J. Plant Biol. (2009) 52:41–48
DOI 10.1007/s12374-008-9002-x

ORIGINAL RESEARCH

Flavonoid *O*-Diglucosyltransferase from Rice: Molecular Cloning and Characterization

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Received: 6 October 2008 / Revised: 21 October 2008 / Accepted: 22 October 2008 / Published online: 21 January 2009

Abstract Among more than 100 rice uridine diphosphate glycosyltransferases (UGTs), *OsUGT-3* was selected as a candidate for producing flavonoid *O*-diglycosyltransferases based on phylogenetic analysis and molecular docking. This gene was functionally expressed in *Escherichia coli*. Analysis of kaempferol, luteolin, quercetin, and tricin reaction products using liquid chromatography-mass spectrometry revealed that these were diglucosylated. The glucosylation positions of kaempferol, which was the best substrate, were determined to be the 3- and 7-hydroxyl groups. This is the first flavonoid *O*-diglucosyltransferase described from rice.

Keywords Flavonoids · Flavonoid *O*-diglucoside · Glycosyltransferase · *Oryza sativa* · UGT

The attachment of sugars to secondary metabolites is a common modification reaction found in microorganisms and plants, modulating the biological activity of antibiotics and plant hormones [1, 13, 15]. Glycosylation of those secondary metabolites also has a role in increasing solubility and stabilizing compounds [4]. This process is mediated by uridine diphosphate-dependent glycosyltransferase (UGT; EC 2.4.1.17), which uses uridine diphosphate sugar (UDP-sugar) as a donor and transfers sugar(s) to an acceptor [2]. UGTs have been found in microorganisms, plants, and humans. Those from humans have been extensively studied due to their metabolism of xenobiotics [17], whereas the UGTs from microorganisms have been targeted for engineering to produce novel drugs [24]. Plant UGTs outnumber those from other sources due to their diversity of secondary metabolism. More than 300 UGTs have already been found in plants, and this number continues to increase [2].

Flavonoids are widely distributed in plants, and more than 9,000 are known [23]. Their diversity arises from a combination of several modification reactions, including methylation, glycosylation, malonylation, and hydroxylation. Most plant flavonoids are glycosylated. The major sugars attached to flavonoids are glucose, galactose, and rhamnose [5, 19]. Some UGTs utilize specific UDP-sugars, whereas most use diverse UDP-sugars [20]. For instance, UGT89C1 from *Arabidopsis thaliana* exclusively uses UDP-rhamnose [27]. Specificity of the UDP-sugar in UDP-galactose: anthocyanin galactosyltransferase is altered in a single amino acid mutation [12]. Some flavonoids contain more than one sugar unit—either at different positions or in tandem [26]. Nevertheless, most of the UGTs studied so far in vitro transfer a single sugar molecule into the flavonoids. A few UGTs from *A. thaliana* produce flavonoid *O*-diglucosides in addition to monoglucosides [16]. For example, UGT71C1 transfers glucoses into the 7-hydroxyl and 3′-hydroxyl groups of quercetin, while UGT76E2 moves them into the 7-hydroxyl and 3-hydroxyl groups.

Rice also contains various flavonoid glucosides. Although some are *O*-glucosides, the major forms are *C*-glucosides [7].

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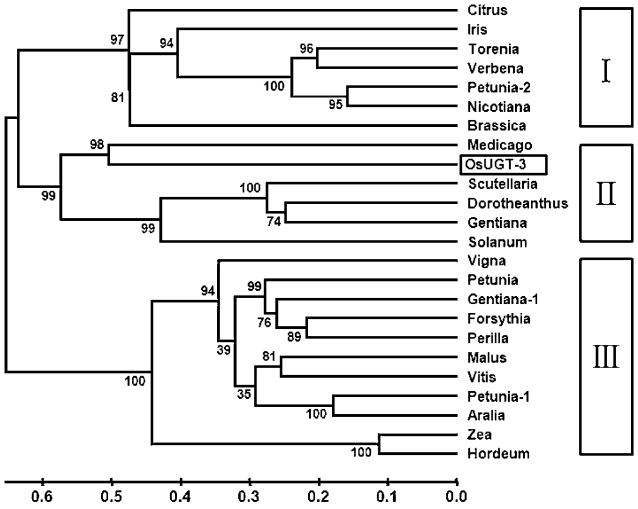


