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Synthesis of flavonol 3-*O*-glycoside by UGT78D1

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Abstract Glycosylation is an important method for the structural modification of various flavonols, resulting in the glycosides with increased solubility, stability and bioavailability compared with the corresponding aglycone. From the physiological point of view, glycosylation of plant flavonoids is of importance and interest. However, it is notoriously complicated that flavonols such as quercetin, kaempferol and myricetin, are glucosylated regioselectively at the specific position by chemical method. Compared to the chemical method, enzymatic synthesis present several advantages, such as mild reaction condition, high stereo or region selectivity, no protection/deprotection and high yield. UGT78D1 is a flavonol-specific glycosyltransferase, responsible for transferring rhamnose or glucose to the 3-OH position *in vitro*. In this study, the activity of UGT78D1 was tested against 28 flavonoids acceptors using UDP-glucose as donor nucleoside *in vitro*, and 5 acceptors, quercetin, myricetin, kaempferol, fisetin and isorhamnetin, were discovered to be glucosylated at 3-OH position. Herein, the small-scale 3-O-glucosylated quercetin, kaempferol and myricetin were synthesized by UGT78D1 and their chemical structures were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS).

Keywords UGT78D1 · Substrate specificity · UDP-glucose · Flavonol

Guangxiang Ren and Jingli Hou have equal contributions.

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Introduction

Flavonoids are a large group of plant natural products, exhibiting bioactivities with important implications for plant, animal and human health [1–5]. They often exist in glycosylated forms, comprising sugar units and an aglycone core. A large portion of this structural variability is due to the attachment of one or more sugar moieties at the different positions of the aglycone core as illustrated in Fig. 1 [6]. Flavonols such as quercetin, myricetin and kaempferol, attract considerable interest due to their diverse biological activities [7–10]. Attachment of one or more sugars units at different positions of the core aglycone structure, can increase their solubility and help to exhibit their inherent functions such as antioxidant capability and potential medicinal properties.

Glycosylated flavonols can be obtained by chemical or enzymatic syntheses. However, chemical synthesis of glycosylated flavonols at the specific position is notoriously complicated, due to several potential glycosylation sites in the flavonol aglycone (Fig. 2). Moreover, the chemical synthesis involves several protection and deprotection steps. Regioselectivity of glycosyltransferases offers an important strategy to overcome the limitation of chemical synthesis, and attracts a lot of attention. The enzymes involved in transferring sugars from a range of nucleoside donors onto acceptor molecules, glycosyltransferases (GTs), have been intensively studied recently [11–16]. Among these enzymes, the family 1 glycosyltransferases has been found to predominantly recognize low-molecular-weight compounds, such as phenolics, and transfer sugar to the 3-OH and 7-OH of the flavonol with high regioselectivity [6, 12, 17–22].

Uridine diphosphate glycosyltransferases (UGTs) catalyze the transfer of a sugar moiety from the uridine diphosphate-

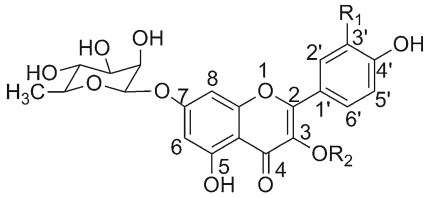


Fig. 1 Flavonol-glycosides that accumulate in wild type arabidopsis. R1, H (Kaempferol), OH (quercetin). R2, glucose (flavonol-3O-glucoside-7-O-rhamnoside), rhamnose (flavonol-3-O-rhamnoside-7-O-rhamnoside)

sugar to low molecular weight acceptors. *in vitro* study indicated that UGT78D1 is able to use UDP-glucose as substrates *in vitro* to transfer D-glucose to 3-OH position of quercetin [18]. Moreover, it can utilize UDP-rhamnose other than UDP-glucose as the donor [6]. While *in vitro* study indicated that quercetin and kaempferol were the substrates for UGT78D1 with UDP-rhamnose as donor nucleoside by screening 9 flavonoids, substrate specificity has not yet been reported for the recombinant protein with UDP-glucose as donor nucleoside. In this study, we tested the activity of UGT78D1 against 28 flavonoid acceptors using UDP-glucose as donor nucleoside *in vitro*, and 5 acceptors, including quercetin, myricetin, kaempferol, fisetin and isorhamnetin, were discovered as the potential substrates. In addition, we also synthesized and characterized the 3-O-glycosylated quercetin, myricetin and kaempferol.

