Journal of Biochemistry and Molecular Biology, Vol. 40, No. 6, November 2007, pp. 870-874

JOURNAL OF Biochemistry and Molecular Biology

Characterization of Uridine-Diphosphate Dependent Flavonoid Glucosyltransferase from *Oryza sativa*

Byoung Seok Hong, Jeong Ho Kim, Na Yeon Kim, Bong-Gyu Kim, Youhoon Chong and Joong-Hoon Ahn*
Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea

Received 30 April 2007, Accepted 18 June 2007

We cloned a uridine-diphosphate dependent glycosyltransferase *RUGT-10* from *Oryza sativa*. The recombinant enzyme was expressed by glutathione-*S* transferase gene fusion system in *Escherichia coli*. RUGT10 showed different regioselectivity depending on the structures of substrates (*e.g.* flavanone, flavonol, and flavone). Apparently, flavanone such as naringenin and eriodictyol gave one 7-*O*-glucoside while flavone and flavonol gave more than two products with preferential glucosylation position of hydroxyl group at C-3 position.

Keywords: Flavonoid, Glycosyltransferase, *Oryza sativa*

Introduction

Attachment of sugar unit to small compounds, called glycosylation is one of the common reactions found in plants. Moreover, sugar acceptors found in plant are diverse. They include flavonoids, antocyanins, cyanidins, terpenoids, hormones and alkaloids. Glycosylation of these compounds has been suggested to play crucial roles in stabilization of antocyanins and cyanidins; storage of flavonoids and terpenoids; and regulation of hormones (Bowles *et al.*, 2005). In addition, glycosylation has been recognized as one of the important mechanisms in detoxification of exogenous compounds (Jones *et al.*, 2001).

Glycosyltransferases (GTs) responsible for transferring a sugar into small compounds are classified as Family 1. This GT family1 uses uridine diphosphate sugar (usually UDP-glucose, UDP-galactose and UDP-rhamnose) as a sugar donor and is also called UDP-glycosyltransferases (UGTs) (Mackenzie *et al.*, 1997; Vogt and Jones, 2000). It has been reported that *Arabidopsis thaliana* contains 120 UGTs (Li *et al.*, 2001; Paquette *et al.*, 2003). Several UGTs using different groups of substrates in *A. thaliana* have been characterized *in vitro* but substrates of most UGTs still remain unknown (Bowles *et al.*, 2005). *Oryza sativa*, one of the model crops, also contains more UGTs than *A. thaliana* but their functional characterization is currently under way to elucidate their functional importance (Ko *et al.*, 2006) However, *in vivo* functional characterization is usually hindered due to lack of available mutants and complexity of metabolites. Thus, *in vitro* characterization of individual UGTs using heterologous expression system proceeds *in vivo*.

Flavonoids are typical phytochemicals having on impact on human (Cornwell, *et al.*, 2004; Usha *et al.*, 2005) and are synthesized via the phenylpropanoid pathway. Attachment of sugar to flavonoid occurs at the last step of biosynthesis pathway. To date, various flavonoid UGT genes have been cloned and characterized (Hirotani *et al.*, 2000; Kramer *et al.*, 2003; Willits *et al.*, 2004; Kim *et al.*, 2006). Some of UGTs have been used for biocatalytic synthesis of flavonoid glycosides (Lim *et al.*, 2004) due to the complex and labor-intensive chemical synthesis. In addition, the nature of sugars and the glycosylation positions in flavonoids affect their absorption and utilization in humans.

In rice, apigenin, luteolin, and kaempferol were found (Chatterjee *et al.*, 1976; Stevenson *et al.*, 1996). Some of them exist as *C*-glucosides (Besson *et al.*, 1985) but it is still unknown what types of flavonoid *O*-glucosides are present in rice. We believe that *in vitro* characterization of UGTs is helpful for *in vivo* studies of UGTs. Previously, we characterized one flavonoid *O*-glucosyltransferease from rice (Ko *et al.*, 2006). Here, we reported *in vitro* characterization of a flavonoid *O*-glucosyltransferase which showed different regioselectivity toward flavonols.

