Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

Functional Characterization of Two Efficient Glycosyltransferases Catalysing the Formation of Rutin from *Sophora japonica* L.

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1. EXPERIMENTAL SECTION

1.1 Materials and Reagents

Reference compounds, substrates and sugar donors used in this study were purchased from YuanYe Biotechnology Co., Ltd. (Shanghai, China). Methanol and acetonitrile (Thermo Fisher Scientific, USA)) were of HPLC grade. The determination of conversion rates were analyzed by HPLC on an Waters Alliance e2695 instrument (Waters, USA) and Agilent HPLC 1260 instrument (Agilent). Samples were separated on a Zorbax SB-C18 column (4.6×250 mm, 5 mm, Agilent, USA). The column temperature was 30°C and the enzymatic products were eluted with conditions given in Table S1. To calculate the conversion rate of the reaction, the peak area of both substrates and products were integrated by Chromeleon® under a certain wavelength. The conversion rates were calculated from peak areas of glycosylated products and substrates (The peak area of glycosylated product divided by the total peak area of the glycosylated product and the substrate). LC/MS analysis was performed on a Q-Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). *Sophora japonica* used in this study was collected from Haidian District of Beijing, China in September 2022. All plant materials were frozen with liquid nitrogen immediately after collection and were stored at -80°C.

1.2 Transcriptome sequencing and candidate genes acquiring

The transcriptome data used in this study was acquired by Novogene Co., Ltd. (Beijing, China). The unigenes were compared with reported GTs using the local Basic Local Alignment Search Tool (Blast) with an e-value of $1e^{-21}$.

1.3 RNA isolation and cloning of candidate genes in *S. japonica*

The total RNA of *S. japonica* was acquired by using the TranZol kit (TransGen Biotech, China) and was reverse-transcribed to cDNA with the SMARTer RACE cDNA Kit (Clontech, USA). Using the cDNA library as a template, the full-length cDNA fragments of candidate genes were cloned by PCR using TransStart KD Plus

DNA polymerase (TransGen Biotech, China) with the gene-specific primer pairs (Table S2) and purified by agarose gel electrophoresis (120 V, 20 min). In the PCR program, the thermal cycling parameters were initially denaturized at 94 °C for 4 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 10 min.

1.4 Heterologous expression of candidate genes and protein purification

Sj3GT and Sj6″RhaT were cloned into expression vector pET-28a(+) (Invitrogen, USA) at BamH I site using homologous recombination method. After identification of the sequences, the recombinant plasmid pET-28a(+)-candidate genes were transformed into *E. coli* BL21(DE3) (TransGen Biotech, China) for heterologous expression. *E. coli* cells were grown in 500 mL Luria-Bertani (LB) medium containing 100 mg/mL Kanamycin at 37°C with shaking (180 rpm). After OD_{600} reached 0.4-0.6, 0.1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to induce protein expression. After 18-24 h of incubation at 16°C, the cell pellets were collected by centrifugation (7,500 rpm for 3 min at 4°C), and resuspended in 20 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole, pH 8.0), and disrupted by sonication on ice. After centrifugation at 7,500 rpm for 45 min at 4°C, the cell debris was removed and supernatant was obtained. The supernatant was then mixed with 1 mL Ni-NTA resin (TransGen Biotech, China) and incubated for 0.5 h in an ice bath with shaking (50 rpm). Then the mixture was applied to an Affinity Chromatographic Column (TransGen Biotech, China) pre-equilibrated with lysis buffer. The endogenous proteins of *E. coli* BL21(DE3) were washed away by 5 mL 30 mM imidazole elution buffer and recombinant protein was eluted by 1.5 mL 300 mM imidazole elution buffer separately. The purified protein solution was added with approximately 0.5 mL glycerol (25%) and stored at -80°C.

1.5 Functional characterization in vitro

To identify functions of the enzymes, assay was performed in a reaction solution composed of 0.5 mM sugar donor (UDP-glucose for Sj3GT, UDP-rhamnose for

Sj6″RhaT), 0.1 mM substrate (quercetin/isoquercitrin) and 50 μg of purified enzymes in reaction buffer (50 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 8.0). The reaction was initiated by adding purified enzyme solution into the reaction solution and incubated at 37°C for 0.5 hours. The reaction was terminated by adding 100 μL methanol and centrifuged at 15,000 rpm for 20 min for HPLC analysis.

1.6 Biochemical properties of Sj3GT and Sj6″RhaT

To acquire the optimal pH of Sj3GT and Sj6″RhaT, the reaction was conducted by changing reaction buffer with pH value in the scope of 4.0-9.0. To measure the best reaction temperature, the reaction was incubated at different temperatures (4-60°C). To test the dependence of divalent metal ions for enzymes' catalytic activity, different divalent metal ions and EDTA in the final concentration of 10 mM were added into the reaction solution. The enzymatic reactions of Sj3GT were conducted using UDP-Glc as sugar donor and quercetin as acceptor, 2.5 ng of purified enzyme was added into the reaction solution. The enzymatic reactions of Sj6″RhaT were conducted using UDP-Rha as sugar donor and isoquercitrin as acceptor, 5 ng of purified enzyme was added into the reaction solution. All reactions were individually conducted in a reaction time of 10 minutes and final volume of 100 μL as described above. The concentration of the buffers used in these experiments was 50 mM. All experiments were performed in triplicate and were analyzed by HPLC.

1.7 Sugar donor selectivity of Sj3GT and Sj6″RhaT

To investigate the sugar donor selectivity of Sj3GT and Sj6″RhaT, glycosylation reactions using different sugar donors (UDP-glucose (UDP-Glc), UDP-rhamnose (UDP-Rha), UDP-galactose (UDP-Gal), UDP-arabinose (UDP-Ara), UDP-*N*-acetyl-glucosamine (UDP-NAG), UDP-galacturonic acid (UDP-GalA), UDP-xylose (UDP-Xyl)) were conducted using quercetin or isoquercitrin as acceptor respectively. All reactions were conducted individually as described above. The reaction mixtures were analyzed by HPLC and LC/MS.

1.8 Kinetic parameters of Sj3GT and Sj6″RhaT

For kinetic assay of Sj3GT, enzymatic reactions were performed in a final volume of 50 μL containing 50 mM Tris-HCl buffer (pH 7.0), 2.5 ng of purified Sj3GT, 0.1mM of UDP-glucose, and varying concentrations (0.5-40 μM) of quercetin. The reactions were conducted at 45°C for 10 min with shaking (400 rpm) and stopped by adding 100 μL pre-cooled methanol. For kinetic assay of Sj6″RhaT, enzymatic reactions were performed in a final volume of 50 μL containing 50 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 7.0), 5 ng of purified Sj6″RhaT, 0.1 mM of UDP-rhamnose, and varying concentrations (1-40 μM) of isoquercitrin. The reactions were conducted at 37°C for 10 min with shaking (400 rpm) and stopped by adding 100 μL cooled methanol. All samples were centrifuged at 15,000 rpm for 20 min and analyzed by HPLC as described above. All experiments were performed in triplicate. The data were analyzed by Michaelis-Menten plots.

1.9 Molecular modeling and mutagenesis experiments

To simulate the structures of Sj3GT and Sj6″RhaT, molecular modeling was conducted using templates provided by SWISS-MODEL. The structure of Ct3GT-A[1] (PDB:3WC4) was used to model Sj3GT. Mutants of Sj3GT (Q336A, H351A, G353F, S356A, D359A, P371A, D375A, Q376A) were designed based on the result of molecular modeling. We modeled the Sj6″RhaT using AlphaFold2[2]. Mutants of Sj6″RhaT (E275A, V134A, V134T, Y135A, Y135T, S136A, S136T) were designed based on the modeled structure and were constructed using Fast Mutagensis System kit (Transgen, China) according to the manufacture's instructions. The primers used to construct the site-directed mutants are listed in Table S2.

Functional characterization of the mutants were performed in a final volume of 100 μL containing 50 mM Tris-HCl buffer (pH 7.0), 5 μg purified mutant enzyme, 0.5 mM of UDP-glucose, 0.1 mM of quercetin. The reaction was conducted at 45 °C for 10 min with shaking (400 rpm) and stopped by adding 100 μL cooled methanol.

2. AMINO ACID SEQUENCES

2.1 Amino acid sequence of SjUGT8

MTNSSEKKHVAVFVFPFGSHSAPLFNLVLKLAHAAPNLSFSFIGTENSNQP
LFSKPNIPNNIKAYSVGDGVPEGHVLGGHPVERVNLFLQAGPENLRKGIDL
AVAGTKQRVTCIIADAFVTPSLIVAQDLNVPWIPVWPPLSCSLSAHFYTDLI
REQCANNSAAHRALDFLPGLSKMRVEDLPEGILNGGEEDILFSKTLPSLGR
VLPQAKAVIINFFEELDPPLFVQDMRSKLQSMLYVGFLTLSLPLLPLPPSDT
DATGCLSWLDKQNARSVAYISFGTVVTPPPHELVAVAEALEASGFPFLWS
LKDNLKGLLPNGFLERTSIRGKIVPWAPQTQLLGHDSVGVFVTHCGCNSV
SDSISNGVPMICRPFFGDQRMTGRMVEDIWEIGVKIEGGVFSKNGLLKSLN
LILVQEEGKKMREKALKVKRIVQDAAGPEGKAAQDFKTLLEIVSSS

2.2 Amino acid sequence of SjUGT9

MSGVNNDELHVVMFPFLAFGHISPFVQLSNKLFSHGVQITFLSAPSNIPRIK
STFNLHPGIHIIPLQLPNSIANTAELPPDMTGNLIHALDLMQPQVKSLLLELKPQ
FVFFDFAQNWLPKLASEVGIKSVHFSVYSAISDAYITVPSRFAGIEGRSITFDDL
KKAPLGYPEKSNISLKAFEATDFMFLFRRFDENLTGYERVLQSLSECSYIVFKT
CKEIEGPYLDYIETQFGKPVLLTGPLVPEPAMDVLDEKWSKWLDSFPAKSVIF
CSFGSETFLNDDQIRELANGLELTGLPFILVLNFPSNLSAQAELDRALPRGFLD
RVKNRGVVHTGWLQQKLILAHSSVGCYVCHAGFSSVIEAMVNDCQLVLLPF
KGDQFFNSKLIAKDLEAGIEVNRKEEDGYFHKEDILEALKTIMVENDKEPGKH
IRENHMKWMKFLLNKEIQNKFITDLVAQLKSMA

3. SUPPLEMENTARY TABLES

Table S1. Candidate genes of UGTs and their expression level in different organs.