Result and discussion

By SDS-PAGE anaylasis, it is found that UGT78D1 was expressed mainly in the supernatant and could be easilly purified via the one-step nickel affinity chromatography. A pre-experiment using the purified UGT78D1 to catalyze the reaction between UDP-Glucose and quercetin indicated the enzyme worked well (data not shown). To explore the substrate specificity of UGT78D1 using UDP-glucose as donor nucleotide, 28 flavonoids were tested *in vitro* and the glycosylated product was monitored by LC-MS. Novel compounds were formed for the substrates: quercetin, myricetin, kaemferol, fisetin and isorhamnetin, according to new LC peaks confirmed by MS data (data not shown). But no novel compound was detected for the other substrates (Table 1). A comparison of the structures among these compounds indicates the following elements are

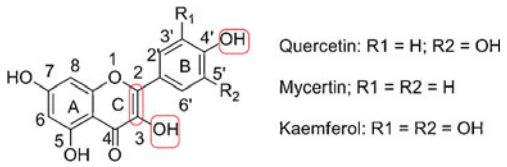


Fig. 2 The chemical structures of quercetin, mycertin and kaemferol

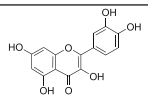
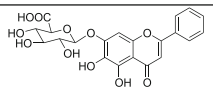
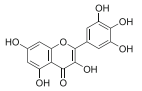
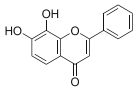
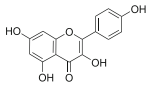
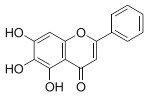
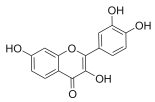
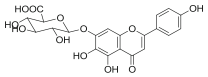
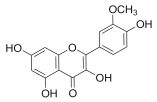
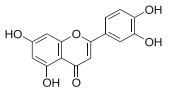
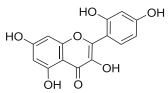
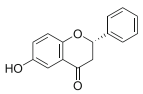
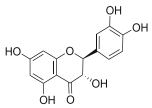
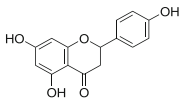
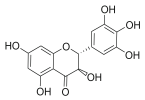
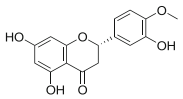
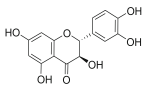
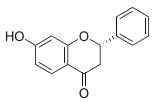
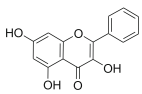
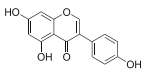
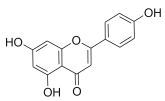
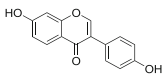
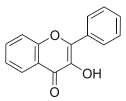
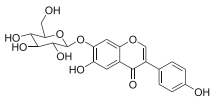
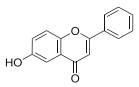
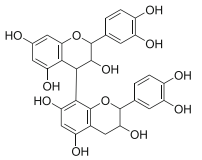
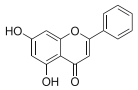
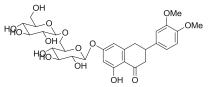
essential to the potential acceptors of UGT78D1: 1) the 3-hydroxyl group, 2) the double bond in the ring C, 3) the 4′-hydroxyl group (Fig. 1). Considering the ambiguity of UGT78D1 in recognizing sugar donors, the donor specificity of UGT78D1 was also examined with UDP-galactose, UDP-glucuronic acid and UDP-*N*-acetylglucosamine. No UGT activity was observed with the three sugar donors.

To obtain a higher yield of the glycosylated products, experiments designed to find the optimal reaction time were conducted using myricein as the substrate acceptor. The reaction was carried out as indicated in methods in a total volume of 500 μl and for a time range of 20 h. The reaction mixtures (40 μl) were sampled at some specific time points (0 h, 0.33 h, 1 h, 2 h, 4 h, 9 h and 20 h). The same volume of methanol was added to each sample and protein was removed from the reaction mixture by filtration. To measure the amount of glycosylated product and the remaining substrate, the filtrate was monitored on HPLC. The result indicates that Myricetin is quantitatively converted into the product within 20 h, the longer the time, the higher the yield of the product (Fig. 3).

To confirm the LC-MS results, which have deduced the generation of specifically linked glycosylated products, 3-*O*-glycosylated quercetin, myricetin and kaempferol were characterized by NMR and HRMS (Figs. 4, 5 and 6). Firstly, these products were synthesized by scaling up the reaction volume to 100 mL, following the indicated reaction condition for 20 h. The protein was removed by filtration and the filtrate was freeze dried. The resulting residue was purified to give the corresponding glucoside. Their structures were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS.