Fig. 1 Phylogenetic tree analysis of OsUGT-3. I anthocyanin glycosyltransferase, II UGTs with diverse regioselectivity and substrate, III flavonoid 3-*O*-glycosyltransferase. GenBank accession numbers for UGTs: Citrus (B033758), Iris (AB113664), Torenia (AB076698), Verbena (AB013598), Petunia-2 (AB027455), Nicotiana (AB072919), Brassica (A62529), Medicago (AY747627), Scutellaria (AB031274), Dorotheanthus (Y18871), Gentiana (AB076697), Solanum (STU82367), Vigna (AB009370), Petunia (AB027454), Gentiana-1 (D85186), Forsythia (AF127218), Perilla (AB013596), Malus (AF117267), Vitis (AB047092), Petunia-1 (AF165148), Aralia (AB103471), Zea (AY167672), and Hordeum (X15694)

We have been studying rice flavonoid UGTs [3, 9, 10], all of which are flavonoid *O*-monoglucosyltransferases. However, rice also appears to possess flavonoid di- or triglucosides [7]. We have searched rice UGTs with the ability to transfer two sugars into flavonoids. Here, we report the in vitro characterization of rice flavonoid *O*-diglucosyltransferase.

Materials and Methods

Cloning of *OsUGT-3*

OsUGT-3 (GenBank accession number CT830931) was cloned by reverse transcription polymerase chain reaction (RT-PCR), using cDNA as a template. Primers were forward, 5′-ATGAAGCAAACCGTCGTCC-3′, and reverse, 5′-TTCGTCAGTTAGTTCTGGACAAG-3′. cDNA was synthesized with total RNA isolated from 3-week-old rice plants (*Oryza sativa* "Nakdong"), using Omniscript reverse transcriptase (Quiagen, Germany) and oligo dT as a primer. The PCR product was subcloned into a pGEM-T easy vector (Promega, USA) and sequenced.

Expressions of *OsUGT*s and other genes were analyzed with real-time PCR, as described by [6]. The *OsUGT-3* primers were 5′-GGTGTTCATGACGGCGGAGA-3′ and 5′-TCCGACTCCATCACCAGCCT-3′, while the other primers were detailed by Kim et al. [6] and Ko et al. [10]. Four-week-old rice plants were exposed to UV irradiation supplied by three lamps (Philips TL 20W/12 UV, 0.15 W m^{-2}). These plants were located 30 to 35 cm below the lamps. Stems and leaves were harvested after 2, 6, or 24 h of treatment.

Expression of *OsUGT-3* in *Escherichia coli*

The open reading frame of *OsUGT-3* was sub-cloned into pGEX 5X-3 (Amersham Biotech, USA). To express *OsUGT-3*, we cultured an *E. coli* transformant harboring *OsUGT-3* overnight in Luria–Bertani (LB) medium containing 50 μg mL^{-1} ampicillin. Afterward, 0.5 mL of this culture was inoculated into 100 mL of the LB/ampicillin medium and grown until its absorbance at 600 nm reached 0.8. Isopropyl-β-D-thio-galactoside was then added at a final concentration of 0.1 mM, and the culture was incubated overnight at 18°C. The recombinant protein was purified as described by Ko et al. [9] then checked via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Analysis of Reaction Products

Flavonoids were obtained from either Indofinechemicals (New Jersey, USA) or our lab collections. The reaction mixture for the UDP-glucosyltransferase enzyme assay contained 10 μg of purified OsUGT-3, 5 mM $MgCl_2$, 1 mM UDP-glucose, and 100 μM of substrate in 500 μl of 10 mM KH_2PO_4 (pH 7.4). This mixture was incubated at 37°C for 30 min, and the reactions were stopped with 10 μl of

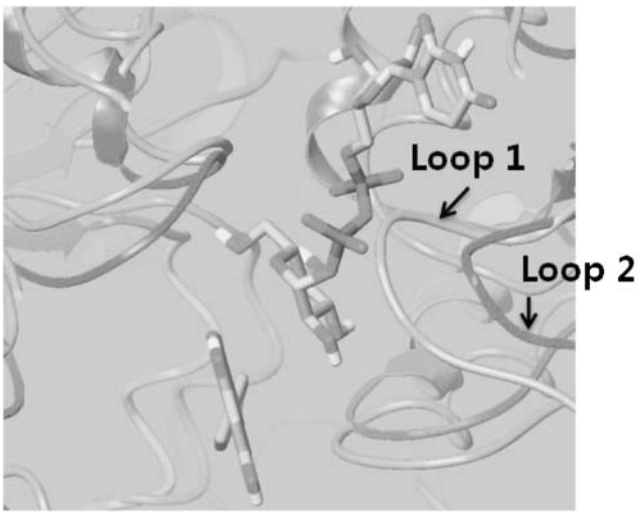


Fig. 2 Substrate binding sites for OsUGT3 (*dark-gray line*) and 706D3 (*light-gray line*). Two structures are overlapped. Kaempferol 3-*O*-glucoside and UDP-glucose fit into binding site. Loops 1 and 2 are shown