Materials and Methods

Cloning of RUGT10. cDNA was synthesized with total RNA isolated from 3-weeks old whole rice plant using omniscript reverse

*To whom correspondence should be addressed.
Tel: 82-2-450-3764; Fax: 82-2-3437-6106
E-mail: jhahn@konkuk.ac.kr

transcriptase (Qiagen, Germany). Isolation of total RNA was carried out with Plant total RNA isolation kit from Qiagen. Two primers, 5'-GCAATGCCGAGCTCTGG-3' (forward) and 5'-TGTCTCAGT ACTCAATTAGTGCGA-3' (reverse) were designed based on the nucleotide sequence of *RUGT-10* (GeneBank accession number AP006584). PCR was carried out by incubating at 94°C for 15 min to activate the hot start Taq DNA polymerase (Qiagen), followed by 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1.5 min at 72°C. The PCR product was subcloned into the pGEMT-easy vector (Promega, Madison, WI, USA) and sequenced.

Expression of RGT10 in *E. coli* and enzyme assay. To construct the expression vector of *RUGT-10*, the open reading frame of *RUGT-10* was amplified with primers containing restriction enzyme sites *Eco*RI at the forward primer and *Not*I at the reverse primer. The resulting PCR product was digested and subcloned into *Eco*RI/*Not*I sites of pGEX 5X-1(Amersham Biotech, USA). Induction and purification of recombinant RUGT-10 were carried out as described in Ko *et al.* (2006). The reaction mixture contained 20 μg of the purified recombinant RUGT-10, 5 mM UDP-glucose, 30 μM of substrate, 5 mM $MgCl_2$ in 10 mM potassium phosphate buffer (pH 7.0). The reaction mixture was incubated at 37°C for 1 h. The reaction was terminated by the addition of equal volume of ethyl acetate. The organic layer was evaporated to dryness. The dried reaction product was dissolved in dimethylsulfoxide (DMSO). Reaction product was analyzed by high performance liquid chromatography (HPLC; Palo Alto) on a Varian 1000 C18 reversed-phase column (Palo Alto, 4.60 × 250 mm, 0.6 μm) and a photodiode array detector. For analytical scale, the mobile phase consisted of 50 mM phosphate buffer (pH 3.0) that was programmed as follows; 10% acetonitrile at 0 min, 30% acetonitrile at 10 min, 60% acetonitrile at 40 min, 90% acetonitrile at 45 min, 10% acetonitrile at 50 min. The flow rate was 1 ml/min and UV detection was performed at 340 nm.

Results and Discussion

Among 226 rice UGTs found in carbohydrate active enzyme site (CAZY; http://afmb.cnrs-mrs.fr/CAZY), one of UGTs (*RUGT-10*) having high similarity with flavonoid glycosyltransferase was cloned using reverse transcription-polymerase chain reaction (RT-PCR) and sequenced. The open reading frame of *RUGT-10* consisted of 1416 bp, which encodes 49.5-kDa protein. It also contains PSPG (plant secondary product UGT consensus sequence) motif at the C-terminal region. Phylogenetic analysis several flavonoid UGTs from rice and *Arabidopsis* showed that RUGT-10 were closely located to flavonoid 3-*O*-glucosyltranferase (Fig. 1). Regioselectivity of RUGT-10 for flavones and flavonols was dependent on the presence of 3'-hydroxyl group (see below). It is worth mentioning that the regioselectivity of flavonoid glucosyltransferases is not always correlated with the clade they belong to. For, example, AtGT-1 showed the flavonoid 3-*O*-glucosyltransferase activity (Kim *et al.*, 2006) but it was classified into other clade.