The HiResMALDI-FTMS analysis of the purified glucosides confirmed only one glucose was transferred to the substrates (monoglucoside of quercetin: calcd. for $C_{21}H_{20}NaO_{12}$ $[M+Na]^+$ 487.0852 found 487.0842; monoglucoside of kaempferol: calcd. for $C_{21}H_{20}NaO_{11}$ $[M+Na]^+$ 471.0903 found 471.0896; monoglucoside of myricetin: calcd. for $C_{21}H_{20}NaO_{13}$ $[M+Na]^+$ 503.0802 found 503.0797.) The NMR data for quercetin 3-*O*-β-glucoside: ^{1}H NMR (CD_3OD, 400 MHz): δ 7.71 (1H, *d*, *J*=2.0 Hz, H-2′); 7.59 (1H, *dd*, J_1=2.0 Hz, J_2=8.5 Hz, H-6′); 6.87 (1H, *d*, *J*=8.5 Hz, H-5′); 6.40 (1H, *d*, *J*=1.9 Hz, H-8), 6.21 (1H, *d*, *J*=1.9 Hz, H-6); 5.25 (1H, *d*, *J*=7.5 Hz, H-1″); 3.19–3.73 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″); ^{13}C NMR (CD_3OD, 100 MHz): δ 179.6, 166.1, 163.1, 159.1, 158.5, 149.9, 146.0, 135.7, 123.2, 123.1, 117.6, 116.1, 105.8, 104.3, 99.9, 94.7, 78.5, 78.2, 75.8, 71.3, 62.6; the NMR data for kaempferol 3-*O*-β-glucoside: ^{1}H NMR (CD_3OD, 400 MHz): δ 8.06 (2H, *d*, *J*=8.6 Hz, H-2′ and H-6′), 6.89 (2H, *d*, *J*=8.6 Hz, H-3′ and H-5′); 6.41 (1H, s, H-8), 6.21 (1H, s, H-6), 5.25 (1H, *d*, *J*=7.0 Hz, H-1″), 3.19–3.71 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″); ^{13}C NMR (CD_3OD, 100 MHz): δ 179.6, 166.0, 163.1, 161.6, 159.2, 158.6,

Table 1 Quantitative substrate specificity of recombinant UGT78D1 using UDP-glucose as sugar nucleotide as analyzed by LC-MS (CR: Conversion rate)

Run	Aglycon	CR (%)	Run	Aglycon	CR (%)
Quercetin		100	Baicalein 7-*O*-β-D-glucuronide		0
Myricetin		90	7,8-Dihydroflavone		0
Kaempferol		69	Baicalein		0
Fisetin		76	Scutellarin		0
Isorhamnetin		20	Luteolin		0
Morin		0	6-Hydroxyflavanone		0
Dihydroquercein		0	Naringenin		0
Dihydromyricetin		0	Hesperitin		0
Taxifoliol		0	7-Hydroxyflavanone		0
Galangin		0	Genistein		0
Apigenin		0	Daidzein		0
3-Hydroxyflavone		0	Puerarin		0
6-Hydroxyflavone		0	Procyanidins		0
Chrysin		0	Compound 1		0

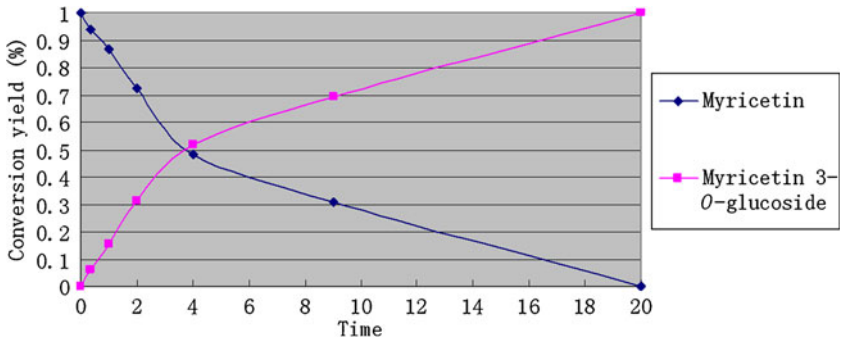


Fig. 3 Changes in the level of Myricetin-3-*O*-glucoside and aglycone in the reaction mixture over a time-course of 0–20 h

135.5, 132.3, 122.8, 116.1, 105.8, 104.1, 99.9, 94.8, 78.5, 78.1, 75.8, 71.4, 62.7; Myricetin-3-O-β-glucoside: ^{1}H NMR (CD_3OD, 400 MHz): δ 7.30 (s, 2H, H-2′ and H-6′), 6.39 (1H, *d*, *J*=2.0 Hz, H-8), 6.21 (1H, *d*, *J*=2.0 Hz, H-6), 5.26 (1H, *d*, *J*=7.7 Hz, H-1″), 3.21–3.78 (6H, m, H-2″, H-3″, H-4″, H-5″, H-6″); ^{13}C NMR (CD_3OD, 100 MHz): δ 179.5, 166.1, 163.1, 1589.1, 158.5, 146.5, 138.1, 135.9, 121.9, 110.0, 105.7, 104.5, 99.9, 94.7, 78.4, 78.2, 75.7, 71.1, 62.5.