Gene ID	NO.	Leaves	Flowers	Roots	Assayed
Cluster-154.21377	SjUGT1	24.78	24.31	13.96	√
Cluster-154.21182	SjUGT2	94.87	32.23	5.63	√
Cluster-154.18213	SjUGT3	14.36	23.02	21.41	√
Cluster-154.28795	SjUGT4	0.6	114.04	1.66	√
Cluster-154.13248	SjUGT5	0	73.61	0.04	√
Cluster-154.24531	SjUGT6	26.75	37.99	50.9	√
Cluster-154.11545	SjUGT7	0.93	48.14	5.94	√
Cluster-154.13112	SjUGT8	2.55	157.39	17.79	√
Cluster-154.12903	SjUGT9	0.32	68.2	24.54	√

Table S2. PCR primers used in this study.

Primers	**Sequences (5' to 3')**
SjUGT8-F	CAGCAAATGGGTCGCGGATCCATGACAAACTCATCGGAGAAGAAAC
SjUGT8-R	GCTCGAGTGCGGCCGCAAGCTTAGAGCTAGAAACTATTTCCAAC
SjUGT9-F	CAGCAAATGGGTCGCGGATCCATGTCTGGTGTGAACAATGATGAG
SjUGT9-R	GCTCGAGTGCGGCCGCAAGCTTAGCCATGGACTTCAACTGAGCAAC
Sj3GT-W333A-F	gcgGCTCCTCAAACTCAACTTTTAGGACATG

Sj3GT-W333A-R	GAGTTTGAGGAGCcgcAGGCACTATTTTCC
Sj3GT-Q336A-F	gcaACTCAACTTTTAGGACATGATTCTGTAG
Sj3GT-Q336A-R	CTAAAAGTTGAGTtgcAGGAGCCCAAGGCAC
Sj3GT-H351A-F	gccTGTGGGTGTAACTCTGTGTCTGACAG
Sj3GT-H351A-R	GTTACACCCACAggcAGTCACAAACACTCC
Sj3GT-G353F-F	tttTGTAACTCTGTGTCTGACAGTATTTCC
Sj3GT-G353F-R	CACAGAGTTACAaaaACAGTGAGTCACAAAC
Sj3GT-S356A-F	gccGTGTCTGACAGTATTTCCAATGGGGTG
Sj3GT-S356A-R	ACTGTCAGACACggcGTTACACCCACAGTG
Sj3GT-D359A-F	gccAGTATTTCCAATGGGGTGCCTATGATC
Sj3GT-D359A-R	CATTGGAAATACTggcAGACACAGAGTTAC
Sj3GT-P371A-F	gccTTCTTTGGAGATCAAAGGATGACTGG
Sj3GT-P371A-R	GATCTCCAAAGAAggcCCTGCAGATCATAGGC
Sj3GT-D375A-F	gcaCAAAGGATGACTGGAAGAATGGTAGAG
Sj3GT-D375A-R	CAGTCATCCTTTGtgcTCCAAAGAAGGGCC
Sj3GT-Q376A-F	gcaAGGATGACTGGAAGAATGGTAGAGGAT
Sj3GT-Q376A-R	CCAGTCATCCTtgcATCTCCAAAGAAGGGC
Sj6″RhaT-E275A-F	gcgACATTTCTGAATGATGATCAAATCAG
Sj6″RhaT-E275A-R	CATTCAGAAATGTcgcACTTCCAAAGGAGC
Sj6″RhaT-V134A-F	gcaTACTCTGCCATTTCTGATGCTTACATT
Sj6″RhaT-V134A-R	AATGGCAGAGTAtgcTGAGAAGTGAACAGAC
Sj6″RhaT-V134T-F	accTACTCTGCCATTTCTGATGCTTACATT
Sj6″RhaT-V134T-R	AATGGCAGAGTAggtTGAGAAGTGAACAG
Sj6″RhaT-Y135A-F	gcaTCTGCCATTTCTGATGCTTACATTAC
Sj6″RhaT-Y135A-R	AGAAATGGCAGAtgcAACTGAGAAGTGAAC
Sj6″RhaT-Y135T-F	accTCTGCCATTTCTGATGCTTACATTAC
Sj6″RhaT-Y135T-R	GAAATGGCAGAggtAACTGAGAAGTGAAC
Sj6″RhaT-S136A-F	gcaGCCATTTCTGATGCTTACATTACTGTG
Sj6″RhaT-S136A-R	CATCAGAAATGGCtgcGTAAACTGAGAAGTG

Sj6″RhaT-S136T-F	accGCCATTTCTGATGCTTACATTACTGTG
Sj6″RhaT-S136T-R	CATCAGAAATGGCggtGTAAACTGAGAAGTG

Table S3. HPLC methods used in this study.

Method	Solvent A	Solvent B	Gradient Analysis	Substrates
A	Water containing 0.1% formic acid	ACN	20% B, 15 min; 20-30% B, 1 min; 30% B, 9min;	Quercetin (Fig. 1B)
B	Water containing 0.1% formic acid	ACN	20% B, 9 min; 20-100% B, 1 min; 100% B, 5 min.	Isoquercitrin (Fig. 1B)
C	Water containing 0.1% formic acid	ACN	20-50% B, 4min 50-100% B, 3min 100% B, 3min	Substrates tested by Sj3GT, except for 2b
D	Water containing 0.1% formic acid	ACN	5-30% B, 20min 30-100% B, 5min	Substrates tested by Sj6″RT , substrate 1g tested by Sj3GT and quercitrin in combinatorial catalysis (Fig. S1)

4. SUPPLEMENTARY FIGURES

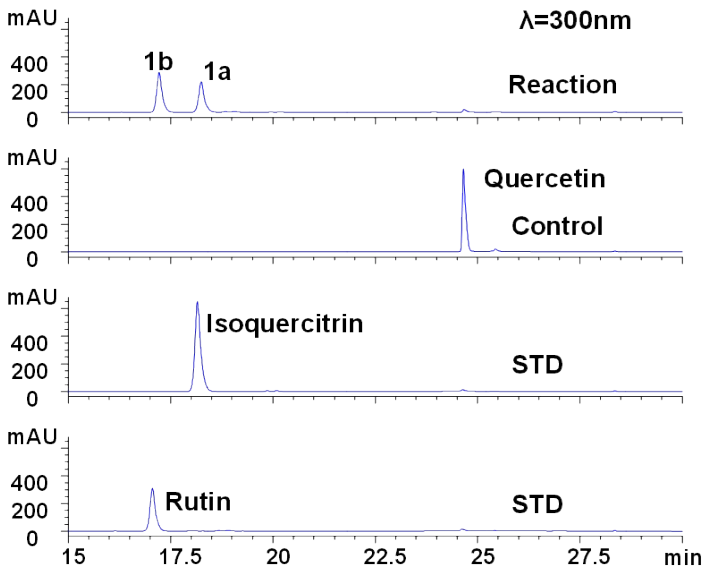

Fig. S1 HPLC analysis of the combinatorial catalysis reaction using quercetin (**1**) as substrate.

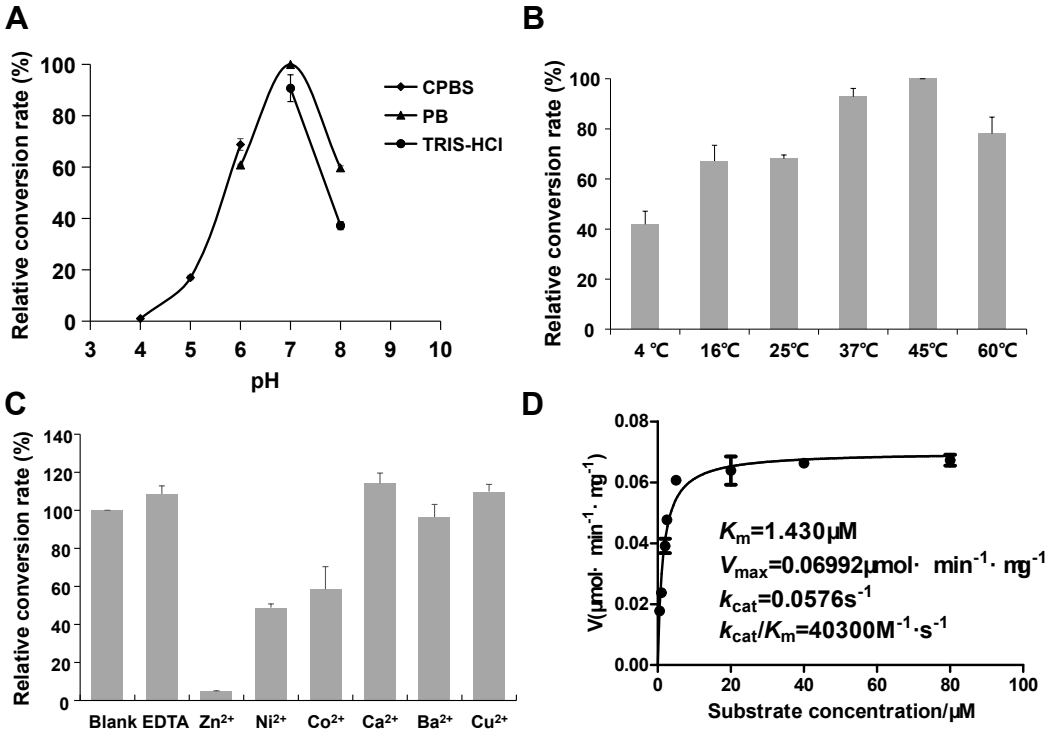

Fig. S2 Biochemical properties of Sj3GT. (A) The catalytic activity of Sj3GT in different reaction buffer. CPBS: Citrate buffer; PB: Phosphate buffer; TRIS-HCl: Tris-hydrochloride buffer. (B) The catalytic activity of Sj3GT at different temperature. (C) The impact of divalent ions on the catalytic activities of Sj3GT ('Blank' represent groups adding the same volume of solvent ddH_2O as the other groups). (D)Kinetic parameters of Sj3GT. All experiments were performed in triplicate (n=3).

