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Glycosylation of Flavonoids with *E. coli* Expressing Glycosyltransferase from *Xanthomonas campestris*

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Abstract Glycosyltransferase family 1 (UGT) uses small chemicals including phenolics, antibiotics, and alkaloids as substrates to have an influence in biological activities. A glycosyltransferase (*XcGT-2*) from *Xanthomonas campestris* was cloned and consisted of a 1,257 bp open reading frame encoding a 45.5 kDa protein. In order to use this for the modification of phenolic compounds, *XcGT-2* was expressed in *Escherichia coli* as a glutathione S-transferase fusion protein. With the *E. coli* transformant expressing *XcGT-2*, biotransformation of flavonoids was carried out. Flavonoids having a double bond between carbons 2 and 3, and hydroxyl groups at both C-3' and C-4', were glycosylated and the glycosylation position was determined to be at the hydroxyl group of C-3', using nuclear magnetic resonance spectroscopy. These results showed that XcGT-2 regiospecifically transferred a glucose molecule to the 3'-hydroxyl group of flavonoids containing both 3' and 4'-hydroxyl groups.

Keywords: Biotransformation, flavonoids, glycosyltransferase

Secondary metabolites from plants and microorganisms are estimated to serve as leading compounds for the development of new drugs [18]. Bioactive natural products include polyketides, alkaloids, and flavonoids, which have a great diversity of chemical structures and biological activities [15]. Structural diversity of secondary metabolites is enhanced by diverse sugars that are attached to specific positions on the aglycon core. Thus, organisms producing these bioactive compounds contain various glycosyltransferases (GTs) that transfer a sugar moiety into the aglycon core. GTs are classified into 78 families, among which family 1 GTs use small molecules such as polyketides, flavonoids, or antibiotics as sugar acceptors and UDP-activated sugar as sugar donors [13]. Thus, Family 1 GTs are called UGTs. Plants contain many types of UGTs because they produce various types of small compounds [1]. UGTs from plants use a certain class of compounds as substrates [20]. On the other hand, some microbial UGTs take part in the synthesis of antibiotics as a part of the antibiotic synthetic gene cluster [6, 18]. Since the completion of genome projects from a myriad of organisms, there are many genes that are annotated as UGTs. However, many of them are still not functionally characterized, so they could be called "orphan" UGTs. Thus, the functional analysis of these UGTs is a main theme to those who are interested in glycosylation of small compounds. For this, using a heterologous expression system is the best way to save time and labor.

Among plant secondary metabolites, flavonoids are known to have roles in plants, microorganism, and human. Various flavonoids serve as a mediator interaction between plants and microorganisms [17]. In addition, the glycosylation position significantly affects absorption and utilization of flavonoids in human [5]. Therefore, regioselective glycosylation and increase of flavonoid solubility by glycosylation or solubility enhancer are studied extensively [7]. We report here the regiospecific modification of flavonoids with UGTs from *Xanthomonas campestris*. *X. campestris pv. campestris* (ATCC 33913) contained two UGTs, according to the CAZy site (http://afmb.cnrs-mrs.fr/CAZY/). These two UGTs are named *XcGT-1* (GenBank Accession Number AE012507) and *XcGT-2* (GenBank Accession Number AE012355). Primers for *XcGT-1* were 5'-ATATGCCTGAGTCCTCCACAG-3' as a forward primer and 5'-GACCAGACAAACGTGCCTG-3' as a reverse primer and primers for *XcGT-2* were 5'-CATGCGCATCGATC-TGAT-3' and 5'-GCTAAAGCGTGGGCACTT-3'. Polymerase chain reaction was carried out under the following condition; 40 cycles of 1-min denaturation at 94°C, 1 min annealing at 55°C, and 1.5 min amplification at 72°C. The PCR products were subcloned into pGEMT-Easy vector (Promega, Madison, WI, U.S.A.) and the resulting plasmid was

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sequenced. *XcGT-1* and *-2* consist of a 1,332 bp and 1,257 bp open reading frame, respectively, encoding a 47.4-kDa and 45.5-kDa protein.

XcGT-1 and *-2* were subcloned into pGEX vector (Amersham, Piscataway, NJ, U.S.A.) to be expressed as glutathione *S*-transferase fusion protein. Both genes were expressed and purified as described in Lee *et al.* [10]. The molecular masses of the expressed proteins were approximately 75 kDa for XcGT-1 and 73 kDa for XcGT-2, which agreed with the sum of the molecular mass of GST plus that of each gene (data not shown).