The NMR data were in agreement with those available in the literature [18, 23, 24], and some of them have been reported for the first time in this work (^{1}H and ^{13}C NMR data for kaempferol 3-*O*-glucoside and myricetin 3-O-

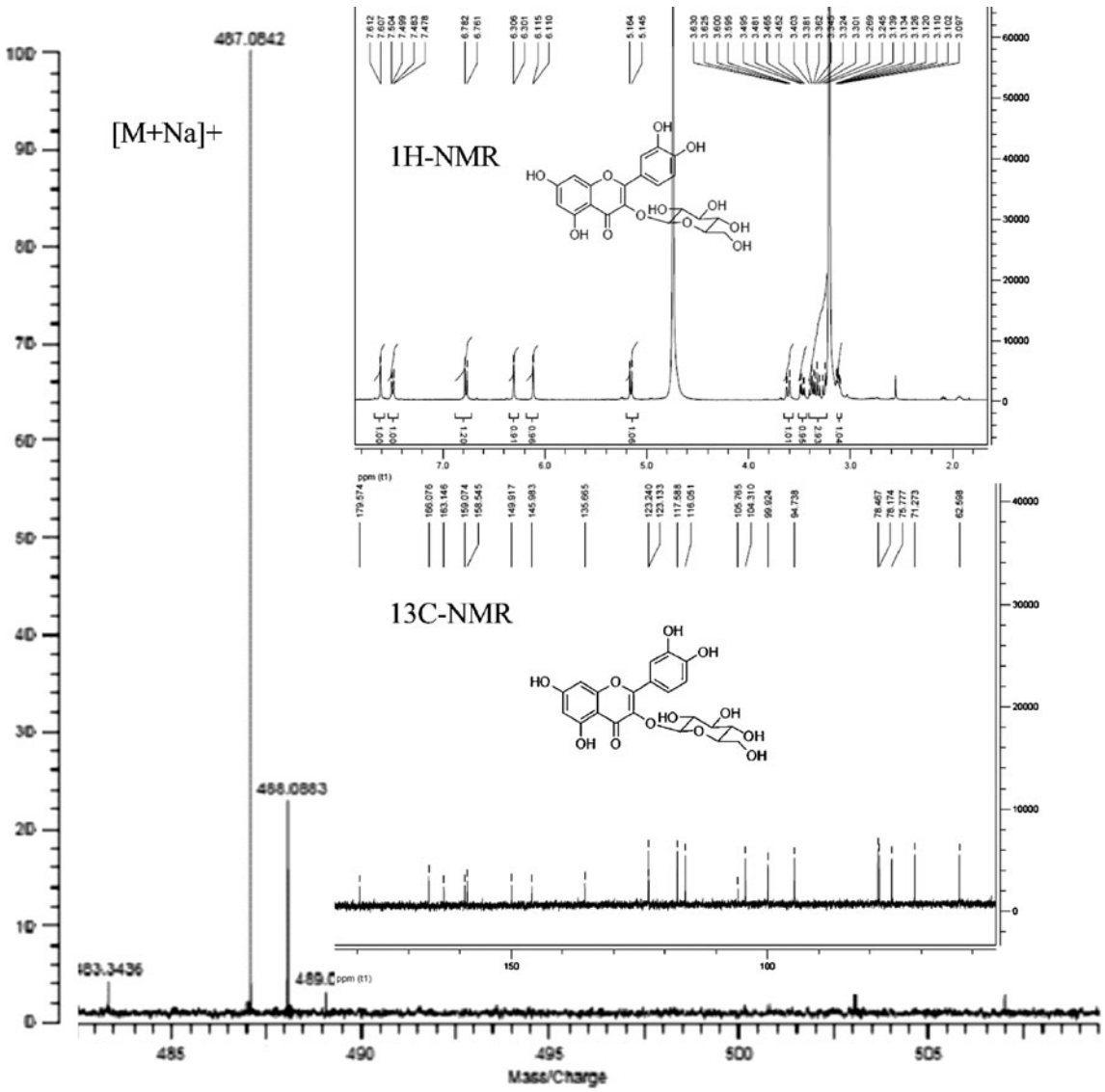


Fig. 4 Characterization of quercetin-3-O-glucoside with HRMS and NMR(^{1}H-NMR, ^{13}C-NMR)

