Master's Thesis

Molecular cloning and Characterization of flavonoid 7-O-glycosyltransferase(*bGT3292*) from *Citrus platymamma hort. Ex Tanaka*

Dong-Shik, YANG

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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병귤의 글루코실트랜스퍼레이즈(*bGT3292*) 클로닝 및 활성 분석

지도교수 김 재 훈

양 동 식

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Molecular cloning and Characterization of flavonoid 7-O-glycosyltransferase(*bGT3292*) from Citrus platymamma hort. Ex Tanaka

Dong Shik, YANG

(Supervised by Professor Jae Hoon Kim)

A thesis submitted in partial fulfillment of the requirement

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This thesis has been examined and approved.

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Chairperson of the supervising committee

Professor Key-Zung Riu, Ph.D., **College of Applied Life Sciences**, **Jeju National University**

_____

Professor Dong-Sun Lee, **Ph.D., College of Applied Life Sciences**, **Jeju National University**

_____

Professor Jae Hoon Kim, **Ph.D.**, **College of Applied Life Sciences**, **Jeju National University**

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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ABSTRACT

An UDP-glucosyltransferase (*bGT3292*) was isolated from the leaf of *Citrus platymamma hort. ex Tanaka* (Rutaceae family) and was expressed in Escherichia coli. The expressed protein was investigated for its specific activity, using various substrates such as apigenin, eriodictyol, kaempferol, luteolin, naringenin, quercetin. *bGT3292* was found to be a flavonoid-7-o-glucosyltrtansferase and was classified into group D that has a glucosyl transferase activity on flavonol. However, different from other flavonoid-7-o-glucosyltrtansferases, *bGT3292* also can produce luteolin-4'-o-glucoside and quercetin -4'-o-luteolin. 7-OH of naringenin was best modification site among substrates used. In citrus plant, *bGT3292* was higher expressed in Flower than in fruit, leaf, and stem.

1. INTRODUCTION

Flavonoids are one of the natural substances in the secondary metabolite derived from high plant and are known to have effects such as anti-inflammantion, anti-cancer, anti-viral, anti-oxidative and UV-sheild[1]. The vast variety of flavonoid compounds have been discovered in the plant. In the plant system, flavonoids protect from oxidative stress and regulate growth of plant. Flavonoid biosynthesis pathway is exclusively enhanced under oxidative stress, Because of their useful biological activity, flavonoids are studied in medical science and many other areas. But some kinds of flavonoid are difficult to extract from plant species. So, Artificial production, using some modification by metabolic engineering, is need for lowering the production cost[2].

Glycosyltransferase(GT) is enable to glycosylate flavonoids and can enhance the stability, bioavailability of flavonoids. Glycosylation consists of enormous kinds of flavonoids and enhance immune system of plant. Depending upon the glycosylation position of flavonoid, flavonoid bioavailability can change. Thus, regioselective glycosylation is important to transfer cellular membrane and accumulate flavonoids in plant.

Glycosyltransferase can improve crops and fruits. The stability and solubility of flavonoids can be increased in the plant by glycosylation, plant can store more flavonoids and flavonoid contents increase. [3].

Citrus platymamma is a Korean native citrus variety cultivated in Jeju island. Citrus platymamma is resistant cold and various plant diseases. It also used an herbal medicine, but it is poorly investigated about its useful genes and application. Some researches for the development of new varieties of citrus are continually in progress. It has been reported that bitter taste of citrus can be regulated by using glycosyltrasferase genes. Limonin is major component of limonoid that causes delayed bitterness [4]. Limonoid glucosyltransferase can

reduce delayed bitterness. Identification and characterization of useful glucosyltransferases is also helpful to improve citrus fruit quality.