Because both *XcGT-1* and *-2* belonged to GT family 1, small compounds such as several phenolics and flavonoids were tested as potential substrates. Phenolic compounds included caffeic acid, esculetin, ferulic acid, pentachloro phenol, salicyclic acid, scopoletin, and orcinol. Flavonoids included eriodictyol, luteolin, kaempferol, quercetin, and taxifolin. Instead of using the purified recombinant proteins, *E. coli* transformant having *XcGT-1* or *XcGT-2* was used [11]. *E. coli* transformant containing the vector pGEX 5X-2 was used as a control. This method could save the UDP-glucose because UDP-glucose can be supplied by *E. coli*. In addition, phenolic compounds used in this experiment could penetrate into *E. coli*. The *E. coli* transformants were grown and the proteins were induced as describe in Kim *et al.* [8]. The transformants were harvested and resupended in half volume of 50 mM phosphate buffer (pH 7.0) containing 1 % glucose. Thirty μM of each flavonoids was added at each culture and the culture was incubated at

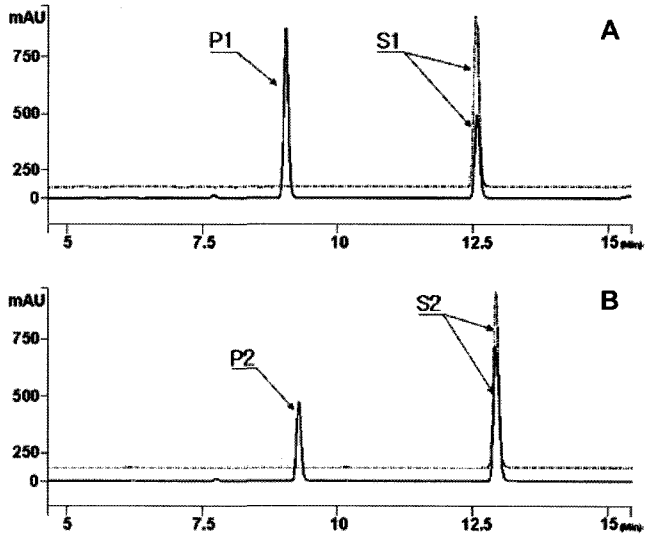
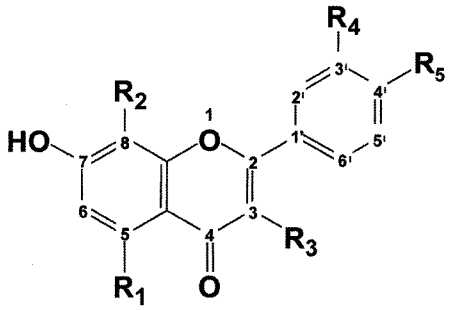


Fig. 1. HPLC profile of luteolin and quercetin reaction product with XcGT-2.

A. HPLC profile of Luteolin (dotted line) and its reaction product (straight line) with XcGT-2 (S1, luteolin; P1, luteolin reaction product with XcGT-2). **B.** HPLC profile of quercetin (dotted line) and its reaction product (straight line) with XcGT-2 (S2, quercetin; P2, quercetin reaction product with XcGT-2).

Table 1. Structure of flavones and relative conversion rate with XcGT-2.



	R1	R2	R3	R4	R5	Relative conversion rate (%)
Fisetin	H	H	OH	OH	OH	7.5
Gossypectin	OH	OH	OH	OH	OH	49.4
Kaempferol	OH	H	OH	H	OH	ND
Luteolin	OH	H	H	OH	OH	100
Quercetin	OH	H	OH	OH	OH	70.7
3'-OH Flavone	H	H	H	OH	H	ND

[1]Coumarin and phenolic acid showed 6.3 and 8% relative activity, respectively, compared with luteolin.
[2]ND, not detected.
[3]Eriodictyol and taxifolin have the same structure with luteolin and quercetin, respectively, except for a double bond between carbon 2 and 3. These two flavonoids were not served as substrates.

28°C for 12 h. Reaction products were analyzed using high performance liquid chromatography (HPLC) as previously described [8]. *E. coli* transformant containing pGEX 5X-2 or *XcGT-1* did not use any of these compounds, whereas XcGT-2 used several compounds as substrates. Thus, only XcGT-2 was used for further study. XcGT-2 could metabolize scopoletin, phenolic acid, luteolin, and quercetin. Luteolin and quercetin were better substrates than phenolic acid and scopoletin, both of which displayed about less than 10% activity compared with luteolin. As shown in Fig. 1, a new product produced from luteolin was eluted at 9.05 min and a new peak from quercetin was eluted at 9.30 min. Eriodictyol and taxifolin were also tested for substrates for XcGT-2 but they did not display any reaction product. Luteolin and quercetin contain a double bond between carbons 2 and 3, which is lacking in eriodictyol and taxifolin. Because XcGT-2 used only luteolin and quercetin, a double bond between carbons 2 and 3 would be important for activity. On the other hand, kaempferol, which also contains a double bond between carbons 2 and 3, was not metabolized by XcGT-2. Kaempferol does not have a hydroxyl group at C-3', which is a difference between kaempferol and quercetin (Table 1). These results strongly suggested that glycosylation is likely to occur at a hydroxyl of C-3' on the B-ring of flavones. We tested further with fisetin and gossypectin, both of which contain a 3'-hydroxyl group and a double bond between C-2 and C-3.