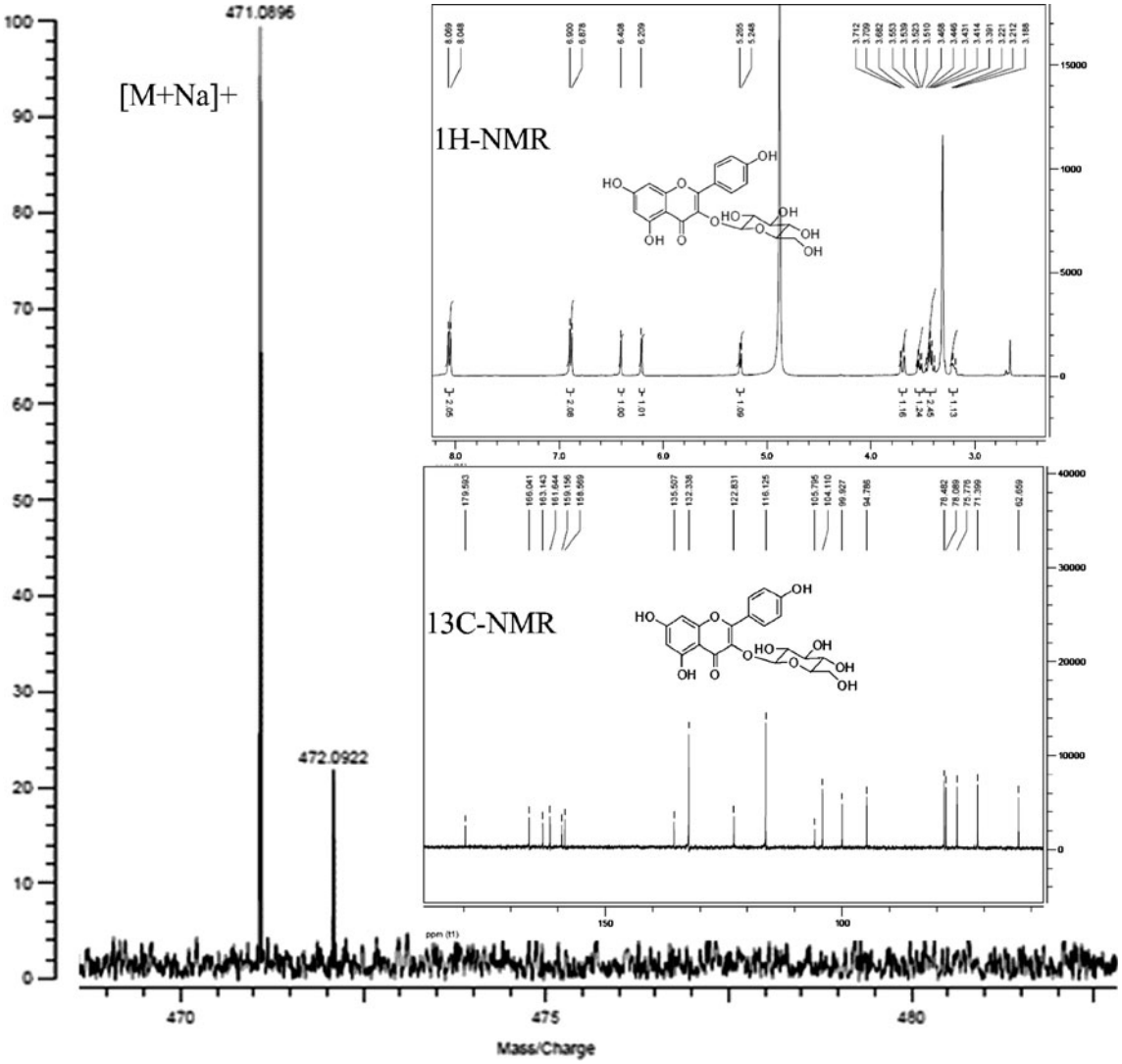


Fig. 5 Characterization of kaemferol-3-*O*-glucoside with HRMS and NMR(^{1}H-NMR, ^{13}C-NMR)

glucoside). The shielding parameters for hydrogen atoms in the substrate flavonol, when compared to the corresponding signals for the aglycon moiety of the product, supported the fact that the glycosidic linkage is by the C-3 position (Table 2). The measured value for the coupling constant (J_{HH}) between the sugar protons H-1″ and H-2″ ranging within 7–8 Hz for the three glucosides, indicated β-configuration for the 3-*O*-glycosidic linkage. In addition, the UV–vis spectra are also consistent with the literature [23] (Table 3).

Conclusion

In summary, the substrate specificity for UGT78D1 using UDP-glucose as sugar donor was studied extensively by screening 28 flavonoids. Five flavonols, quercetin, myricetin, kaempferol, fisetin and isorhamnetin, were identified as the preferred substrates. In addition, quercetin 3-*O*-glucose, keampferol 3-*O*-glucoside and myricetin 3-*O*-glucoside were synthesized using UGT78D1, and their structures were confirmed by HRMS and NMR.