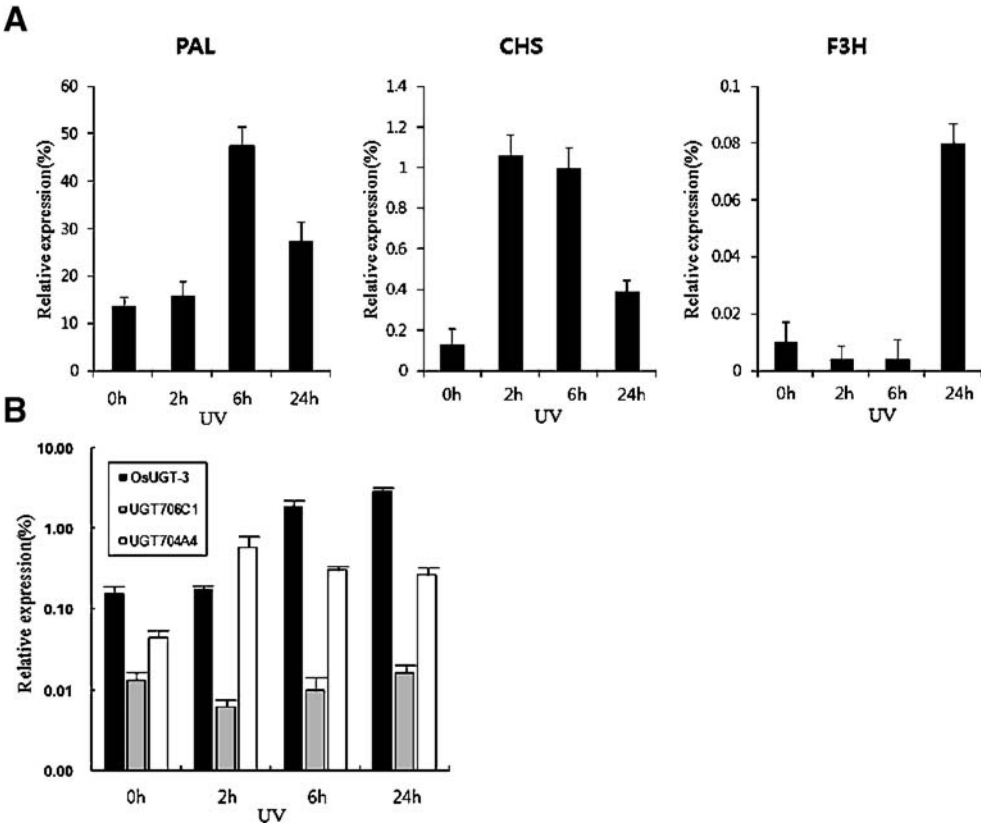


Fig. 3 Expression of genes involved in flavonoid biosynthesis (PAL, CHS, and F3H; **a**) and rice UGTs (**b**) upon UV-B irradiation. RNA was isolated from rice after 2, 6, or 24 h of treatment. Expression was analyzed by real-time PCR. Reaction was performed three times

trichloroacetic acid. The reaction products were analyzed by high-performance liquid chromatography (HPLC), as described by Ko et al. [9]. Analysis of liquid chromatography-tandem mass spectroscopy (LC-MS/MS) was carried out as described in Kim et al. [7, 8].

We used the *Medicago truncatula* UGT71G1 structure (PDB: 2acv) as a template to model the structures of OsUGT-3 and 706D3 [22] according to the method of Park et al. [21].