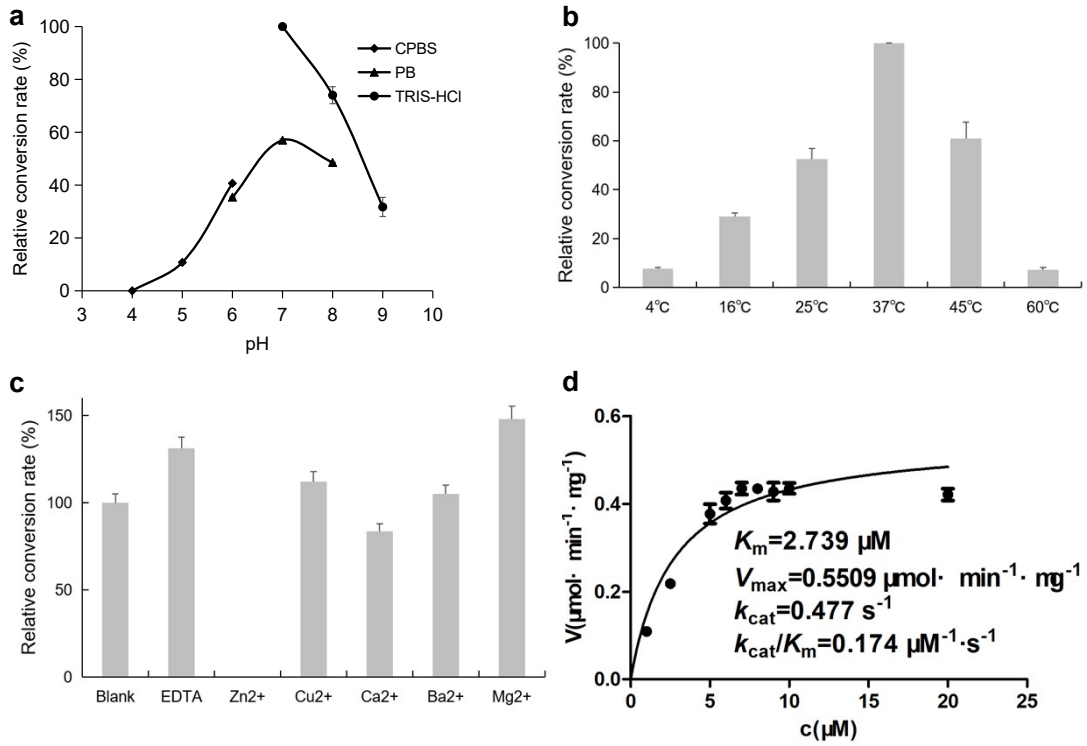

Fig. S3 Biochemical properties of Sj6″RhaT. (A)The catalytic activity of Sj6″RhaT in different reaction buffer. CPBS: Citrate buffer; PB: Phosphate buffer; TRIS-HCl: Tris-hydrochloride buffer. (B) The catalytic activity of Sj6″RhaT at different temperature. (C) The impact of divalent ions on the catalytic activities of Sj6″RhaT ('Blank' represent groups adding the same volume of solvent ddH_2O as the other groups). (D) Kinetic parameters of Sj6″RhaT. All experiments were performed in triplicate (n=3).

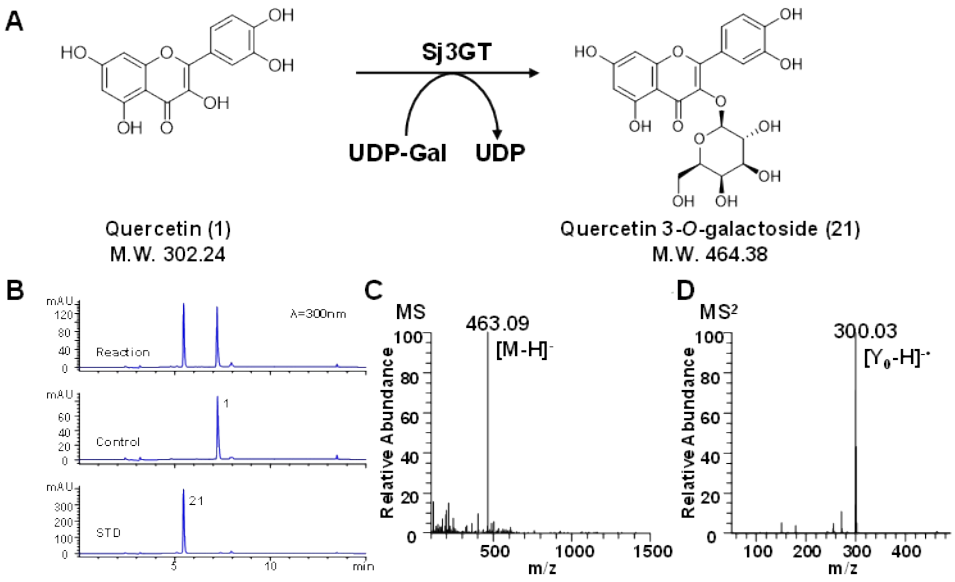

Fig. S4 The catalytic activity of Sj3GT utilizing UDP-Gal. (A) The reaction catalysed by Sj3GT utilizing UDP-Gal. (B) HPLC analysis of the reaction. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

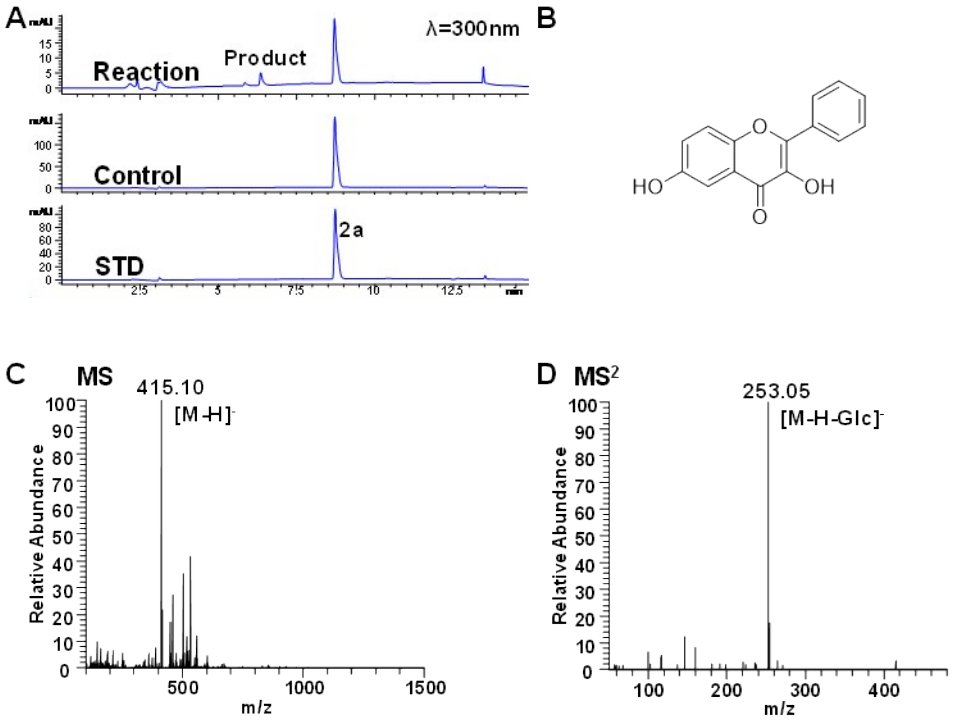

Fig. S5 The catalytic activity of Sj3GT converting substrate **2a**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **2a**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

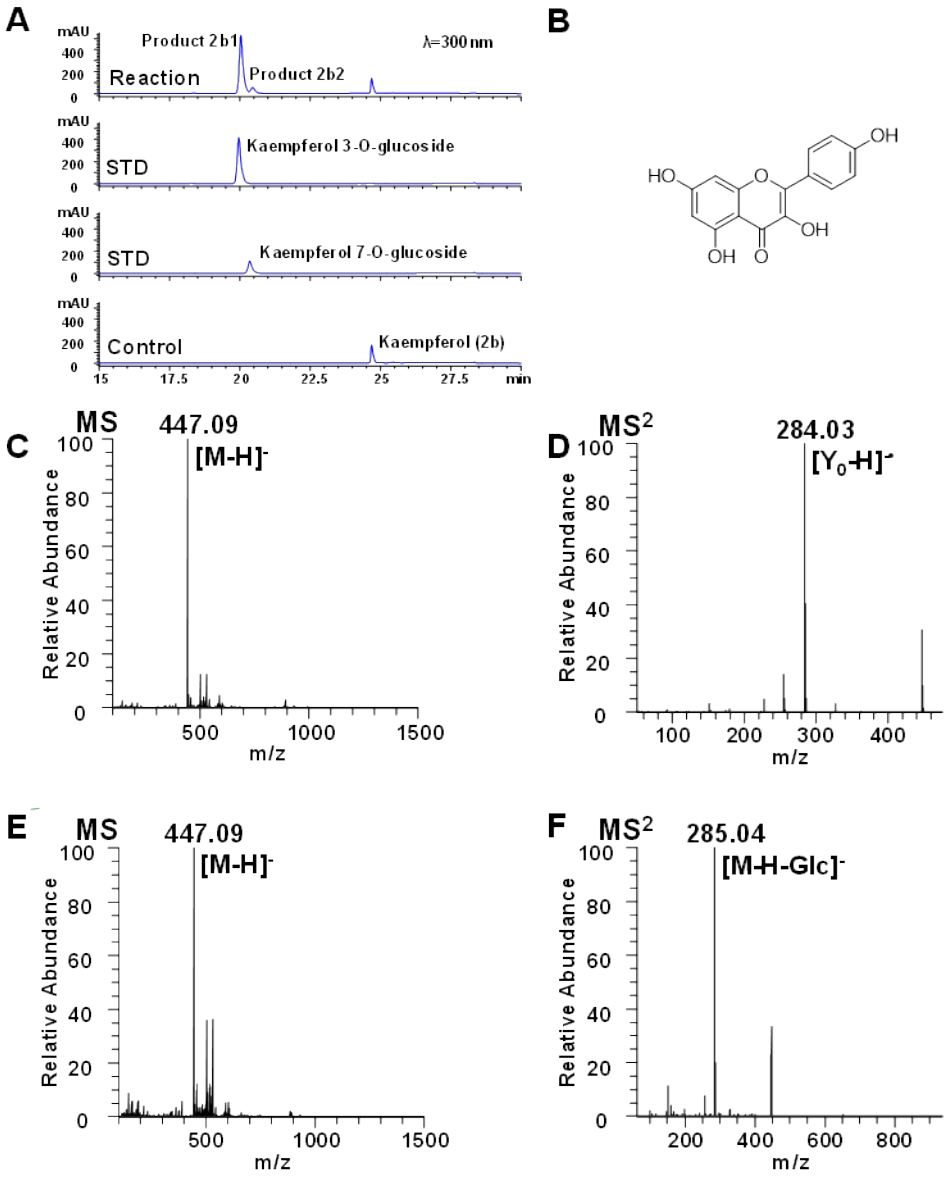

Fig. S6 The catalytic activity of Sj3GT converting substrate **2b**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **2b**. (C) (-)-ESI-MS spectra of the reaction product **2b1** (kaempferol 3-*O*-glucoside). (D) MS/MS spectra of the reaction product **2b1**. (E) (-)-ESI-MS spectra of

the reaction product **2b2** (kaempferol 7-*O*-glucoside). (F) MS/MS spectra of the reaction product **2b2**.