Analysis of the reaction product using HPLC showed that both flavonoids also underwent reactions and revealed a new peak. However, fisetin was not a good substrate compared with luteolin, probably because of the lack of a hydroxyl group at C-5 (Table 1). Results indicated that the 3'-hydroxyl group and the double bond between carbons 2 and 3 are important for the activity of XcGT-2. It seems that XcGT-2 transfers a glucose molecule into the 3'-hydroxyl group of flavonoids. However, XcGT-2 did not use a 3'-hydroxyl flavone as a substrate. Taken together, the minimum structural requirements for XcGT-2 are 1) a double bond between C-2 and C-3 on the C-ring, and 2) hydroxyl group at both C-3' and C-4' on the B-ring of flavones.

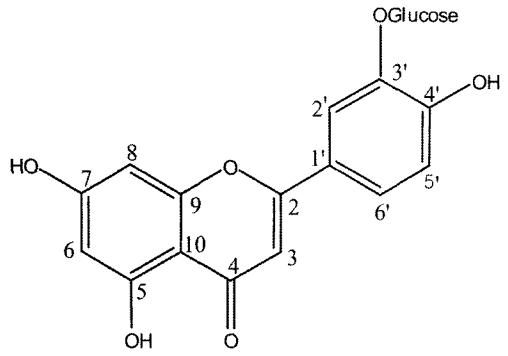
Because no commercial flavonoid 3'-*O*-glucosides are available, the glycosylation position of the luteolin reaction product was determined by nuclear magnetic resonance (NMR) spectroscopy and the resulting ^{1}H NMR spectrum was compared with luteolin 3'-glucoside, which was predicted to be the reaction product of XcGT-2. The eluent containing the reaction product was collected from HPLC and evaporated. The dried remnant was dissolved in ethylacetate. The supernatant was separated by a centrifuge and evaporated again under reduced pressure. The final remnant was dissolved in dimethylsulfoxide-d6 for the NMR experiments. NMR spectroscopy was carried out as described in Kim *et al.* [8]. Comparison of the ^{1}H NMR data of the reaction product with those of luteolin-3'-*O*-β-glucopyranoside published previously [15] showed that their chemical shifts were exactly identical to each other (Table 2). It was confirmed that the glycosylation position was the 3'-hydroxyl group of luteolin.

Luteolin was the best substrate tested, followed by quercetin and gossypectin (Table 1). The presence of the hydroxyl group at C-3 on the C-ring has a negative influence on the XcGT-2 activity, whereas the hydroxyl group at C-5 on the A-ring has a positive effect.

Flavonoid 3'-*O*-glucosides are rare in nature. Most glycosylation positions are at the hydroxyl group of either C-3, C-7, or C-4' [4]. In addition, diglycosylation products at C-3 and C-7 or C-3' and C-7 are observed. There was a report that UGTs from *Arabidopsis thaliana* glycosylated the 3'-hydroxyl group of quercetin [12]. However, these glycosyltransferases also produced glycosylated products at other positions and quercetin 3'-*O*-glucoside as a minor product. It is commonly found that UGTs from plant usually produced more than one glycosylated flavonoids when several glycosylation positions are available [10]. Thus, XcGT-2 has a distinct characteristic compared with other UGTs studied so far. As far as we know, XcGT-2 is the first UGTs from microorganisms that transfer a glucose into the 3'-hydroxyl group of flavones with regioselectivity.

It is not clear why *X. campestris* contains UGTs. In consideration that *X. campestris* is a plant pathogen, this microorganism confronts several compounds from plants and xenobiotics. Plants produced various types of compounds that contain antibacterial activities including flavonoids [2, 14, 19]. Glycosylation is considered one of the detoxification mechanisms against toxic chemicals. Therefore, XcGT-2 might have *in vivo* function to detoxify foreign chemicals.

Table 2. Assignments of the ^{1}H NMR data of the luteolin reaction product produced by XcGT-2.



Position	Luteolin	
	Luteolin-3'-*O*-β-glucopyranoside ^{1}H/ppm(Hz)[a]	Metabolite ^{1}H/ppm(Hz)
3	6.84(s)	6.81(s)
6	6.20(d;2.5)	6.19(d;2.2)
8	6.53(d;2.5)	6.52(d;2.2)
2'	7.80(d;2.5)	7.79(d;2.2)
5'	6.98(d;7.5)	6.97(d;8.5)
6'	7.65(dd;7.5,2.5)	7.65(dd;8.5,2.2)
1"	4.92(d;8.0)	4.90(d;7.5)
2"-6"	3.3–3.8(m)	3.3–3.8(m)

[a]Ref. 12.

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