselectivity as it glucosylates (+) and (−) SECO with similar efficiency.

Inhibition of LuUGT74S1 by Divalent Cations Hinders Its Use in Whole-Cell Biocatalysts. The inability of LuUGT74S1 to produce SDG when used as a whole-cell biocatalyst in *E. coli* prompted us to study the cause of inhibition in more detail. Knowing the inhibitory effect of 10 mM divalent ions (Mg^{2+}, Ca^{2+}, and Mn^{2+}) on LuUGT74S1 activity,[33] we established dose−response curves of these ions and tested the effect of EDTA. While it is well known that divalent cations, particularly Mg^{2+} and Mn^{2+}, play a very important role in carbohydrate biocatalysis, the actual mechanism by which the metal interacts with the ligands and enzymes is not well understood.[47] LuUGT74S1 does not require Mg^{2+} for the enzymatic activity (Supporting Information Figure S9) but is equally inhibited by Ca^{2+} and Mg^{2+} ions (Figure 7). Here, too, LuUGT74S1 shows a peculiarity. While numerous glycosyltransferases of plant origin are stimulated by divalent metal ions or remain unaffected,[48] the enzyme from flax is inhibited by Ca^{2+} and Mg^{2+} ions in a concentration-dependent manner. It is proposed that the formation of an Mg^{2+}-UDP complex reduces product inhibition by UDP and thus stimulates UGTs.[49] There have been only a few studies on the inhibitory effect of metal ions on glycosyltransferases. For example, a flavonol UGT from *Citrus paradise* was inhibited by 1 and 10 mM Zn^{2+}, Cu^{2+}, and Fe^{2+} but remained unaffected by 1 and 10 mM Ca^{2+}, Mg^{2+}, and Mn^{2+}.[50] Mg^{2+} is essential for living cells. It is required for the activity of many enzymes and for maintaining the structure of ribosomes.[51] Since only submillimolar concentrations of Ca^{2+} were quantified in *E. coli* cells, whereas up to 100 mM Mg^{2+} could be determined in bacterial cells, it is reasonable to conclude that the high Mg^{2+} concentration is mainly responsible for the lack of SDG formation by LuUGT74S1 in *E. coli*.[39−44] In contrast, CsUGT74DG1 and FvUGT74DH1 showed good in vivo activity against the other substrates tested, demonstrating that these enzymes are not inhibited by the Mg^{2+} concentrations present in *E. coli*.

The glycosyltransferases LuUGT74S1 from *L. usitatissimum* L., CsUGT74DG1 from *C. sinensis*, and FvUGT74DH1 from *F. vesca* subsp. *vesca* were successfully expressed in *E. coli*. The recombinant LuUGT74S1 is the only functional enzyme capable of converting SECO to SDG, while the homologous proteins from tea plant and strawberry glucosylate chemicals structurally related to SECO. Whereas CsUGT74DG1 and FvUGT74DH1 showed activity as whole-cell biocatalysts in *E. coli*, this was not the case for LuUGT74S1. The Mg-sensitivity of the flax enzyme is suggested to be the cause of the inhibited catalysis ability. Mutations of the *LuUGT74S1* gene to reduce Mg-sensitivity could eliminate this peculiarity of LuUGT74S1 in the future and make the enzyme accessible for use in whole-cell biotransformations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.4c00429.

The gene sequence of pGEX4T1-LuUGT74S1 [*L. usitatissimum*] optimized for expression in *E. coli* and the protein sequence (Figure S1); the gene sequence of pGEX4T1-CsUGT74DG1 [*C. sinensis*] optimized for expression in *E. coli* and the protein sequence (Figure S2); the gene sequence of pGEX4T1-FvUGT74DH1 [*F. vesca* subsp. *vesca*] optimized for expression in *E. coli* and the protein sequence (Figure S3); cloning of the UGT genes and protein production (Figure S4); biotransformation experiment using *E. coli* Waksman cells expressing LuUGT74S1 (Figure S5); optimization of the reaction conditions for LuUGT74S1 (Figure S6); LC/MS analysis showing the effect of 10 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, and NaCl on the enzymatic activity of LuUGT74AY1 (Figure S7); LC/MS analysis showing the inhibition of LuUGT747S1 with increasing amounts of Mg^{2+} (Figure S8); LC/MS analysis showing the effect of Mg^{2+} and EDTA on the activity of LuUGT74S1 (Figure S9); BLAST results for LuUGT74S1 (Table S1); substrates and the corresponding *m/z* values of the glucoside adducts for LC-MS detection (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Cs, *Camellia sinensis*; Fv, *Fragaria vesca*; GT, glycosyltransferase; LC, liquid chromatography; Lu, *Linum usitatissimum*; MS, mass spectrometry; PSPG, plant secondary product glycosyltransferases; SDG, SECO diglucoside; SECO, secoisolariciresinol; SMG, SECO monoglucoside; UGT, uridine diphosphate dependent glycosyltransferases

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