To determine substrates of RUGT-10, the open reading frame of *RUGT-10* was sucloned into the *E. coli* expression vector

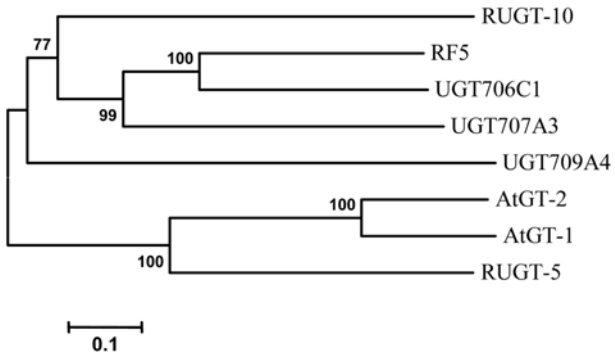


Fig. 1. Phylogenetic tree of flavonoid glucosyltransferases from rice and Arabidopsis. The tree was constructed using MEGA version 3.0 (Kumar *et al.*). The scale bar indicates a distance of 10 changes per 100 amino acid positions. Accession numbers are: RUGT-10(AP006584), RF5(NP_001044170.), UGT706C1(BAB68090), UGT709A4(BAC80066), AtGT-2(NP_567955), AtGT-1(NP_567954), and RUGT-5(XM_463383).

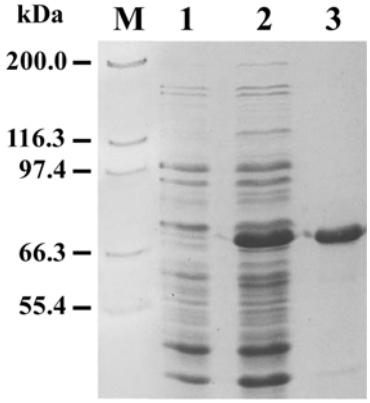


Fig. 2. Expression and purification of the recombinant RUGT-10. 1.5 μg of the purified RUGT-10 was loaded. M, molecular weight size maker; 1, *E. coli* lysate before induction; 2, *E. coli* lysate after induction; 3, Soluble fraction of *E. coli* lysate soluble fraction after induction; 4, purified recombinant RUGT-10.

pGEX 5X-1. The expressed recombinant RUGT-10 was purified and analyzed using SDS-PAGE (Fig. 2). The expressed RUGT-10 was purified to near homogeneity and the molecular weight of the expressed protein was about 66-kDa, which well agrees to the sum of molecular weight of the predicted RUGT and that of the GST.

According to sequence homology of RUGT-10 with other UGTs, the predicted substrates would be flavonoids. Typical flavonoids representing flavanone (naringenin), flavone (apigenin), and flavonol (kaempferol) were tested as potential substrates of RUGT-10. The reaction products from each reaction were analyzed using HPLC (Ko *et al.*, 2006) Naringenin reaction product gave one peak that had the same retention time (7.85 min) and the UV-spectra with naringenin 7-*O*-glucoside (Fig. 3C). On the other hand, apigenin and kaempferol reaction products generated more than two new peaks. In case of