Material and methods

General information

Flavonol chemicals were purchased from Chengdu PuRuiFa Technology & Develop. Co. Ltd., China. Other materials were purchased from Beijing Dingguo Changsheng Biotechnology Co. Ltd., China.

Expression and purification of UGT78D1

UGT78D1 (GI: 839933) was codon optimized for E. *coli* heterologous expression and synthesized in Beijing Augct

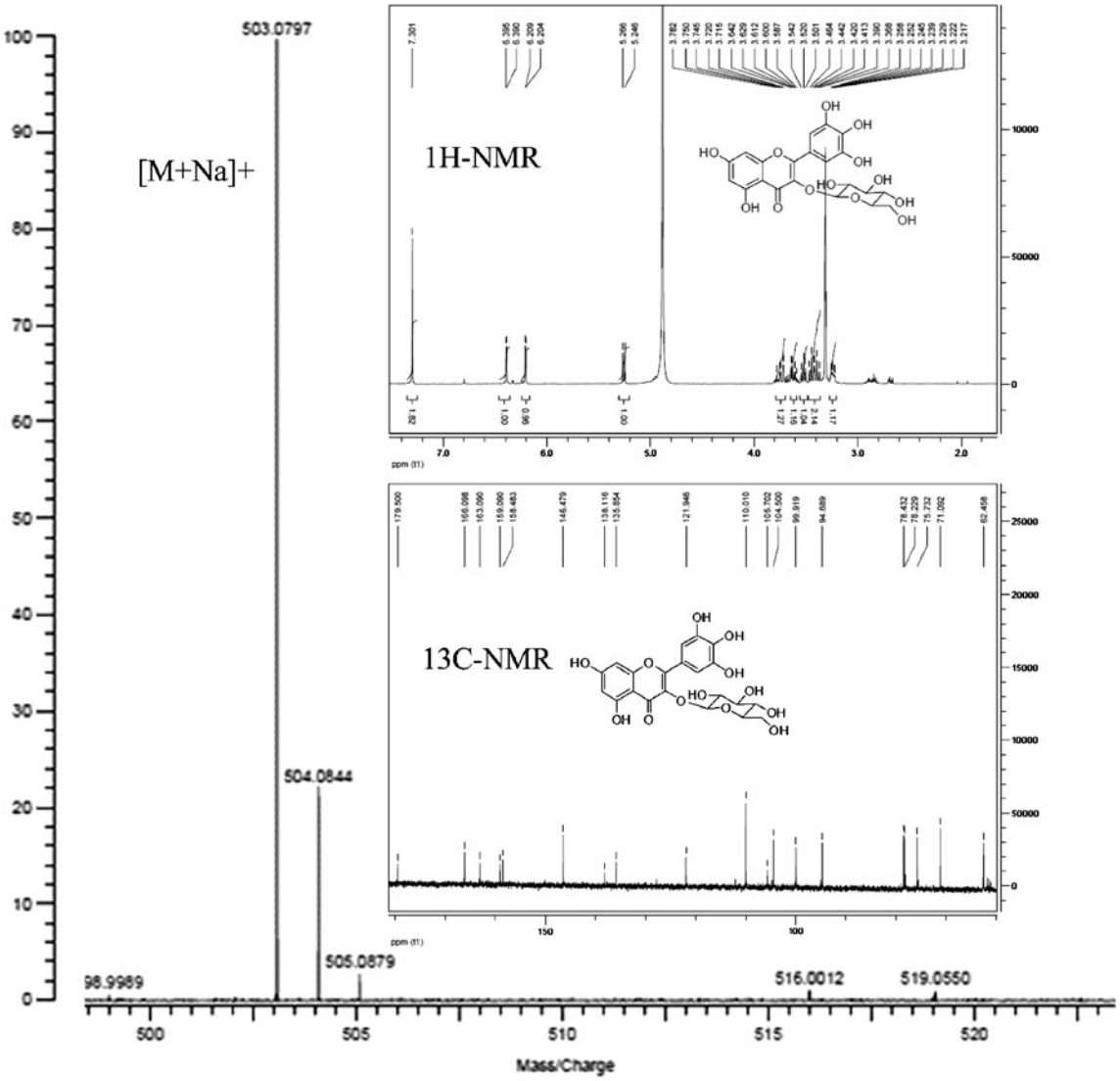


Fig. 6 Characterization of myricetin-3-O-glucoside with HRMS and NMR(^{1}H-NMR, ^{13}C-NMR)

Table 2 The ^{1}H NMR spectral data of Quercetin, Kaempferol, Myricetin and their monoglucosides