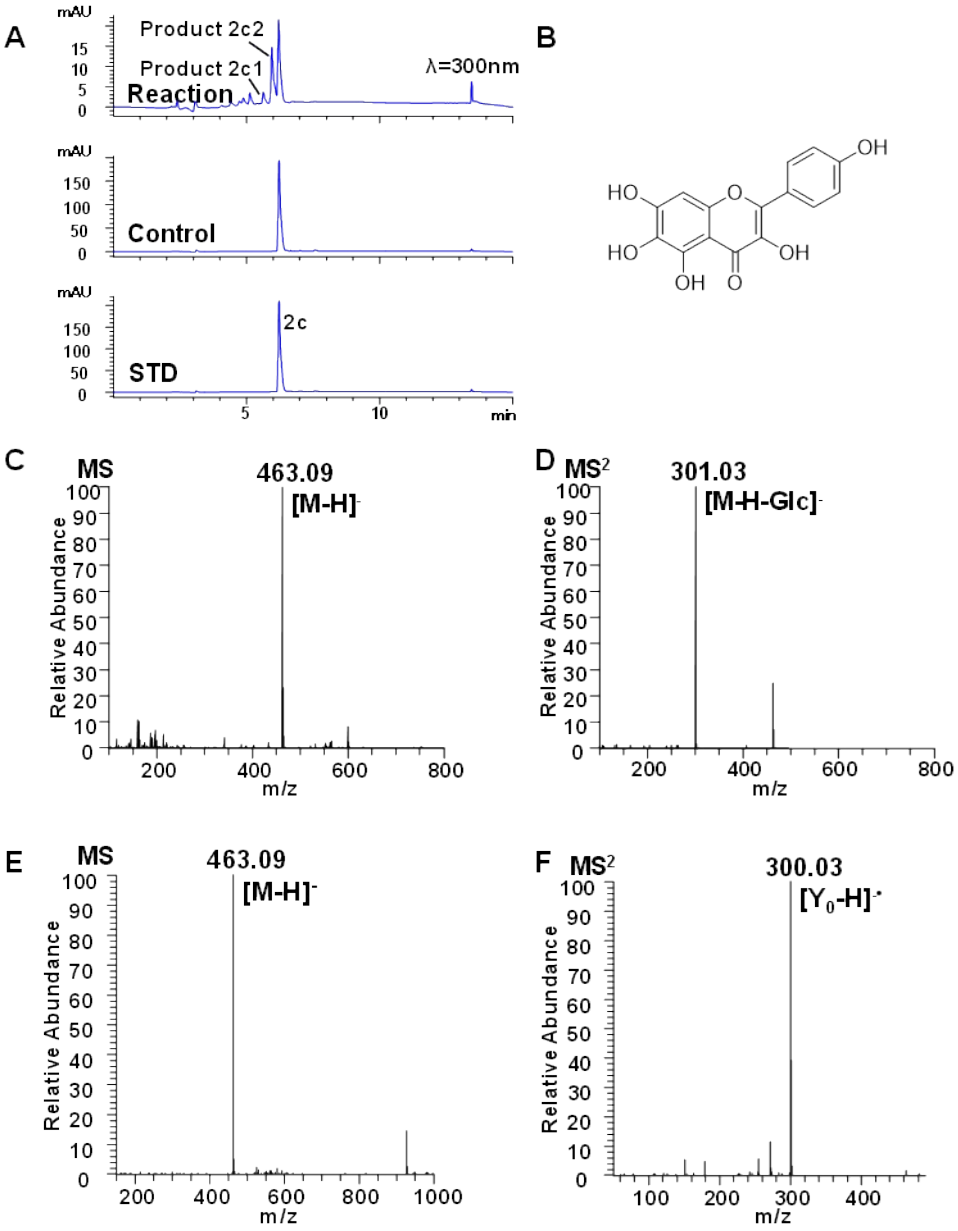

Fig. S7 The catalytic activity of Sj3GT converting substrate **2c**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **2c**. (C) (-)-ESI-MS spectra of the reaction product **2c1**. (D) MS/MS spectra of the reaction product **2c1**. (E) (-)-ESI-MS spectra of the reaction product **2c2**. (F) MS/MS spectra of the reaction product **2c2**.

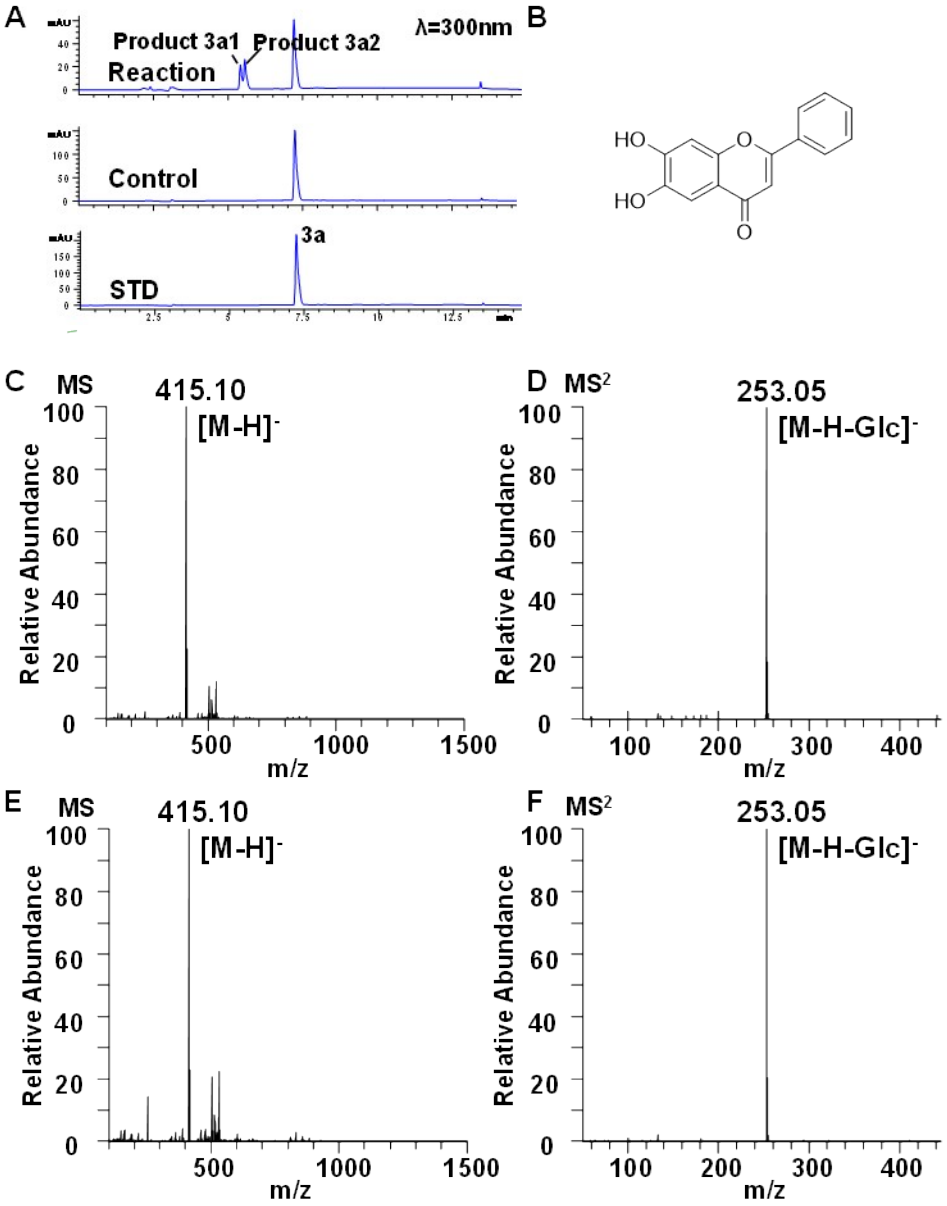

Fig. S8 The catalytic activity of Sj3GT converting substrate **3a**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **3a**. (C) (-)-ESI-MS spectra of the reaction product **3a1**. (D) MS/MS spectra of the reaction product **3a1**. (E) (-)-ESI-MS spectra of the reaction product **3a2**. (F) MS/MS spectra of the reaction product **3a2**.

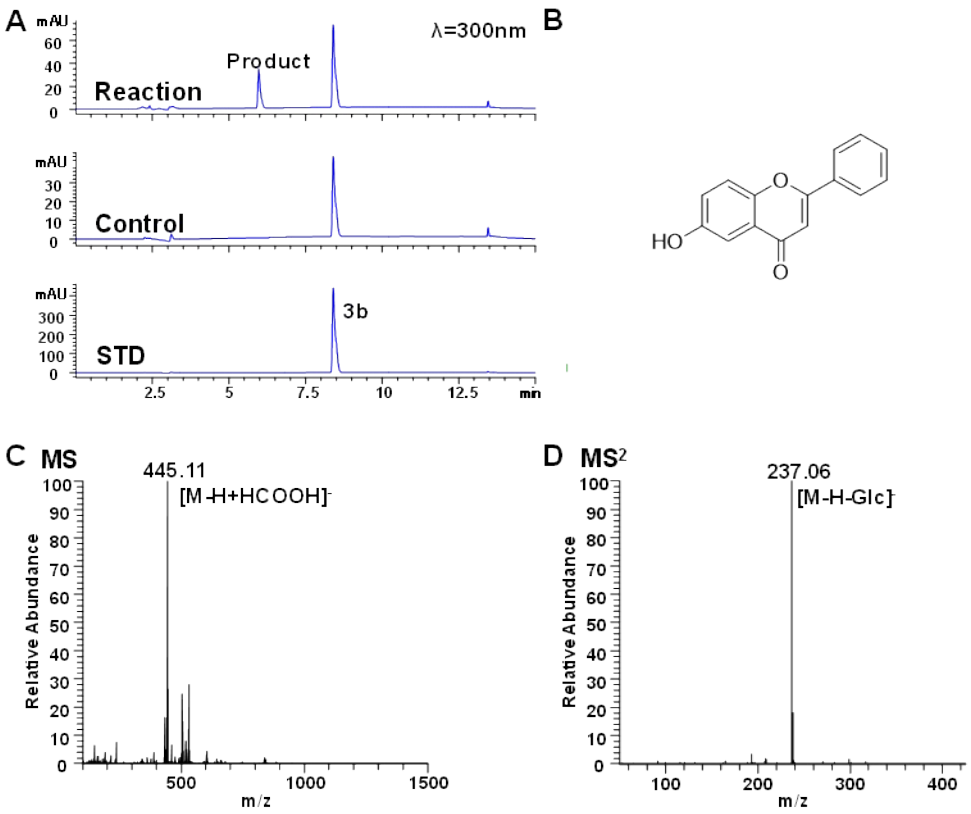

Fig. S9 The catalytic activity of Sj3GT converting substrate **3b**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **3b**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