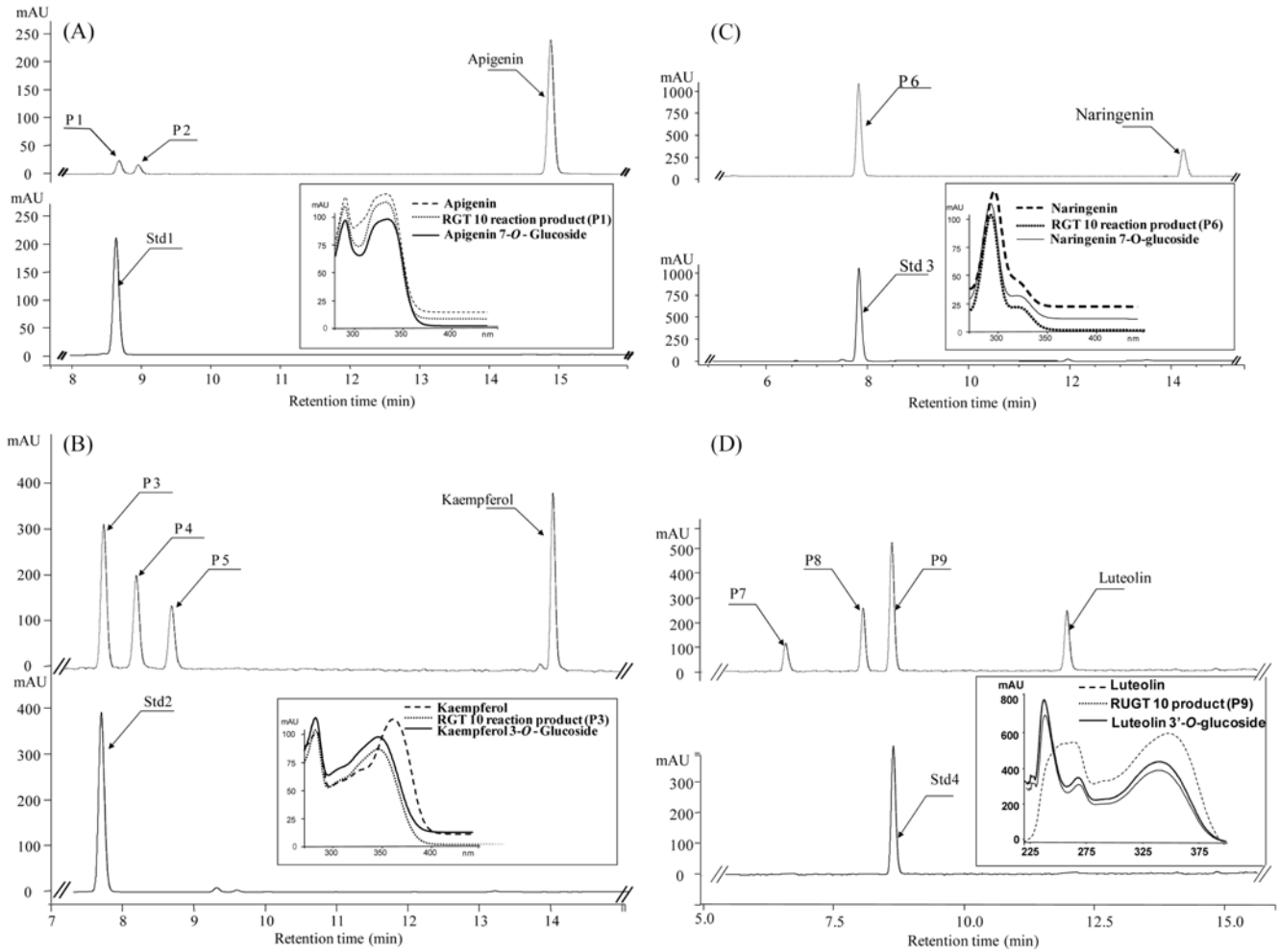
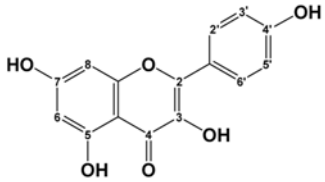
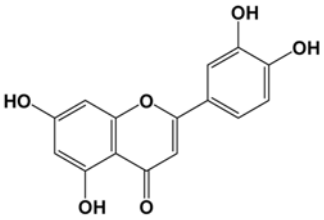
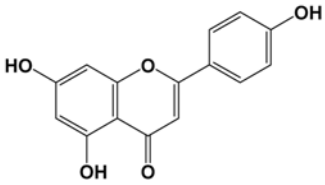
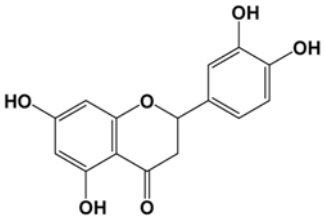
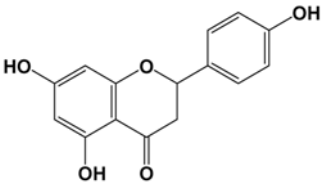