Results and Discussion

Search for Flavonoid *O*-Diglucosyltransferase in Rice

Although rice contains more than 150 UGTs, based on the database for Carbohydrate-Active Enzymes (CAZy; http://afmb.cnrs-mrs.fr/CAZY/), only a few, especially those involved in flavonoid biosynthesis, have been characterized [3, 9, 10]. All are flavonoid mono *O*-glycosyltransferases. However, analysis of rice phenolic compounds via LC-MS/MS has revealed several flavonoid di- or triglucosides, including 8-*C*-hexosyl-tricin *O*-rhamnoside-*O*-hexoside [7]. Thus, the UGTs that transfer two or three sugar units into flavonoids are likely to be present in rice. To select the candidate UGTs for flavonoid *O*-diglucoside among those >150 rice UGTs, we utilized a phylogenetic tree of UGTs (Fig. 1). These UGTs were classified into three groups: anthocyanin glycosyltransferases, flavonoid 3-*O*-glycosyltransferases, and UGTs with diverse substrates and regioselectivity. OsUGT-3 belongs to that last group. To confirm that it is a flavonoid *O*-diglucosyltransferase, we performed molecular modeling of OsUGT-3. Its overall structure differed greatly from that of UGT706D1, a flavonoid *O*-monoglucosyltransferase [10]. First, the substrate entry site of UGT706D1, which is formed by two

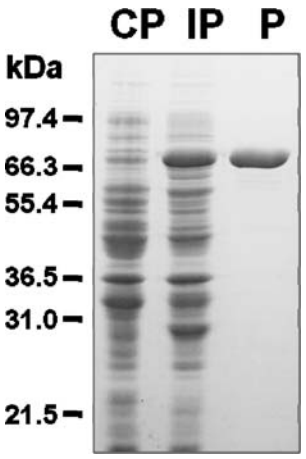
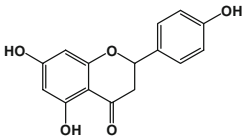
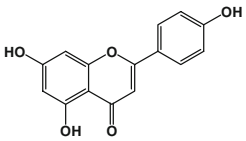
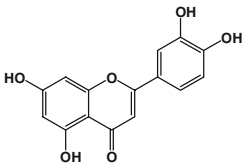
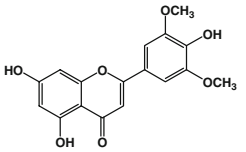
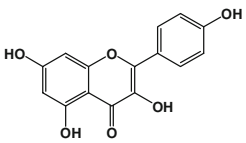
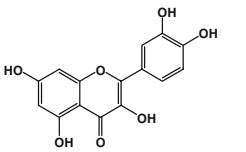


Fig. 4 Expression and purification of recombinant OsUGT-3. CP proteins from uninduced *E. coli* transformant, IP proteins from induced *E. coli* transformant, P purified recombinant protein via GST affinity chromatography

Table 1 HPLC retention times and *m/z* $[M-H]^-$ of substrates and OsUGT-3 reaction products

Substrate	Structure	Relative activity (%)	HPLC retention time (min)[*1]		$[M-H]^-$
Naringenin		81	S	16.5	271
			P1	11.7	433
Apigenin		5	S	17.3	269
			P1	11.8	431
			P2	8.8	609
Luteolin		9	L2	15.0	285
			LP1	12.3	447
			LP2	10.7	447
			LP3	9.6	609
Tricin		nd[*2]	T2	16.7	329
			TP1	11.8	491
			TP2	9.3	653
Kaempferol		100	K2	17.5	285
			KP1	11.5	447
			KP2	7.8	609
Quercetin		89	Q2	15.1	301
			QP1	12.4	463
			QP2	9.3	635

S and P in naringenin and apigenin indicate substrate and product, respectively. Labels for retention times of other substrates are referred to in Figs. 4, 5, and 6

nd not determined

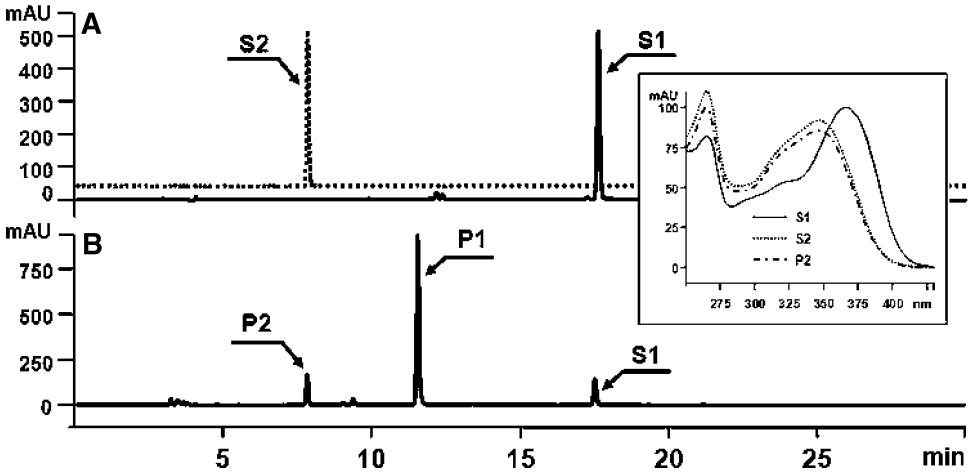


Fig. 5 Analysis of kaempferol reaction product by HPLC. Inset contains UV spectra of kaempferol (*S1*), kaempferol 3,7-*O*-diglucoside (*S2*), and reaction product (*P2*)