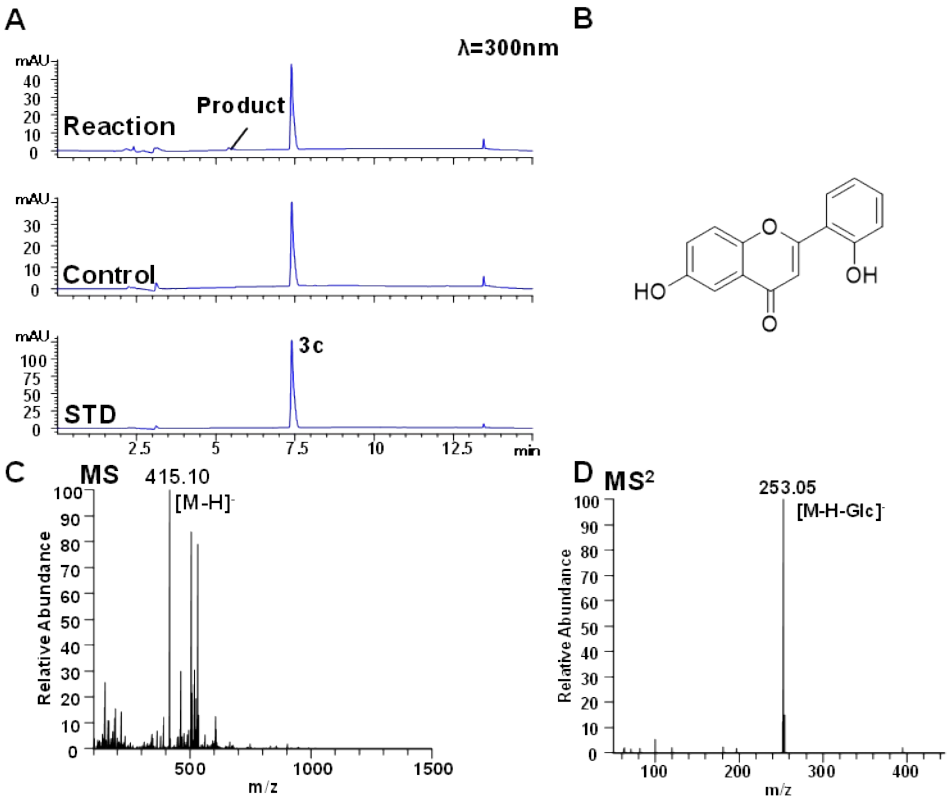

Fig. S10 The catalytic activity of Sj3GT converting substrate **3c**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **3c**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

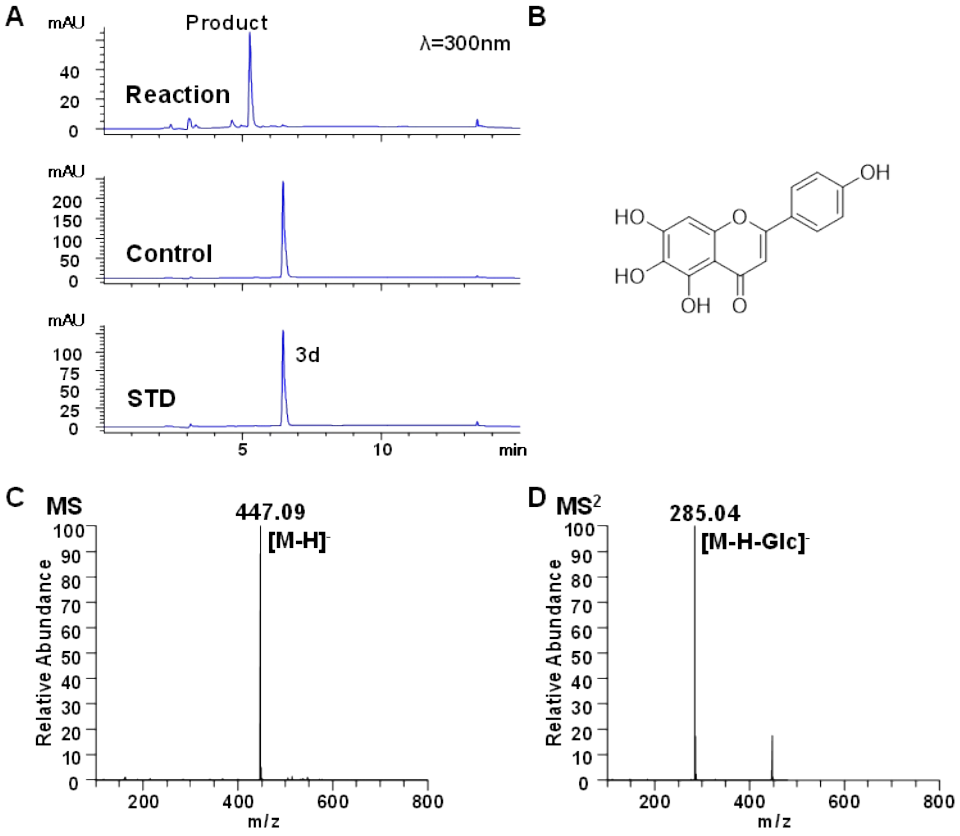

Fig. S11 The catalytic activity of Sj3GT converting substrate **3d**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **3d**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

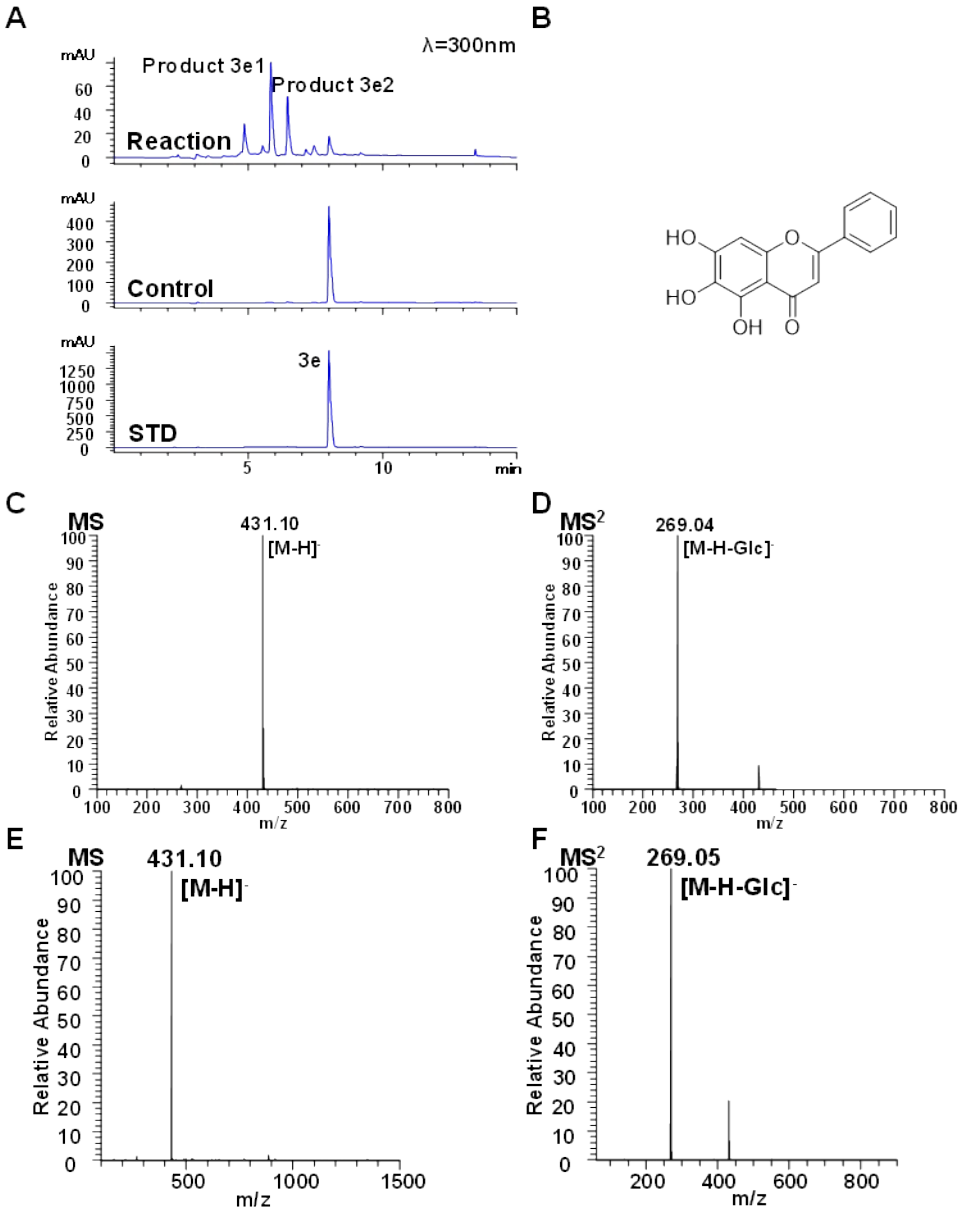

Fig. S12 The catalytic activity of Sj3GT converting substrate **3e**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **3e**. (C) (-)-ESI-MS spectra of the reaction product **3e1**. (D) MS/MS spectra of the reaction product **3e1**. (E) (-)-ESI-MS spectra of the reaction product **3e2**. (F) MS/MS spectra of the reaction product **3e2**.