Fig. 3. HPLC elution profiles of RUGT10 assay mixture with apigenin (A), kaempferol (B), naringenin (C), or luteolin (D). Std1, apigenin 7-*O*-glucoside; Std2, kaempferol 3-*O*-glucoside; Std3, naringenin 7-*O*-glucoside; Std4, luteolin 3'-*O*-glucoside From P1 to P9 are reaction products. The structure of these reaction products were determined by comparison of standard or hypsochromic shift. P1, apigenin 7-*O*-glucoside; P2, apigenin 4'-*O*-glucoside; P3, kaempferol 3-*O*-glucoside; P4, kamepferol 7-*O*-glucoside; P5, kamepferol 4'-*O*-glucoside; P6, narigenin 7-*O*-glucoside; P7, luteolin 7-*O*-glucoside; P8, luteolin 4'-*O*-glucoside; P9, luteolin 3'-*O*-glucoside

apigenin, two new peaks were observed (Fig. 3A). The first peak at 8.5 min had the same retention time and the UV-spectra with authentic apigenin 7-*O*-glucoside. The maximum UV-absorbances of the second peak (at 8.8 min) were observed at 268 nm and 324 nm, which showed the hypsochromic shift from apigenin itself (268 nm and 338 nm). It indicated that the glycosylation position of the second peak was likely to be the hydroxyl group at 4'-carbon. It is generally known that there is hypsochromic shift when glycosylation occurs at hydroxyl group of either 3 or 4' carbon (Vogt *et al.*, 1997). Moreover, kaempferol generated three reaction products based on the HPLC analysis (Fig. 3B). The first peak at 7.7 min corresponded to the kaempferol 3-*O*-glucoside by comparing with retention time and UV-spectra. The second peak at 8.2 min was determined to be kaempferol 7-*O*-glucoside because it did not show the hypsochromic shift. The third peak at 8.7 min was likely to be kaempferol 4'-*O*-glucoside based on the hypsochromic shift. These results suggested that number of products and position of glucosylation of RUGT-10 are dependent on the type of flavonoids (flavanone, flavone, and flavonol) because flavanone gave a single glycosylated product and flavone and flavonol gave more than two products. To verify it further, more flavonoids representing three groups were tested. As a result, eriodictyol (flavanone) gave one peak, luteolin (flavone) gave three peaks and quercetin (flavonol) generated four peaks (data not shown). Glucosylation position of eriodictyol was at the 7-hydoxyl group, indicating that RUGT-10 transfers a glucose group into 7-hydroxyl group of flavanone. In case of flavone and flavonol, the number of reaction products is correlated with the number of hydroxyl groups. The position of glucosylation is governed by the presence or absence of 3'-hydroxyl group. The most preferable glucosylation position of luteolin (Fig. 3D), which contain the 3'-hydroxyl group, was at the 3'-hydroxyl group followed by 4'- and 7-hydroxyl groups.

Table 1. Substrate Preference of RUGT-10

Compound	Structure	Relative activity (%)	Glycosylated OH-group
Kaempferol		100	3-OH (1), 7-OH (2), 4'-OH (3)
Luteolin		90	7-OH (3), 3'-OH (1), 4'-OH (2),
Apigenin		70	7-OH (1), 4'-OH (2)
Eriodictyol		89	7-OH
Naringenin		94	7-OH

*Numbers in the parenthesis indicate the order of the reaction product.