2. MATERIALS & METHODS

2.1 Cloning and expression of bGT 3292

Total RNA was extracted from leaves of Byungkyool (*Citrus platymamma*, Hort. ex. Tanaka) using Trizol reagent (life technologies). The cDNA was synthesized and amplified using RT-PCR kit (Takara). PCR condition consisted of an initial denaturation at 94℃ for 2min, and followed by 29 cycles (denaturation at 94℃ for 30s, annealing at 55℃ for 30s, and extention at 72℃ for 1min 30s, with final 10 min extension at 72℃). PCR product was digested with *Bam*HⅠ and *Xho*Ⅰ and cloned into pGEX4T-1 plasmid.

Transformed *E.coli* BL21 (DE3) cells were pre-cultured at 37℃ over night in Luria-Bertani (LB) broth medium (20ml) containing ampicillin (100 μg/ml). These cells were grown in LB medium at 37℃ until the cell concentration reached to OD_{600nm} of 0.7, and IPTG (isopropyl-1-thio-β-D-galactopyranoside) was add to a final concentration of 0.1mM and 2% Ethanol, followed by growing for additional 20 hours at 18℃.

2.2 Phylogenetic analysis

bGT3292 amino acid sequence and other UGT amino acid sequence from Citrus sinensis and arabidopsis were used for this study. Amino acid sequences of UGT were extracted from Arabidopsis Glycosyltransferase 1 (http://www.p450.kvl.dk/gst.shtml) and NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acids were aligned using the Multiful sequence alignmnet by CLUSTALW (http://www.genome.jp/tools/clustalw/). Phylogenetic

tree was constructed using MEGA6. The evolutionary history of *bGT3292* was inferred using the Neighbor-Joining method.

2.3 The analysis of *bGT3292* Gene Expressions using Real-Time PCR

The relative expression level of *bGT3292* transcript in leaves, stem, fruit and flower of C. platymamma were analyzed for using real-time PCR. Total RNA was isolated from these tissues using Trizol agent (life technologies). The cDNAs were synthesized using RT-PCR kit (Takara). The primers were designed as 5'-GCGCGGATCCATGGCATCCGAAGCCAG CCAGGTT and 3'- CGCCGCCTCGAGTTAAATCCCTTCTGAGCTTGGC. A reaction was performed using SYBR-Green® PCR Kit (Bio-rad, USA) on the MiniOpticon™ Real-Time PCR Detection System. Pre-incubated at 95℃ for 2min and followed by 40 cycles of 30 sec at 95℃, 30 sec at 60℃. The results obtained from different samples were standardized to the constitutive GAPDH gene expression level.

2.4 Enzyme assay

To analysis enzyme substrate specificity, E.coli cells harboring GT gene were harvested by centrifugation. And then the recation mixture (500μl) containing 50mM potassium phosphate (pH 7.0), 1% glucose and 50μM flavonoid was added to cell s. It was incubated for 20hr at 28℃ and extracted using Ethyl acetate. Ethyl acetate was evarporated and then reaction product was dissolved in methanol.

2.5 SDS-PAGE and Immunobloting assay

The total protein was quantified using Bradford assay (Bradford reagent, Bio rad). The protein bands were separated by electrophoresis and were electrotransferred to 0.45um Nitrocellulose Blotting Membrane (GE Healthcare Life science) at 60V 3hr in 4℃. After transfer, the membrane was blocked for 1hr in a 5% skim milk in TBS-0.05% Tween 20(TBST) and washed 5 times in TBST for 5 ~ 10min. The membrane was incubated for 16hr with GST primary antibody (Santa Cruz, USA) that was diluted 1:1000 in TBST, and then incubated for 2hr with secondary antibody (Santa Cruz, USA) that was diluted 1:1000 in TBST. We used an enhanced chemiluminescent substrate to detect band of GST fused protein (WEST –ZOL iNtRON Biotechnology Inc., Korea).