Position	Quercetin	Quercetin 3-*O*-glucoside	Myricetin	Myricetin 3-*O*-glucoside	Kaempferol	Kaempferol 3-*O*-glucoside
C2′	7.73(1H, *d*, *J*=2.1 Hz	7.71(1H, *d*, *J*=2.0 Hz)	7.34 (2H,s)	7.30(2H, s)	8.05(2H, *d, J*=8.5 Hz)	8.06(2H, *d*, *J*=8.6 Hz)
C6′	7.63(1H, *d*, J_1=2.1 Hz, J_2=8.5 Hz	7.59(1H, *dd*, J_1=2.0 Hz, J_2=8.5 Hz)				
C3′	—	—	—	—	6.88 (2H, *d*, *J*=8.5 Hz)	6.89 (2H, *d*, *J*=8.6 Hz)
C5′	6.88 (1H, *d*, J=8.5 Hz)	6.87 (1H, *d*, *J*=8.5 Hz)	—	—		
C8	6.38 (1H, *d*, *J*=2.0 Hz)	6.40 (1H, *d*, *J*=1.9 Hz)	6.36 (1H, *d*, *J*=2.0 Hz)	6.39 (1H, *d*, *J*=2.0 Hz)	6.35 (1H,s)	6.41 (1H,s)
C6	6.17(1H, *d*, *J*=2.0 Hz)	6.21(1H, *d*, *J*=1.9 Hz)	6.16 (1H, *d*, J=2.0 Hz)	6.21(1H, *d*, *J*=2.0 Hz)	6.15(1H,s)	6.21(1H, s)
Glc-1	—	5.25 (1H, *d*, *J*=7.5 Hz)	—	5.26(1H,*d*, *J*=7.7 Hz)	—	5.25 (1H, *d*, *J*=7.0 Hz)
Glc-2	—	3.19–3.73 (6H, m)	—	3.21–3.78 (6H, m)	—	3.19–3.71 (6H, m)
Glc-3	—		—		—	
Glc-4	—		—		—	
Glc-5	—		—		—	
Glc-6	—		—		—	

Table 3 UV–VIS of flavonol 3-*O*-glycosides

Flavonol	UV–VIS maxima (nm)	Flavonol	UV–VIS maxima (nm)
Myricetin 3-*O*-glucoside	260, 360	Quercetin 3-*O*-glucoside	260, 360
Kaempferol 3-*O*-glucoside	260, 340		

biotech. Co. Ltd., China. Then, it was constructed into pET-21a expression vector. The expression and purification of the protein was as described previous [6].

Screening assay

The reactions were performed at 30 °C for 45 min, in a total volume of 200 μL containing 150 μg/mL recombinant protein, 50 mM Tris–HCl (pH=7.2), 14 mM 2-mercaptoethanol, 4 mM UDP-glucose, and variant flavonols (0.5 mM). After quenching by adding equal volume of methanol, the reaction mixtures are centrifuged at 12, 000 × g for 30 min and filtered with a 0.22 μm filter. 40 μL of the reaction mixtures were determined on LC-20A or LCMS-2020 (Shimadzu, Japan) to identify and quantify the products, using a Synergi Polar-RP column(250*4.60 mm) with a linear gradient elution of 25 % to 85 % acetonitrile in H2O (all containing 0.1 % trifluoroacetic acid) at 0.8 mL/min over 20 min and monitored at 360 nm. The yield was analyzed based on the integrated areas of products and substrate acceptor.

Characterization of flavonol-glucosides

Scaled-up reactions were conducted for three acceptor substrates with high yields of the product (quercetin, myricetinand and kaempferol). After purification of the flavonol-glucosides by reverse phase chromatography, the glycosylated products were characterized by HiResMALDI-FTMS (Varian 7.0T FTMS), NMR, and UV–VIS.

Reverse phase chromatography was eluted with a gradient of acetonitrile in H_2O (containing 0.1 % trifluoroacetic acid).

NMR was conducted on BRUKER-AV400 in deuterated methanol (CD_3OD).

UV–VIS was conducted on Varioskan Flash Multimode Reader (Thermo Scientific) using 96 well UV plates. Each flavonol glucoside was dissolved in water and the concentration was 0.1 mM.