The preferable glucosylation position of RUGT-10 toward flavonols is in contrast with that of RUGT-5. The preferable glucosylation position of RUGT-5 toward flavonol was the 3'-hydroxyl group (Ko *et al.*, 2006). In case of kaempferol, which does not contain 3'-hydroxyl group, 3-hydroxyl group is the most preferable glucosylation site (Fig. 3B) and followed by 7- and 4'-hydroxyl groups (Table 1). In summary, the number of glycosylated products resulted from the presence of double bond between C2 and C3 in the C-ring. RUGT-10 produced one 7-*O*-glucoside when flavanones, which do not contain double bond between C2 and C3 in the C-ring, were used as substrates irrespective of the number of available hydroxyl groups. However, when flavone and flavonol, both of which contain a double bond, were substrates for RUGT-10, the major reaction product was decided by the presence of 3'-hydroxyl group; 3'-*O*-glucosides would be a major product if 3'-hydroxyl group is present. It indicates that the 3'-hydroxyl group seems to play a critical role in the enzyme-substrate interaction compared with the other hydroxyl groups. However, the reason why flavones and flavonols produced multiple products remains elusive. The difference of regioselectivity upon different kinds of flavonoids also observed in UGT73A4 and UGT71F1 from *Beta vulgaris* (Isayenkova *et al.*, 2006) and UGT75L4 from *Malclura pomifera* (Tian *et al.*, 2006). UGT73A4 produced a single 7-*O*-glucoside when flavones and flavanones were substrates, whereas it produced multiple products when flavonol was a substrate. Moreover, UGT71F1 was similar to UGT73A except that it did not use flavanone as a substrate. UGT75L4 produced a 7-*O*-glucoside using isoflavone and a single *O*-glucoside with flavanone while it produced two different multiple products with flavone or flavonol. Until now, the crystal structures of two flavonoid UGTs have been determined (Shao *et al.*, 2005; Offen *et al.*, 2006) of which structural analyses

clearly show that specific interactions around the flavonoid substrate play the key role in determining the regioselectivity of the enzymatic glycosylation. While a multifunctional triterpene/flavonoid glycosyltransferase from *Medicago truncatula* (MtGT, PDB access code: 2acv, Shao *et al.*, 2005) was found no specific interaction around the quercetin substrate, amino acid residues of a red grape enzyme UDP-glucose: flavonoid 3-*O*-glycosyltransferase (VvGT1, PDB access code: 2c1z, Offen *et al.*, 2006) such as Gln84 and His150 specifically recognize flavonol O7 and O4', respectively, to result in specific glycosylation at the O3 position. Thus, homology model construction of various UGTs followed by binding mode analyses and site directed mutagenesis might provide invaluable information to understand the complex substrate specificity of RUGT-10 and regioselectivity underlying glycosylation to facilitate the rational design of substrates.

Acknowledgments This work was supported by a grant from Rural Development Administration, Republic of Korea and R01-2004-000-10688-0 (KOSEF).

References

Besson, E., Dellaminica, G., Chopi, J., Markhama, K. R., Kimb, M., Knob, H. S. and Fukamib, H. (1985) *C*-glycosylflavones form *Oryza sativa*. *Phytochemistry* **24**, 1061-1064.

Bowles, D., Lsayenkova, J., Lim, E. -K. and Poppenberger, B. (2005) Glycosyltransferases: managers of small molecules. *Curr. Opin. Plant Biol.* **8**, 254-263.

Chatterjee, A., Saha, A. P. K., Gupta, P. D., Ganguly, S. N. and Sircar, S. M. (1976) Chemical examination of viable and non-viable rice seeds. *Physiol. Plant.* **38**, 307-308.

Cornwell, T., Cohick, W. and Raskin, I. (2004) Dietary phytoestrogens and health. *Phytochemistry* **65**, 995-1016.

Hirotani, M., Kuroda, R., Suzuki, H. and Yoshikawa, T. (2000) Cloning and expression of UDP-glucse: flavonoid 7-*O*-glucosyltransferase from hairy root cultures of *Scutellaria baicalensis*. *Planta* **210**, 1006-1013.