2.6 HPLC analysis

Samples were analyzed with a Waters HPLC e2695 Alliance separations module (Waters, Milford, MA) and photo didode array (PDA) detector. Phenomenex C18 column (5.05um particle size, 250x4.6mm) was used for all analysis. HPLC grade Acetonitrile and Water (Fisher Scientific,Fair Lawn, NJ) were used as a mobile phases. The products were separated with a linear(A:B) elution gradient : 20% acetonitrile at 0 min, 40% acetonitrile at 20 min, 70% acetonitrile at 25 min, 20% acetonitrile at 30min.

3. RESULTS

3.1 Cloning and gene expression

Sequence information was gained from genomic sequcence of Citrus platymamma Hort.ex. Tanaka. Some kinds of glucosyltransferase genes were identified from Citrus platymamma hort. Ex Tanaka. These genes were expressed in *E.coli*(fig. 1), and then studied about enzyme activity. *bGT 1884, 3292, 3591, 1888, 629-3, 2282*. *bGT629-3* was highest expressed. and then *1884*, *1888*. *bGT2282* did not showed glucosyltransferase activity.

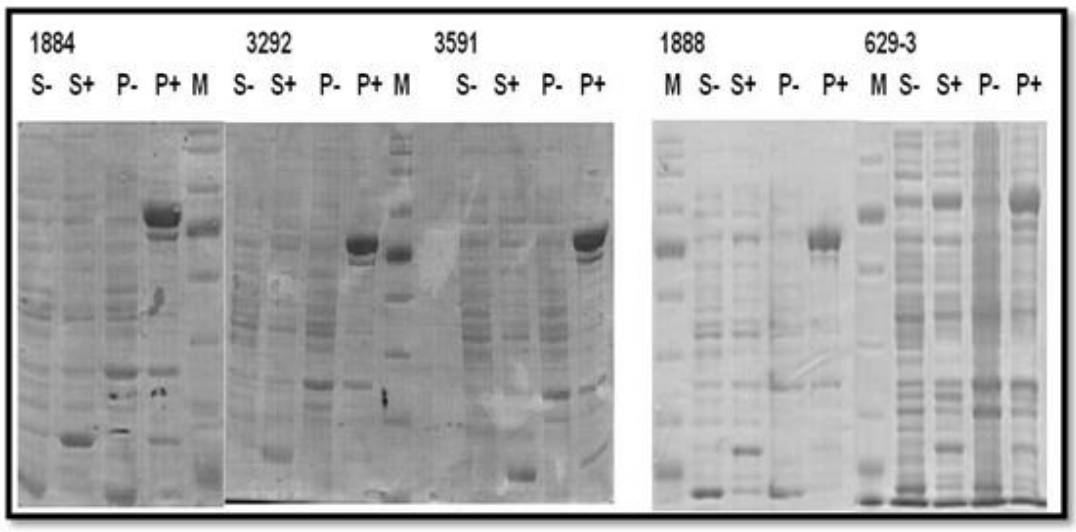


Figure 1. SDS-PAGE of *bGT3292* and other GTs

M, Protein Marker; S(-), uninduced supernatant; S(+), induced supernatant; P (-), uninduced pellet; P(+), induced pellet.