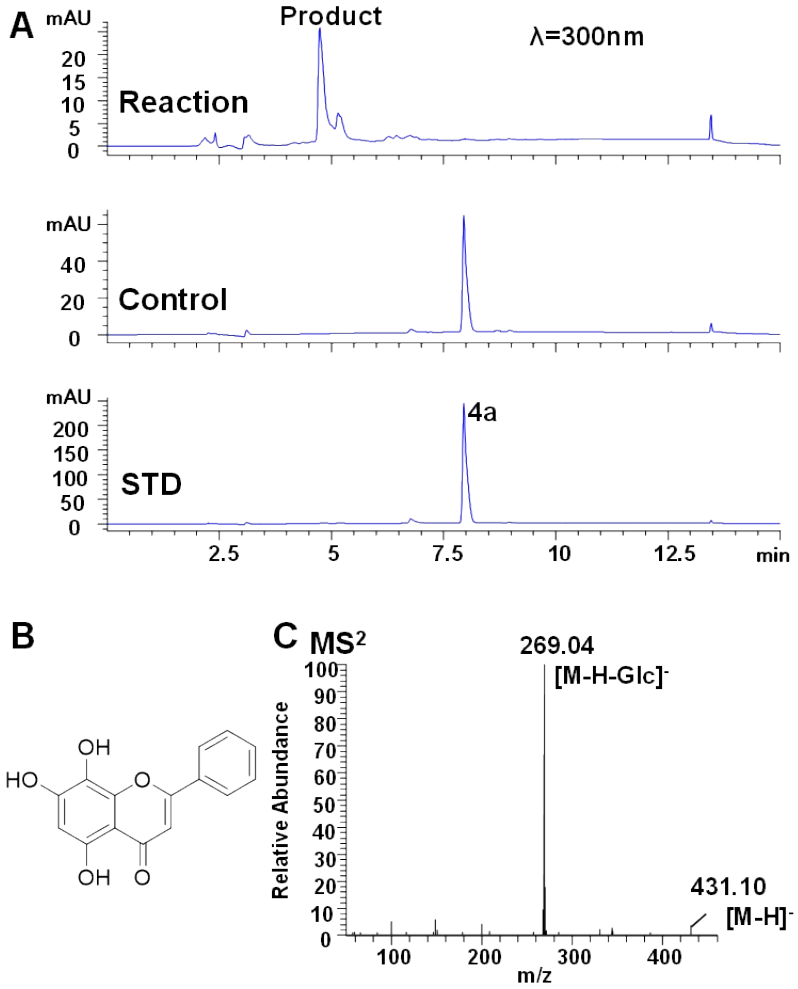

Fig. S13 The catalytic activity of Sj3GT converting substrate **4a**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **4a**. (C) MS/MS spectra of the reaction product.

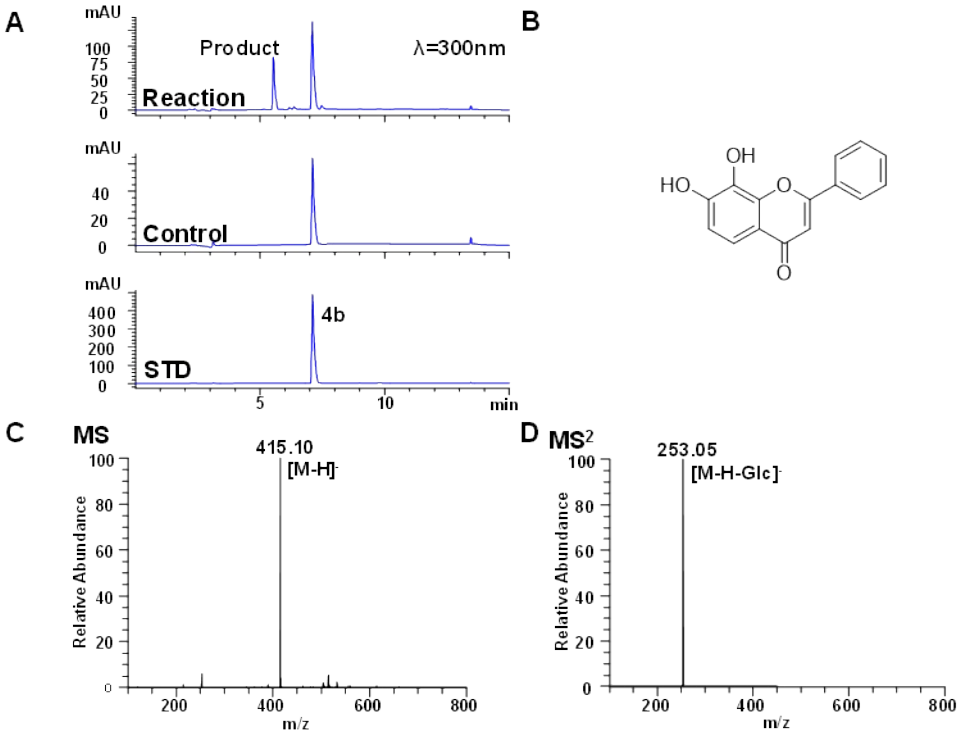

Fig. S14 The catalytic activity of Sj3GT converting substrate **4b**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **4b**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

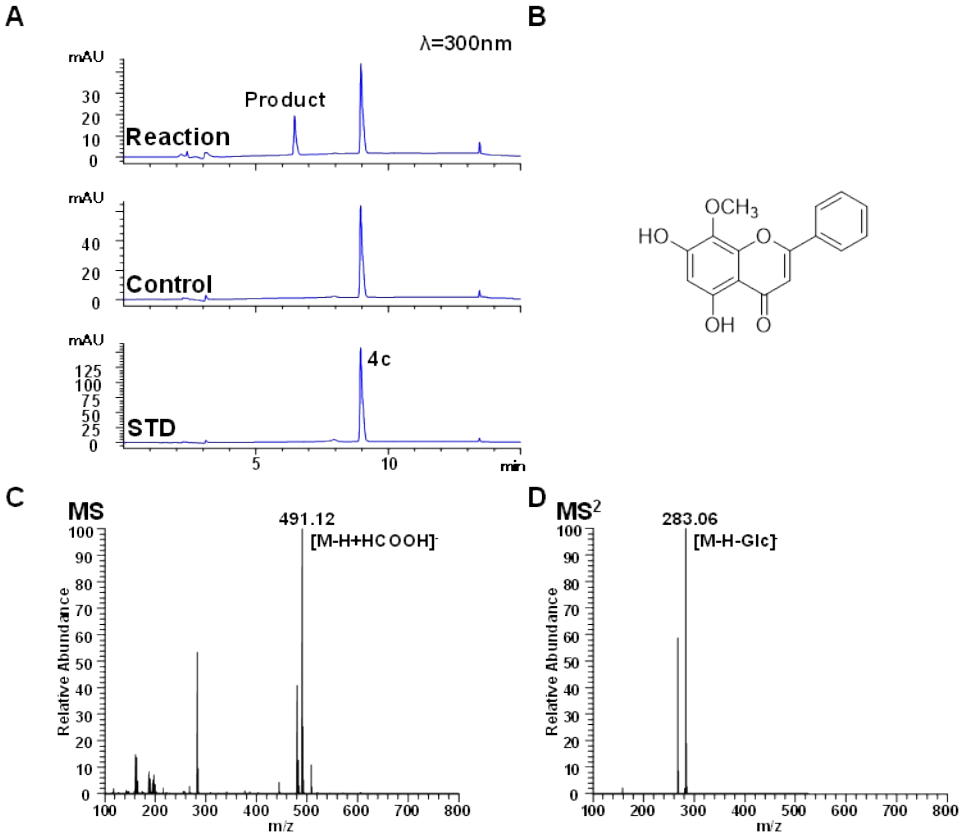

Fig. S15 The catalytic activity of Sj3GT converting substrate **4c**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **4c**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

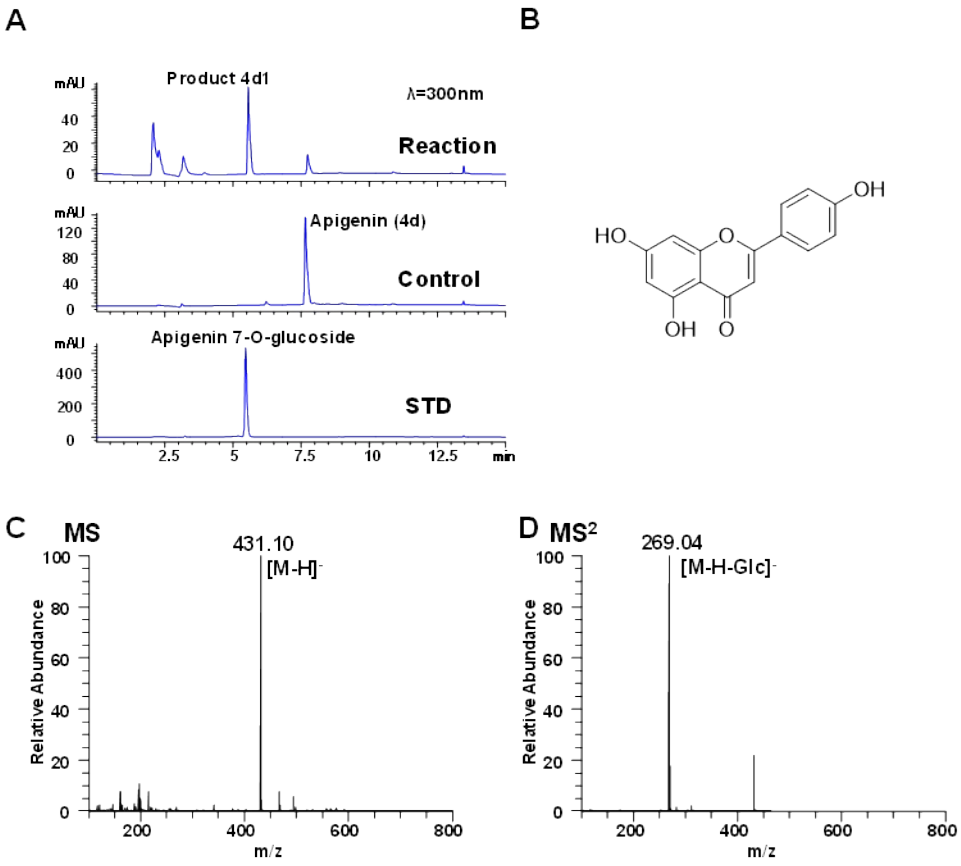

Fig. S16 The catalytic activity of Sj3GT converting substrate **4d**. (A) HPLC analysis of the reaction mixture and the reference standard of the product (apigenin 7-*O*-glucoside). (B) The structure of substrate **4d**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

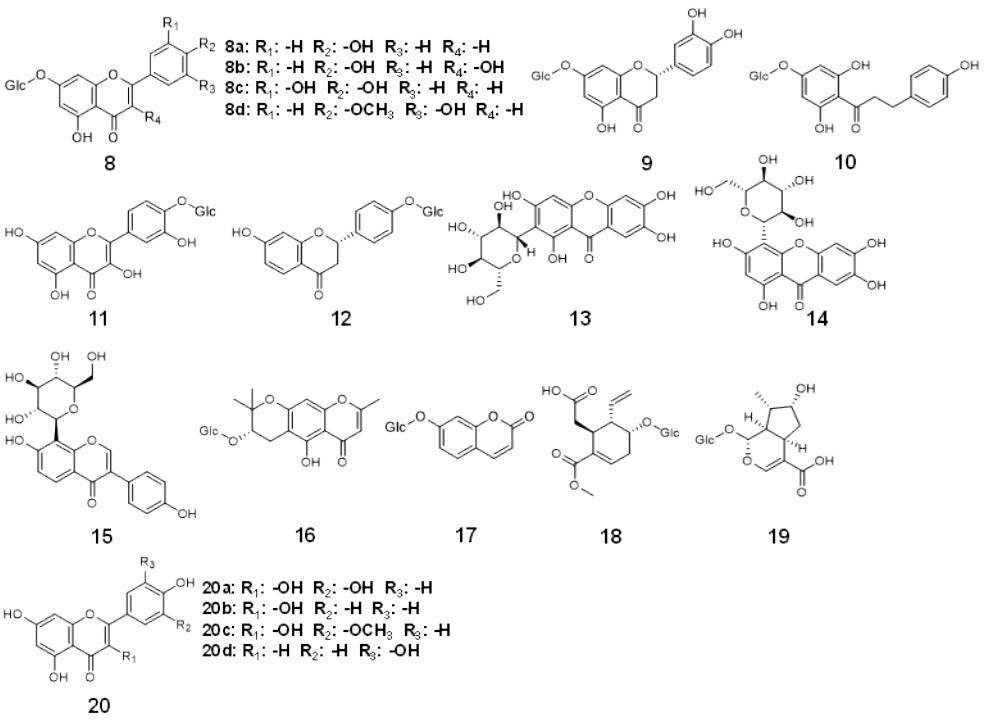

Fig. S17 Substrates not converted by Sj6′′RhaT.