Isayenkova, J., Wray, V., Nimtz, M., Strack, D. and Vogt, T. (2006) Cloning and functional characterization of two regioselective flavonoid glucosyltransferases from *Beta vulgaris*. *Phytochemistry* **67**, 1598-1612.

Jones, P. and Vogt, T. (2001) Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. *Planta* **213**, 164-174.

Kim, J. H., Kim, B. G., Ko, J. H., Lee, Y., Hur, H. -G., Lim, Y., and Ahn, J. -H. (2006) Molecular cloning, expression, and characterization of a flavonoid glycosyltransferase from *Arabidopsis thaliana*. *Plant Sci.* **170**, 897-903.

Ko, J. H., Kim, B. G., Hur, H. -G., Lim, Y. and Ahn, J. -H. (2006) Molecular cloning, expression and characterization of a glycosyltransferase from rice. *Plant Cell Rep.* **25**, 141-146.

Kramer, C. M., Prata, R. T. N., Willits, M. G., De Luca, V., Steffens, J. C. and Graser, G. (2003) Cloning and regiospecificity studies of two flavonoid glucosyltransferases from *Allium cepa*. *Phytochemistry* **64**, 1069-1076.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform.* **5**, 150-163.

Li, Y., Baldauf, S., Lim, E. -K. and Bowles, D. J. (2001) Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana*. *J. Biol. Chem.* **276**, 4338-4343.

Lim, E. -K., Ashford, D. A., Hou, B., G. Jackson, R. G., and Bowles, D. J. (2004) Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. *Biotechnol. Bioeng.* **87**, 623-631.

Mackenzie, P. I., Owens, I. S., Burchell, B., Bock, K. W., Bairoch, A., Belanger, A., Fournel-Gigleux, S., Green, M., Hum, D. W., Iyanagi, T., Lancet, D., Louisor, P., Magdalow, J., Chowdhury, J. R., Ritter, J. K., Schachter, H., Tephly, T. R., Tipton, K. F., and Nebert, D. W. (1997) The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* **7**, 255-269.

Offen, M., Marinez-Fleites, C., Yang, M., Lim, E. K., Davis, B. G., Tarling, C. A., Ford, C. M., Bowles, D. J. and Davies, G. J. (2006) Structure of a flavonoid glucosyltransferase reveals the basis for plant natural product modification. *EMBO J.* **25**, 1396-1405.

Paquette, S., Møller, B. L., and Bak, S. (2003) On the origin of family 1 plant glycosyltransferases. *Phytochemistry* **62**, 399-413.

Shao, H., He, X., Achnine, L., Blount, J. W., Dixon, R. A., and Wang, X. (2005) Crystal structure of a multifunctional trerpene/flavonoid glycosyltransferase from *Medicago trucatula*. *Plant Cell* **17**, 3141-3154.

Stevenson, P. C., Kimmins, F. M., Grayer, R. J. and Raveendranath S. (1996) Schaftosides from rice phloem as feeding inhibitors and resistance factors to brown planthoppers, *Nilaparvata lugens*. *Entomol. Exp. Appl.* **80**, 246-249.

Tian, L., Blount, J. W. and Dixon, R. A. (2006) Phenylpropanoid glycosyltransferases from orange (*Maclura pomifera*) fruit. *FEBS Lett.* **580**, 6915-6920.

Usha, S., Johnson, I. M. and Malathi, R. (2005) Interaction of resveratol and genistein with nucleic acids. *J. Biochem. Mol. Biol.* **38**, 198-205.

Vogt, T., Zimmermann, E., Grimm, R., Meyer, M. and 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.

Vogt, T. and Jones, P. (2000) Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. *Trends Plant Sci.* **5**, 380-386.

Willits, M. G., Giovanni, M., Prata, R. T. N., Kramer, C. M., Luca, V. D., Steffens, J. C. and Grase, G. (2004) Bio-fermentation of modified flavonoids: an example of *in vivo* diversification of secondary metabolites. *Phytochemistry* **65**, 31-41.