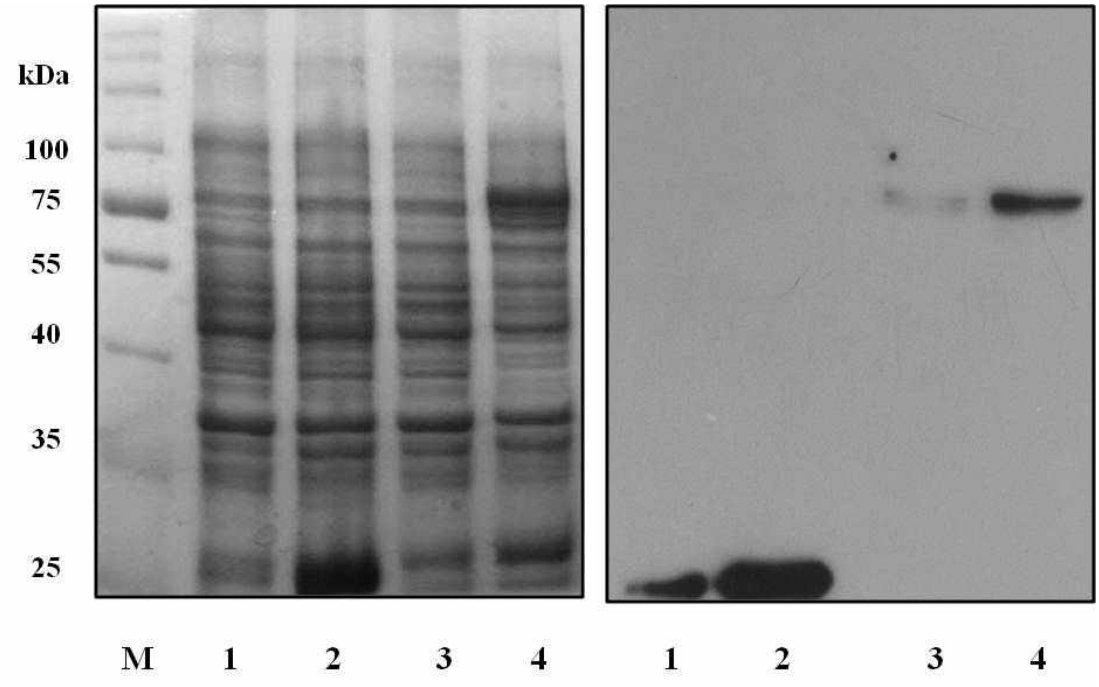


Figure 2. Expression of the recombinant *bGT3292*.

M, Molecular weight marker; 1, uninduced lysate of vector; 2, induced lysate of vector; 3, uninduced of *bGT3292*; 4, induced of *bGT3292*.

bGT3292 (1470bp) contains an open reading frame (ORF) that encodes a protein with 490 amino acid. *bGT3292* gene was expressed using glutathione-S-transferase fusion protein system. To verify protein expression of *bGT3292*, we used SDS-PAGE and Western blot analysis. The molecular weight of the recombinant *bGT3292* was approximately 79.9 kDa containing a fused GST. One band(GST-fused *bGT3292*) was detected by Western blot analysis (Fig.2). To access the expression levels in Citrus plant, the real time PCR was performed with Citrus plant tissues. The expression level of *bGT3292* was highest in flower. However, *bGT3292* was lowly expressed in stem and fruit (fig. 3).

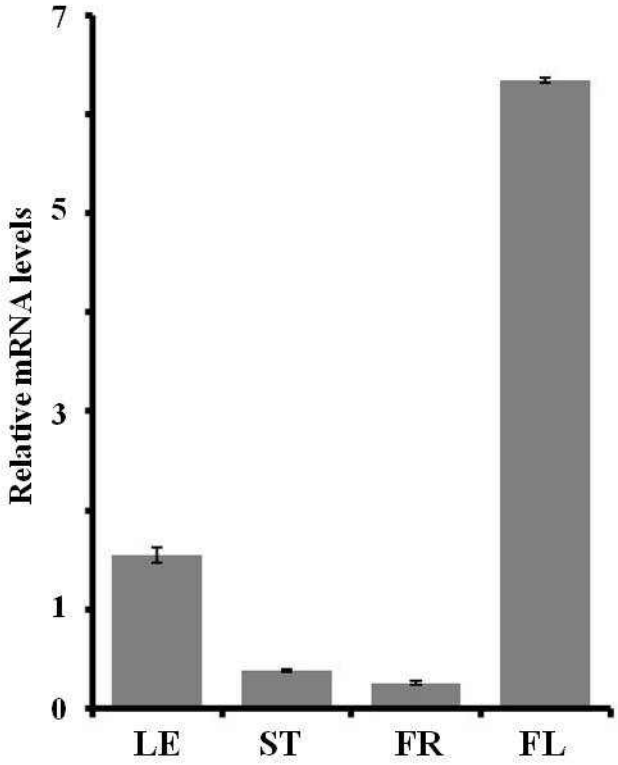


Figure 3. Real time PCR analysis of *bGT3292*.