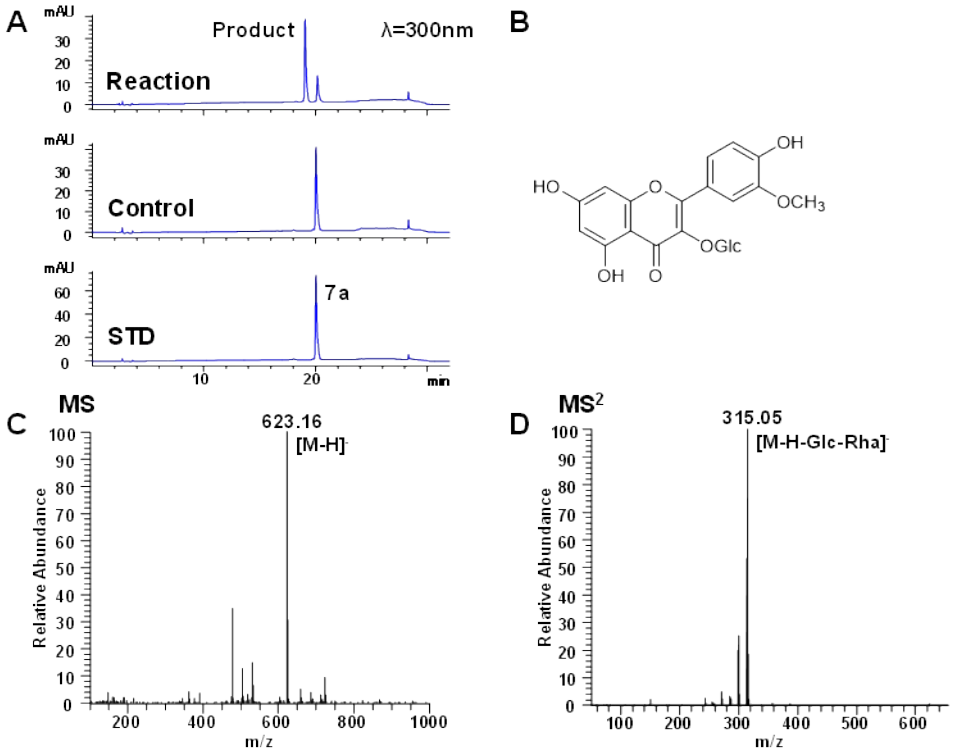

Fig. S18 The catalytic activity of Sj6′′RhaT converting substrate **7a**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **7a**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

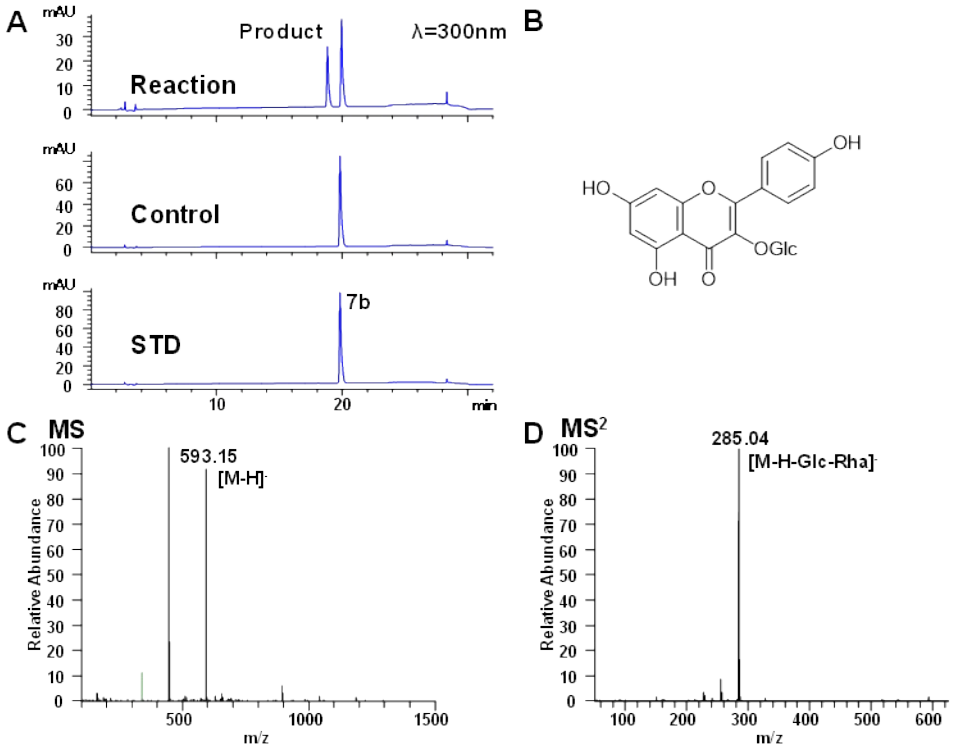

Fig. S19 The catalytic activity of Sj6′′RhaT converting substrate **7a**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **7a**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

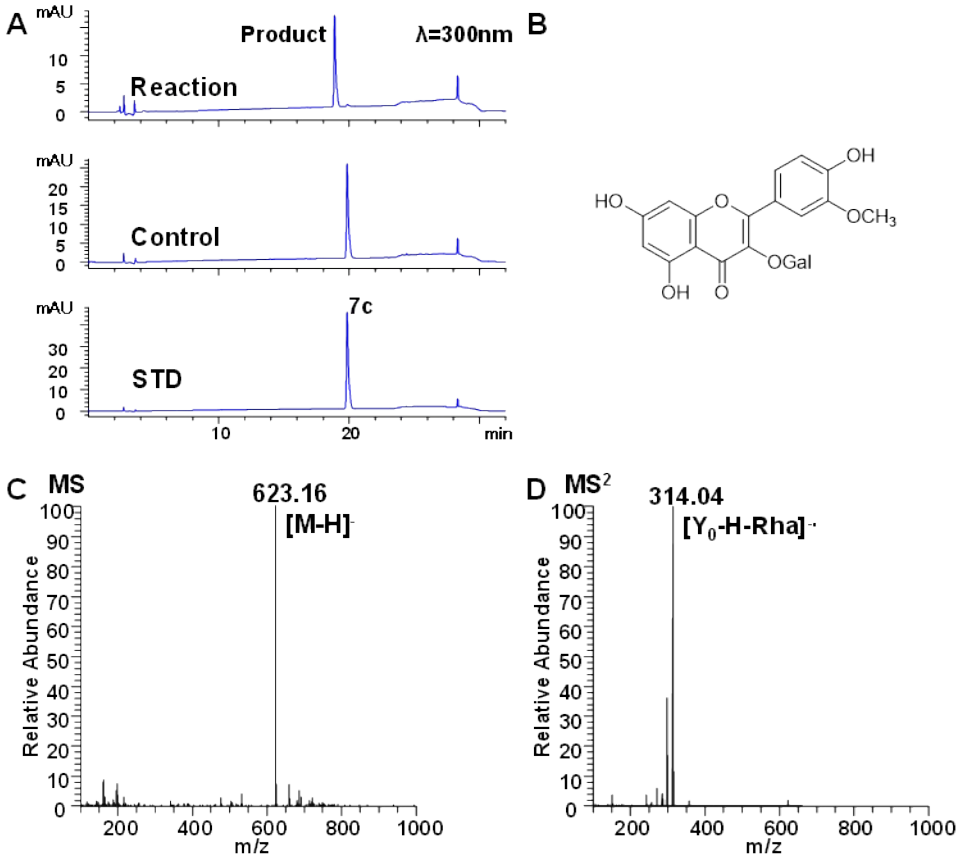

Fig. S20 The catalytic activity of Sj6′′RhaT converting substrate **7c**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **7c**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

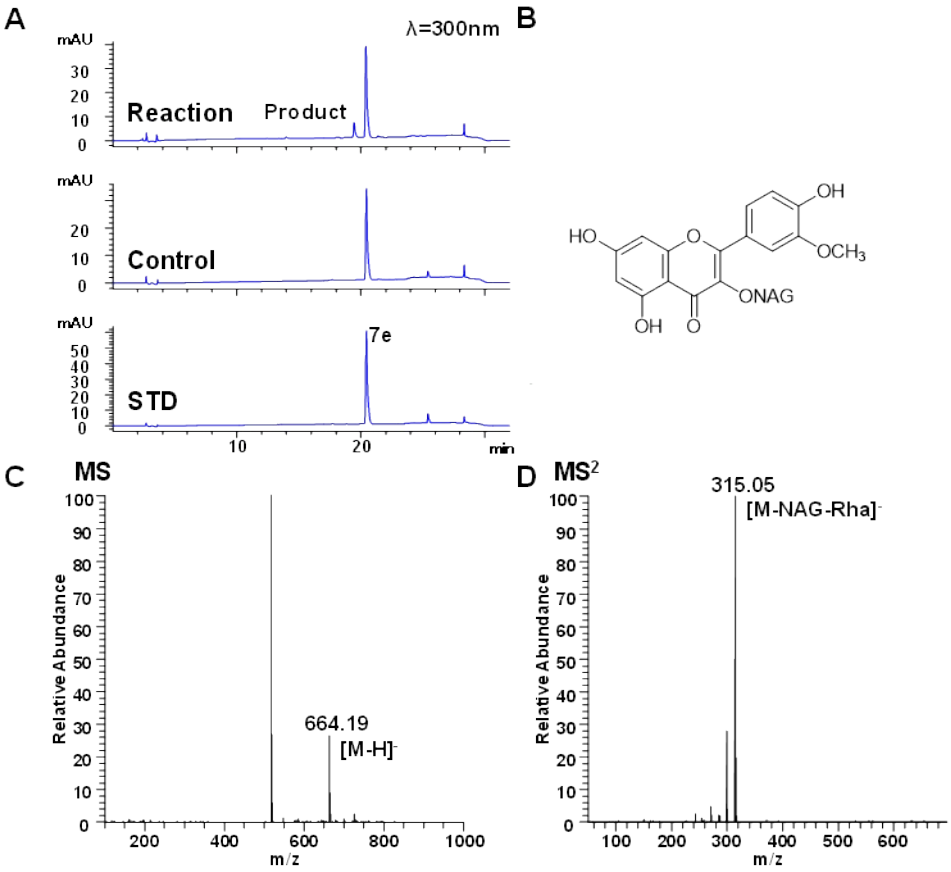

Fig. S21 The catalytic activity of Sj6′′RhaT converting substrate **7e**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **7e**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