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References

1. Dixon, R.A., Steele, C.L.: Flavonoids and isoflavonoids - a gold mine for metabolic engineering. Trends Plant Sci. **4**, 394–400 (1999)
2. Dixon, R.A.: Natural products and disease resistance. Nature **411**, 843–847 (2001)
3. Ross, J.A., Kasum, C.M.: Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu. Rev. Nutr. **22**, 19–34 (2002)
4. Halliwell, B., Rafter, J., Jenner, A.: Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? Am. J. Clin. Nutr. **81**, 268S–276S (2005)
5. Nicolle, E., Souard, F., Faure, P., Boumendjel, A.: Flavonoids as promising lead compounds in type 2 diabetes mellitus: molecules of interest and structure-activity relationship. Curr. Med. Chem. **18**, 2661–2672 (2011)
6. Jones, P., Messner, B., Nakajima, J.-I., Schäffner, A.R., Saito, K.: UGT73C6 and UGT78D1, glycosyltransferase involved in flavonol glycoside biosynthesis in Arabidopsis thliana. J. Biol. Chem. **278**(45), 43910–43918 (2003)
7. Tamara, P.K., John, M.P.: Natural product polyphenols of relevance to human health. Pharm. Biol. **42**(Suppl), 46–63 (2004)
8. Scalbert, A., Johnson, I.T., Saltmarsh, M.: Polyphenols: antioxidants and beyond. Am. J. Clin. Nutr. **81**, 215–217 (2005)
9. Manach, C., Williamson, G., Moland, C., Scalbert, A., Rémésy, C.: Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. **81**, 230–242 (2005)
10. Williamson, G., Manach, C.: Bioavailability and bioefficacy of polyphenols in humans. II. Review of 97 intervention studies. Am. J. Clin. Nutr. **81**, 243–255 (2005)
11. Jones, P., Vogt, T.: Glycosyltransferases in secondary plant metablolism: tranquilizers and stimulant controllers. Planta **213**, 164–174 (2001)
12. Vogt, T., Jones, P.: Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. Trends Plant Sci. **5**, 380–386 (2000)
13. Li, Y., Baldauf, S., Lim, E.-K., Bowles, D.J.: Phylogenetic analysis of UDP-glycosyltransferases multigene family of Arabidopsis thaliana. J. Biol. Chem. **276**, 4338–4343 (2001)
14. Paquette, S., Moller, B.L., Bak, S.: On the origin of family 1 plant glycosyltransferases. Phytochem. **62**, 399–413 (2003)
15. Simkhada, D., Lee, H.C., Sohng, J.K.: Genetic engineering approach for the production of rhamnosyl and allosyl flavonoids from Escherichia coli. Biotechnol. Bioeng. **107**, 154–162 (2010)
16. Choi, S.H., Ryu, M., Yoon, Y.J., Kim, D.-M., Lee, E.Y.: Glycosylation of various flavonoids by recombinant oleandomycin glycosyltransferase from Streptomyces antibioticus in batch and repeated batch modes. Biotechnol. Lett. **34**, 499–505 (2012)
17. Lim, E.K., Doucet, C.J., Elias, L., Worrall, D., Li, Y., Ross, J., Bowles, D.J.: Activity of the group 1 glycosyltransferases of Arabidopsis towards salicylic acid, para-hydroxybenzoic acid and other benzoates. J. Biol. Chem. **277**, 586–592 (2002)
18. Lim, E.-K., Ashford, D.A., Hou, B., Jackson, R.G., Bowles, D.J.: Arabidopsis Glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. Biotech. Bioeng. **87**, 623–631 (2004)
19. Ford, C.M., Boss, P.K., Høj, P.B.: Cloning and characterization of Vitis vinifera UDP-glucose: flavonoid 3-O-glucosyltransferases, a homologue of the enzyme encoded by the maize Bronze-1 locus

that may primarily serve to glucosylate anthocyanidins in vivo. J. Biol. Chem. **273**, 9224–9233 (1998)
20. Kramer, C.M., Prata, R.T.N., Willits, M.G., De Luca, V., Steffens, J.C., Graser, G.: Cloning and regiospecificity studies of two flavonoid glucosyltransferases from Allium cepa. Phytochemistry **64**, 1069–1076 (2003)
21. Miller, K.D., Guyon, V., Evans, J.N.S., Shuttleworth, W.A., Taylor, L.P.: Purification, cloning, and heterologous expression of a catalytically efficient flavonol 3-O-galactosyltransferase expressed in the male gametophyte of Petunia hybrida. J. Biol. Chem. **274**, 34011–34019 (1999)
22. Willits, M.G., Giovanni, M., Prata, R.T.N., Kramer, C.M., Luca, V.D., Steffens, J.C., Graser, G.: Bio-fermentation of modified flavonoids: an example of in vivo diversification of secondary metabolites. Phytochemistry **65**, 31–41 (2004)
23. Castillo-Muñoz, N., Gómez-Alonso, S., García-Romero, E., Gómez, M.V., Velders, A.H., Hermosín-Gutiérrez, I.: Flavonol 3-Glycosides series of vitis 1 vinifera cv. petit verdot red wine grapes. J. Agric. Food Chem. **57**, 209–219 (2009)
24. Agrawal, P.K.: Carbon-13 NMR of flavonoids. Elsevier Science, New York (1989)