LE; leaf, ST; stem, FR; fruit, FL; flower. The GAPDH was used as a internal control

3.2 Phylogenetic analysis

BlAST analysis showed that *bGT3292* is similar with genes of *Citrus sinensis* and *clemintina* (Fig. 4). But these genes were not studied about their enzyme activity. *bGT3292* had 83% similarity with UDP-glycosyltransferase Family 1 from *Citrus x paradish*. This UDP gene is named *CsUGT2*. *CsUGT2* contained in UGT73 group that is known to have an activity to flavonoids, betacyanin, terpenoid [5]. PSPG motif ,consisting of 44 amino acids, is a high conserved region and binds various activated sugar[6]. In the PSPG motif, sequence of *bGT3292* is very similar with *CsUGT2* (only one different amino acid, *CsUGT2* is methionine and *bGT3292* is Lysine). According to the research of this paper, *CsUGT1* and

CsUGT3 is belong to GroupL, *CsUGT2* is groupD. GroupL from Arabidopsis was known to exhibit an activity to hydroxycinamoyl glucose ester. GroupD from Dorotheanthus bellidiformis was known to represent flavonol activity[6]. *CsUGT2* and *bGT3292* was found to be belong to Group D in the phylogenetic tree (Fig. 5).

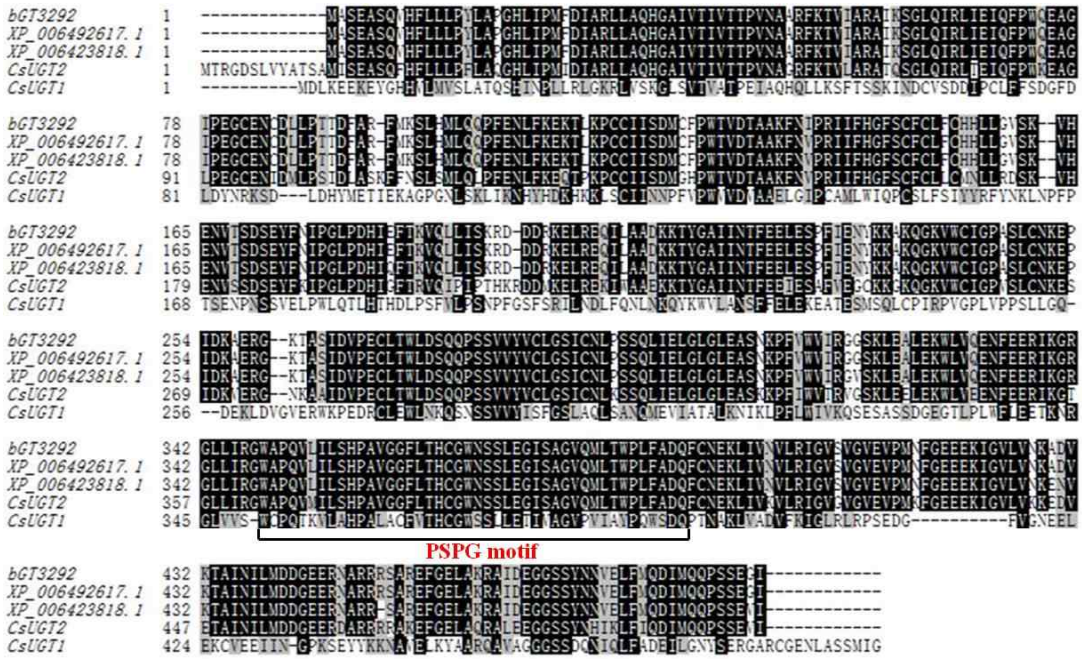


Figure4. Alignments of *bGT3292* and other Plant GTs. The alignment was created using Multiple Sequence Alignment by CLUSTALW.