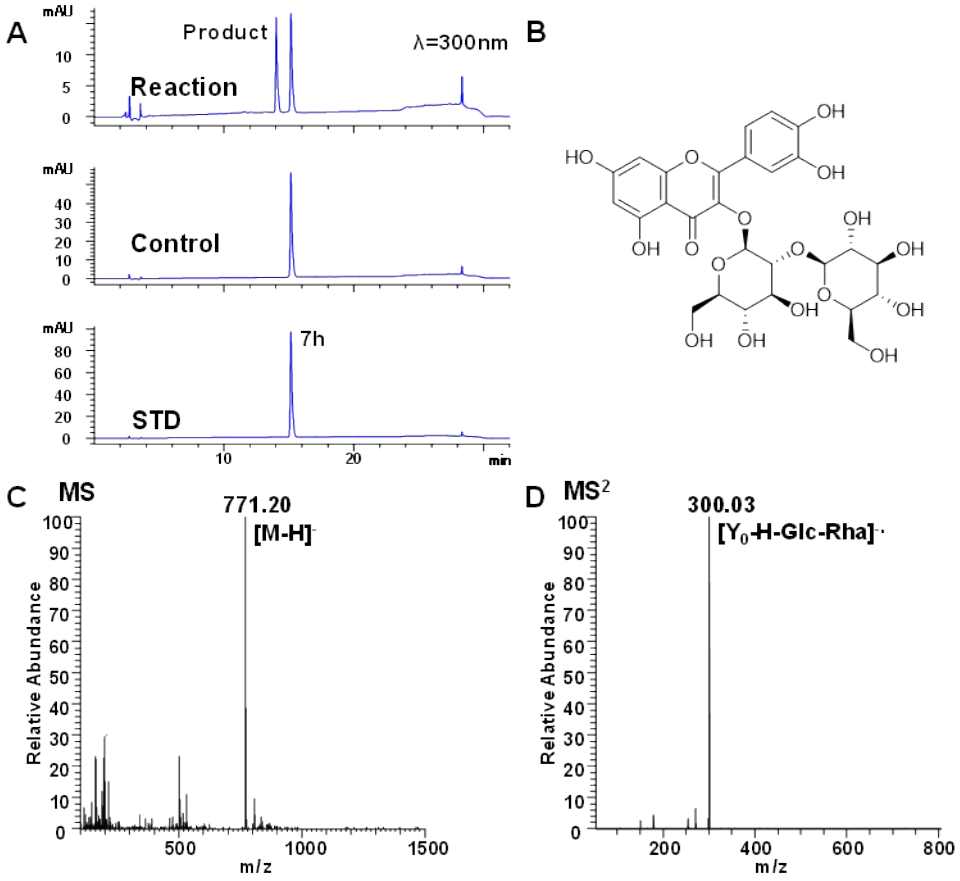

Fig. S22 The catalytic activity of Sj6′′RhaT converting substrate **7h**. (A) HPLC analysis of the reaction mixture and the reference standard. (B) The structure of substrate **7h**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

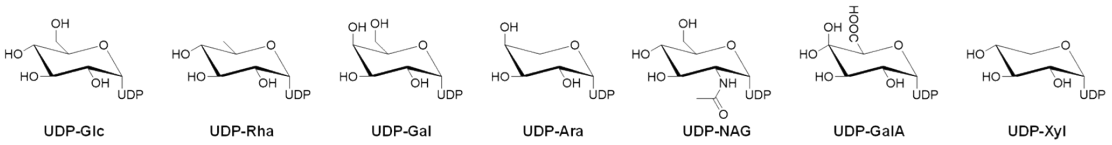

Fig. S23 Sugar donors used in this study.

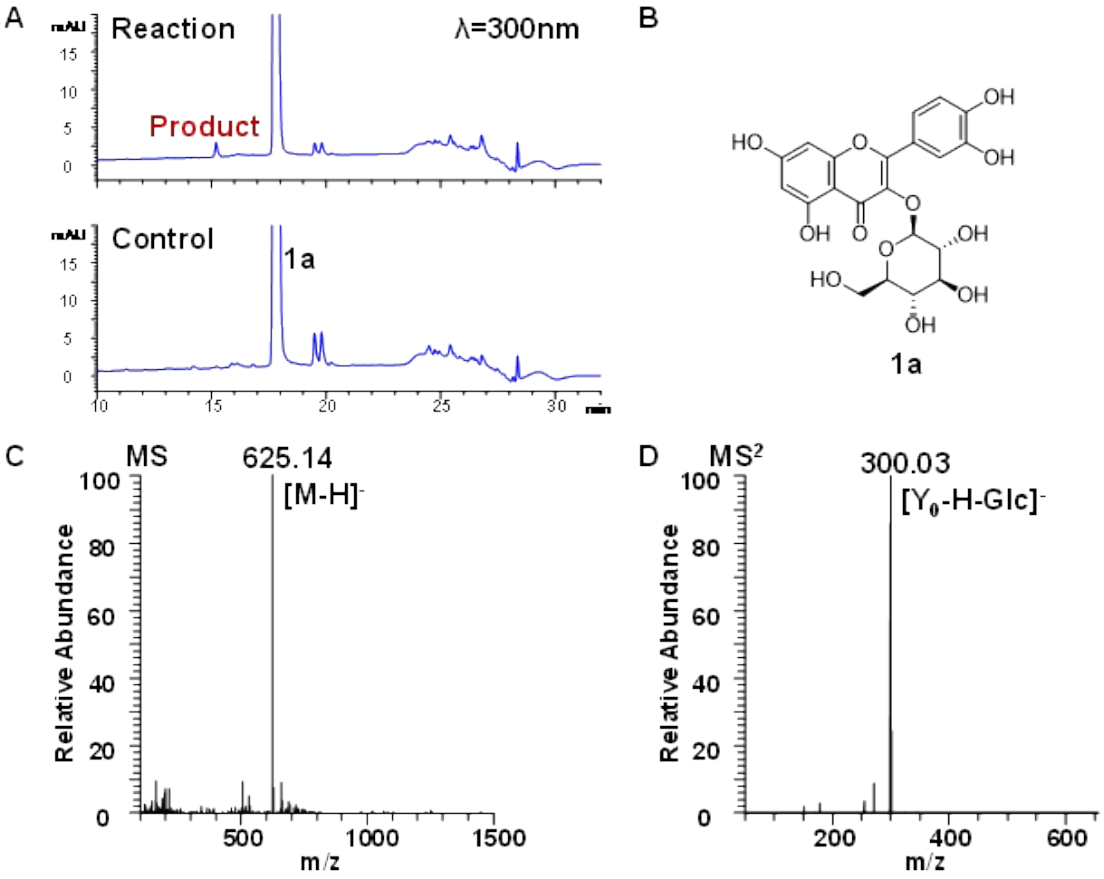

Fig. S24 The catalytic activity of Sj6″RhaT-Y135T utilizing UDP-Glc (A) HPLC analysis of the reaction mixture. (B) The structure of substrate **1a**. (C) (-)-ESI-MS spectra of the reaction product. (D) MS/MS spectra of the reaction product.

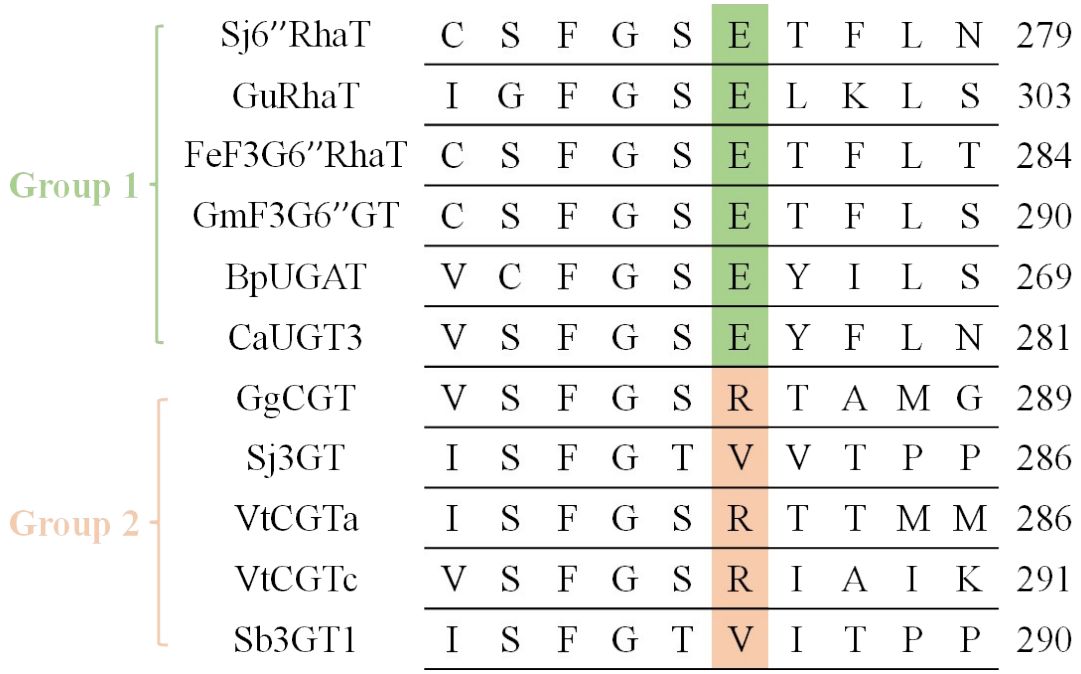

Fig. S25 Sequence alignment of glycosyltransferases catalyzing glycosylation on sugar residues (Group 1) and glycosyltransferases functioning on aglycones (Group 2).

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