loops (Loop 1: Pro14 ~ His18 in UGT706D1, Val17 ~ Leu22 in OsUGT-3; Loop 2: Gly278 ~ Ser289 in UGT706D1, Ala286 ~ Ile292 in OsUGT-3) was narrower than that of OsUGT-3. Therefore, flavonoid *O*-glucoside could not easily enter into the substrate binding site in UGT706D1. Second, the pocket binding was smaller for UGT706D1. Thus, the flavonoid *O*-glucoside could be placed in the substrate-binding pocket of OsUGT-3 but not as easily into UGT706D1 (Fig. 2). Two quercetin *O*-diglucoside transferases are found in *A. thaliana*—UGT71C1 (AC005496) is quercetin 3,7-*O*-diglucose transferase, whereas UGT76E2 is quercetin 7,3′-*O*-diglucose transferase [16]. OsUGT3 shares 54% and 46% amino acid similarity with UGT71C1 and UGT76E2, respectively.

Upon UV-B irradiation, the rice genes involved in flavonoid biosynthesis, i.e., phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and flavone 3-β-hydroxylase (F3H), were induced, albeit differently. CHS was induced after 2 h of treatment and PAL after 6 h, whereas induction of F3H was not observed until after 24 h (Fig. 3a). We also investigated OsUGTs expression. Rice accumulates flavonoids upon UV-B irradiation [18], but alterations in UGT expression under such conditions have not been investigated previously. In addition to *OsUGT-3*, we also examined the expression of *UGT706C1* and *UGT709A4* because they belong to different phylogenetic groups [10]. That of *OsUGT-3* was induced by UV irradiation, while the same treatment led to increased expression by *UGT709A4*. However, that of *UGT706C1* was not altered (Fig. 3b). These results indicate that some rice flavonoid UGTs are regulated like other genes in flavonoid biosynthesis, while others behave differently. By comparison, expression of flavonoid UGTs from *A. thaliana* upon nutrient depletion is not correlated with that of other flavonoid biosynthetic genes such as PAL and CHS [14]. Hence, our understanding about the regulation of UGTs is still premature.

Determination of Substrates

OsUGT-3 was cloned using RT-PCR and sequenced for verification. Its nucleotide sequence was perfectly matched with that from GenBank. This gene was expressed in *E. coli* and partially purified in a glutathione *S*-transferase (GST) affinity column. SDS-PAGE revealed that the recombinant protein was expressed and purified nearly to homogeneity (Fig. 4). Its molecular weight was about 80 kDa, as had been predicted from OsUGT-3 (52.5 kDa) and GST (26 kDa).

Because OsUGT-3 transfers glucose(s) into flavonoids, flavanone (naringenin), flavones (apigenin and luteolin), and flavonols (kaemferol and quercetin), these were tested as potential substrates. As expected in the phylogenetic tree, OsUGT-3 utilized all of these flavonoids based on HPLC analysis. The molecular mass of the naringenin reaction product was increased by 162 Da, indicating that one glucose molecule was attached. In contrast, the apigenin, kaempferol, luteolin, and quercetin reaction products showed that either one or two glucoses were attached (Table 1). Kaempferol was the most preferred substrate, followed by quercetin and naringenin. To determine the positions of glucosylation, we compared the kaempferol reaction product with authentic kaempferol (Fig. 5). The reaction product (P2) had the same retention time and UV spectrum as the authentic kaempferol 3,7-*O*-diglucoside (S2). In addition, the molecular mass of P2 was increased by 324 Da compared with kaempferol. Therefore, these results indicate that OsUGT-3 is a flavonoid *O*-diglucosyltransferase.

We determined that the naringenin reaction product was naringenin 7-*O*-glucoside by comparing its HPLC retention time and UV spectrum with those of the authentic flavonoid glucosides (data not shown).