The *C.sinensis* (XP_006492617.1), *C.clementina* (XP_006423818.1), *CsUGT1* (GQ221686.1), CsUGT2 (GQ221687.1).

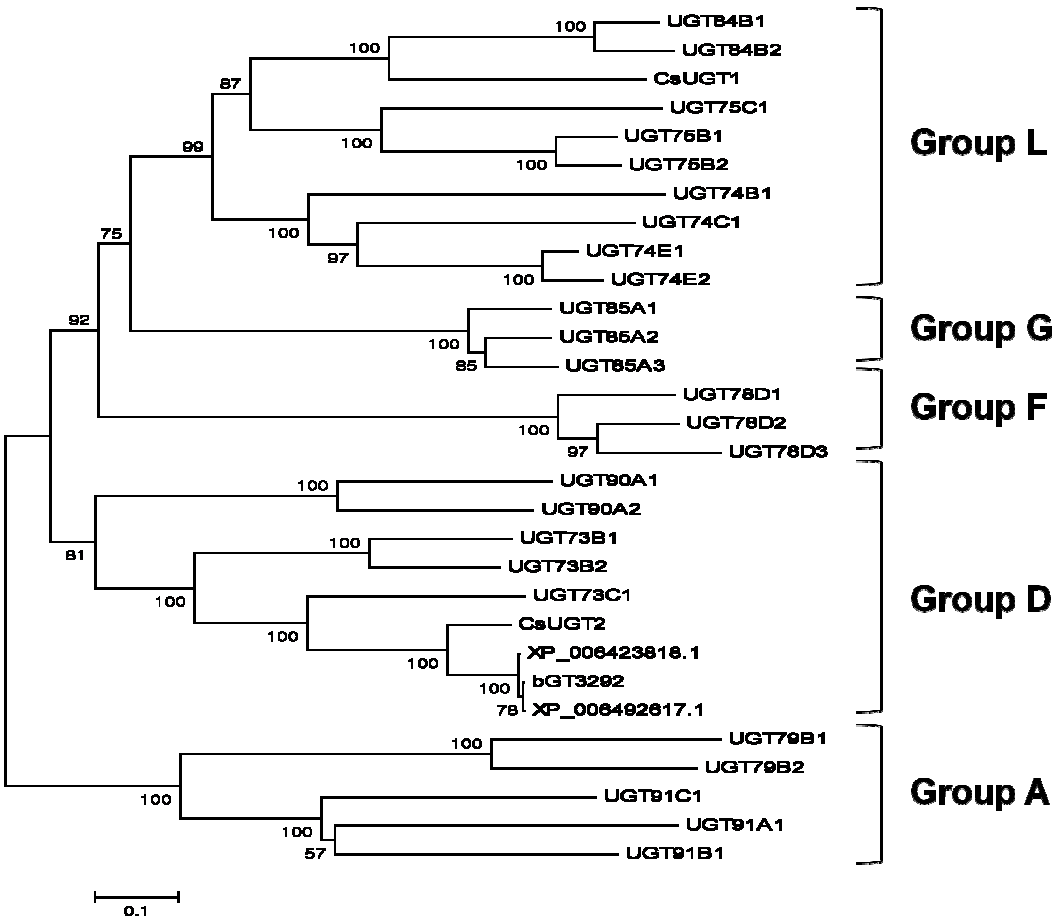


Figure5. Phylogenetic analysis of *bGT3292* and other Plant GTs. Phylogenetic analysis were conducted in MEGA 6.

3.3 Characterization of *bGT3292* activity

We used biotransformation to make flavonoid glycoside. Six substrates including apigenin, eriodictiyol, kaempferol, luteolin, naringenin, quercetin was tested for glucosyltransfrease activity. After biotransformation assay, reaction products were extracted and analyzed by HPLC.