Flavones produced diglucosides (Table 1). One, apigenin, was first glucosylated at the 7-hydroxyl group, then

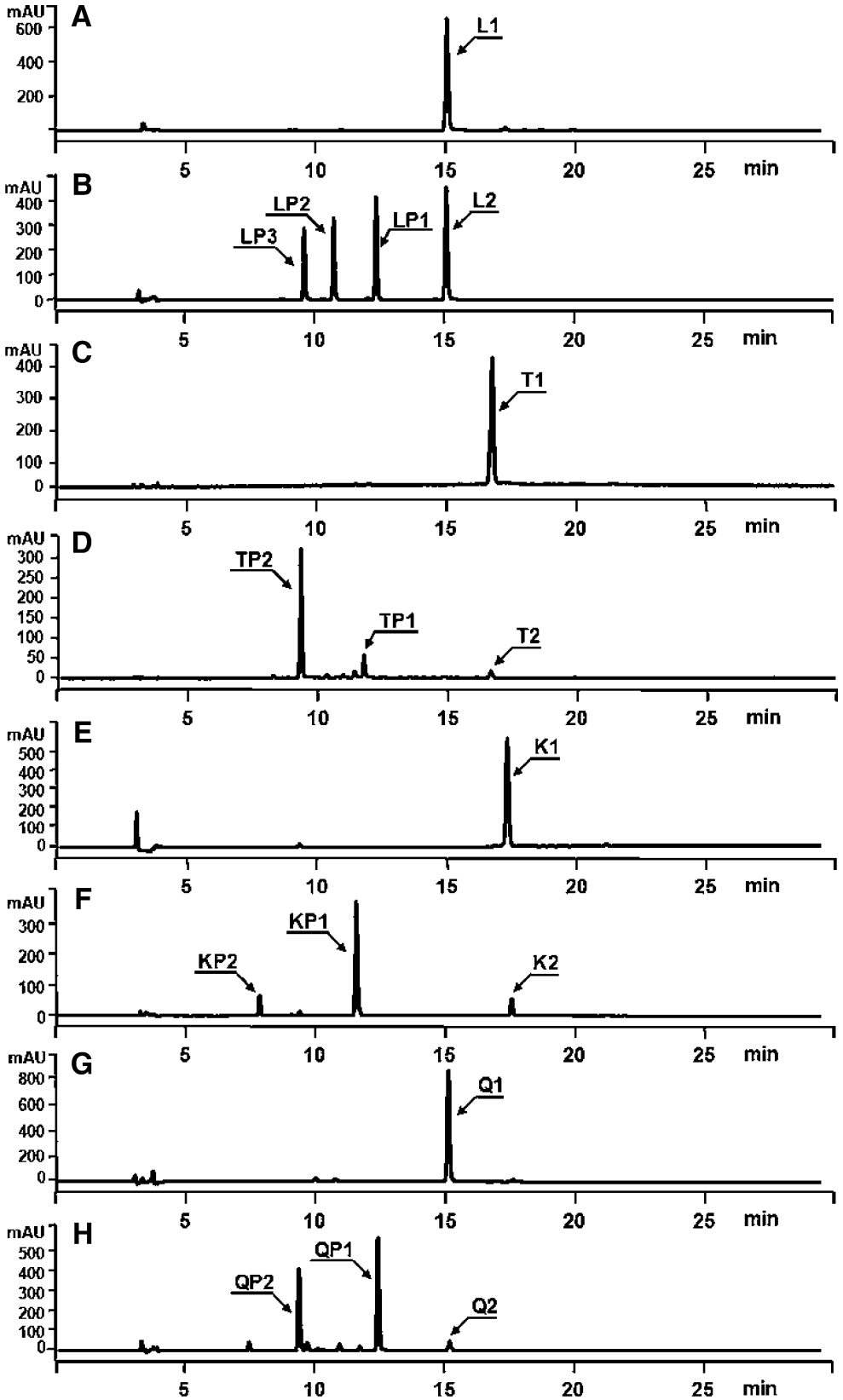


Fig. 6 HPLC analysis of flavonoid reaction products with OsUGT-3. **a** Authentic luteolin (*L1*); **b** luteolin reaction products (*L2* luteolin, *LP1* and *LP2* luteolin *O*-monoglucosides, *LP3* luteolin *O*-diglucoside); **c** authentic tricin; **d** tricin reaction products (*TP1* tricin *O*-monoglucoside, *TP2* tricin *O*-diglucoside); **e** authentic kaempferol; **f** kaempferol reaction products (*KP1* kaempferol *O*-monoglucoside, *KP2* kaempferol *O*-diglucoside); **g** authentic quercetin; **h** quercetin reaction product (*QP1* quercetin *O*-monoglucoside, *QP2* quercetin *O*-diglucoside)

converted into apigenin 7,4′-*O*-diglucoside (data not shown). The other, luteolin, produced 3′-*O*-glucoside (LP1 in Fig. 6a) and 7-*O*-glucoside (LP2 in Fig. 6a), both of which were converted into 7,3′-*O*-diglucoside (LP3 in Fig. 6a). The flavonols also were converted into diglucosides—kaempferol into kaempferol 3-*O*-glucoside, then kaempferol 3,7-*O*-diglucoside; and quercetin initially into 3′-*O*-glucoside (QP1 in Fig. 6f), then to 7,3′-*O*-diglucosides (QP2 in Fig. 6f). The position of glucosylation in each reaction product was determined either through comparison of HPLC retention times with authentic compounds or a hypsochromic shift of UV absorbance [11, 25].

Rice contains 8-*C*-hexosyl-tricin *O*-rhamnoside-*O*-hexoside, but we did not test whether OsUGT-3 has affinity for UDP-rhamnose because the latter is not commercially available. However, most UGTs display a range of affinities toward various UDP-sugars. Here, when tricin (3′,5′-*O*-dimethyltricetin) was used as a sugar acceptor and UDP-glucose as a sugar donor, the molecular mass of the reaction product was increased by 324 Da, indicating that OsUGT-3 transferred two glucose molecules into tricin and formed tricin *O*-diglucoside (Table 1; Fig. 6d). This suggests that in vivo functioning of OsUGT-3 might produce tricin *O*-rhamnoside-*O*-hexose. To explain how 8-*C*-hexosyl-tricin *O*-rhamnoside-*O*-hexoside is synthesized in vivo, we propose two possible scenarios: tricin *C*-glucose serves as a substrate for *O*-glycosylation or tricin *O*-diglucoside serves for *C*-glycosylation. Here, molecular docking of the modeled OsUGT-3 with flavonoid *O*-diglucoside revealed that the latter did not fit into the substrate binding site of the former. Thus, we conclude that tricin *O*-diglucoside is synthesized first, and this tricin *O*-diglucoside is likely converted into *C*-glucoside.

This molecular docking, followed by molecular modeling, is an effective way to predict substrates. Because several genome projects have now been completed, myriad gene sequences are available. Some classes contain more than 100 genes involved in secondary metabolism, one example being the UGTs. However, because using in vitro characterization to predict substrates is a time-consuming process, our approaches that involve such docking might be a useful tool.

Acknowledgments This work was supported by a Korea Research Foundation grant funded by the Korean Government (MOEHRD) (KRF-2005-214-00029).

References

1. Fischbach MA, Lin H, Liu DR, Walsh CT (2005) In vitro characterization of *IroB*, a pathogen-associated *C*-glycosyltransferase. Proc Natl Acad Sci U S A 102:571–576
2. Gachon CMM, Langlois-Meurinne M, Saindrenan P (2005) Plant secondary metabolism glycosyltransferases: the emerging functional analysis. Trends Plant Sci 11:542–549
3. Hong BS, Kim JH, Kim NY, Kim BG, Chong Y, Ahn J-H (2007) Characterization of uridine-diphosphate dependent flavonoid glucosyltransferase from *Oryza sativa*. J Biochem Mol Biol 40:870–874
4. Jones P, Vogt T (2001) Glycosyltransferase in secondary plant metabolism: tranquilizers and stimulant controllers. Planta 213:164–174
5. Jones P, Messner B, Nakajima JI, Schäffner A, Satito K (2003) UGT73C6 and UGT78D1-glycosyltransferases involved in flavonol glycoside biosynthesis in *Arabidopsis thaliana*. J Biol Chem 278:43910–43918
6. Kim BG, Kim JH, Min SY, Shin KH, Kim JH, Kim HI, Ryu SN, Ahn J-H (2007) Anthocyanin content in rice is related to expression level of anthocyanin biosynthetic genes. J Plant Biol 50:156–160
7. Kim JH, Cheon YM, Kim BG, Ahn J-H (2008a) Analysis of flavonoids and characterization of the *OsFNS* gene involved in flavone biosynthesis in rice. J Plant Biol 51:97–101
8. Kim BG, Kim JH, Kim J, Lee C, Ahn J-H (2008b) Accumulation of flavonols by ultraviolet-B is related to induction of flavanone 3-β-hydroxylase and flavonol synthase in soybean. Mol Cell 25:247–252
9. Ko JH, Kim BG, Hur H-G, Lim Y, Ahn J-H (2006) Molecular cloning, expression and characterization of a glycosyltransferase from rice. Plant Cell Rep 25:741–746
10. Ko JH, Kim BG, Kim JH, Kim H, Lim CE, Lim J, Lee C, Lim Y, Ahn J-H (2008) Four glucosyltransferases from rice: cDNA cloning, expression, and characterization. J Plant Physiol 165:435–444
11. Kramer CM, Prata RTN, Willits MG, De Luca V, Steffens JC, Graser G (2003) Cloning and regiospecificity studies of two flavonoid glucosyltransferases from *Allium cepa*. Phytochemistry 64:1069–1076
12. Kubo A, Arai Y, Nagashima S, Yoshikawa T (2004) Alteration of sugar donor specificities of plant glycosyltransferases by a single point mutation. Arch Biochem Biophys 429:198–203
13. Liang D, Qiao J (2007) Phylogenetic analysis of antibiotic glycosyltransferases. J Mol Evol 64:342–353
14. Lillo C, Lea US, Ruoff P (2008) Nutrient deletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. Plant Cell Environ 31:587–601
15. Lim E-K, Bowles DJ (2004) A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J 23:2915–2922
16. Lim E-K, Ashford DA, Hou B, Jackson RG, Bowles DJ (2004) *Arabidopsis* glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. Biotech Bioengr 87:623–631
17. Mackenzie PI, Owens IS, Burchell B, Bock KW, Bairoch A et al (1997) The UDP glycosyltransferase gene superfamily: Recommended nomenclature update based on evolutionary divergence. Pharmacogenetics 7:255–269
18. Markham KR, Tannera GJ, Caasi-Lit M, Whitecross MI, Nayudu M, Mitchell KA (1998) Possible protective role for 3′,4′-dihydroxyflavones induced by enhanced UV-B in a UV-tolerant rice cultivar. Phytochemistry 49:1913–1919
19. Miller KD, Guyon V, Evans JNS, Shuttleworth WA, Taylor LP (1999) Purification, cloning, and heterologous expression of a catalytically efficient flavonol 3-*O*-galactosyltransferase expressed in the male gametophyte of *Petunia* hybrid. J Biol Chem 274:34011–34019
20. Offen W, Martinez-Fleites C, Yang M, Lim E-K, Davis BG, Tarling CA, Ford CM, Bowles DJ, Davies GJ (2006) Structure of

a flavonoid glucosyltransferase reveals the basis for plant natural product modification. EMBO J 25:1396–1405
21. Park SH, Kim BG, Lee SH, Lim Y, Cheong Y, Ahn J-H (2007) Molecular modeling and site directed mutagenesis of the *O*-methyltransferase, SOMT-9, reveals amino acids important for its reaction and regioselectivity. Bull Kor Chem Soc 28:2248–2252
22. Shao HS, He X, Achnine L, Blount JW, Dixon RA, Wang X (2005) Crystal structures of a multifunctional triterpene/flavonoid glycosyltransferase from *Medicago truncatula*. Plant Cell 17:3141–3154
23. Tahara S (2007) A journey of twenty-five years through the ecological biochemistry of flavonoids. Biosci Biotechnol Biochem 71:1387–1404
24. Torson JS, Hosted TJ Jr, Jiang JQ, Buggubs JB, Ahlert J (2001) Nature's carbohydrate chemists: the enzymatic glycosylation of bioactive bacterial metabolites. Curr Org Chem 5:139–167
25. Vogt T, Zimmermann E, Grimm R, Meyer M, Stack D (1997) Are the characteristics of betanidin glucosyltransferases from cell suspension cultures of *Dorotheanthus bellidiformis* indicative of their phylogenetic relationship with flavonoid glucosyltransferases? Planta 203:349–361
26. Williams CA (2006) Flavone and flavonol *O*-glycosides. In: Andersen ØM, Markham KR (eds) Flavonoids; chemistry, biochemistry and application. CRC Press, Boca Raton, FL, USA, pp 749–856
27. Yonekura-Sakakibara K, Tohge T, Niida R, Saito K (2007) Identification of a flavonol 7-*O*-rhamnosyltransferase gene determining flavonoid pattern in *Arabidopsis* by transcriptome coexpression analysis and reverse genetics. J Biol Chem 282:14932–14941