First, we determined the substrate specificity and conversion rate. *bGT3292* was efficiently covert aglycone to glycoside in the all substrates used. Apigenin, eriodictiyol, kaempferol, and naringenin showed one major peak in the reaction product (fig. 6) (fig. 7) (fig. 8) (fig. 9). In case of luteolin and quercetin, reaction products showed two distinct peaks(fig. 10) (fig. 11). Reaction products were compared with each authentic 7-glucoside. Both retention times and UV spectrum for the reaction product of four substrates (apigenin, eriodictiyol, kaempferol, and naringenin) were indistinguishable from those of the authentic 7-glucoside, demonstrating that apigenin, eriodictiyol, kaempferol, naringenin were specifically glucosylated at 7-hydroxyl group. The first peaks of reaction products of luteolin and quercetin determined as 7-glycosides, respectively. As the same method, we also determined the second peaks of reaction products of luteolin and quercetin as 4`-glycoside. The identities of these compounds were also confirmed by using liquid chromatography-mass spectrometry (LC-MS). LC-MS analysis showed the same molecular weight to the synthezed monoglucosides

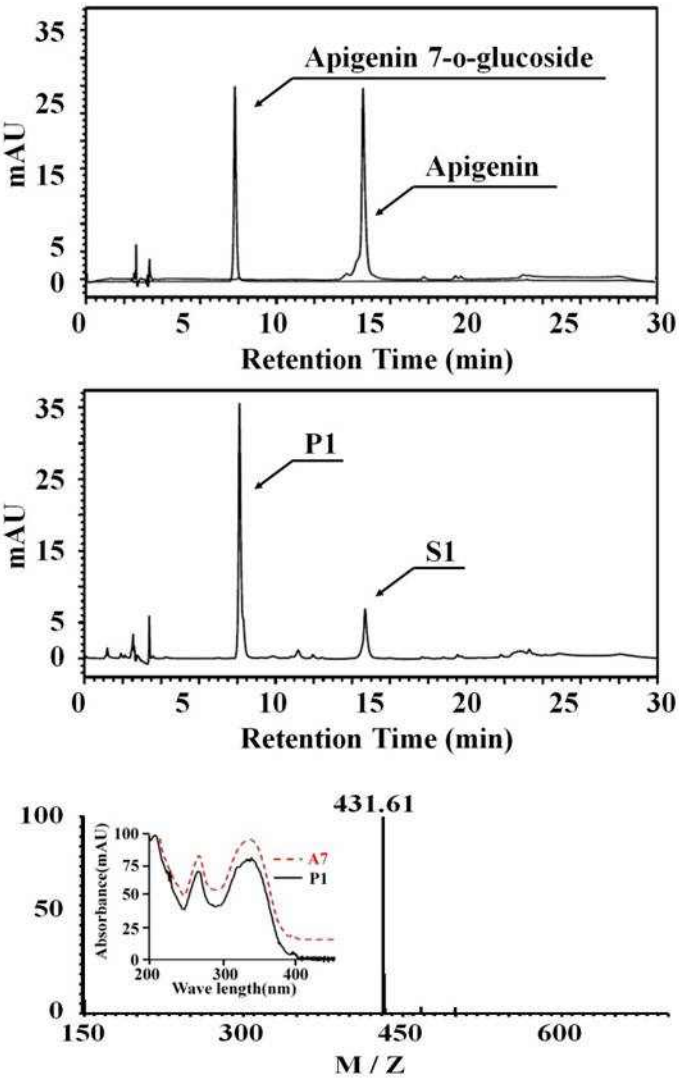


Figure 6.High-performance liquid chromatography(HPLC) of *bGT3292* Reaction Products.

Apigenin : Reaction product of apigenin (P1) and authentic apgenin(S1).

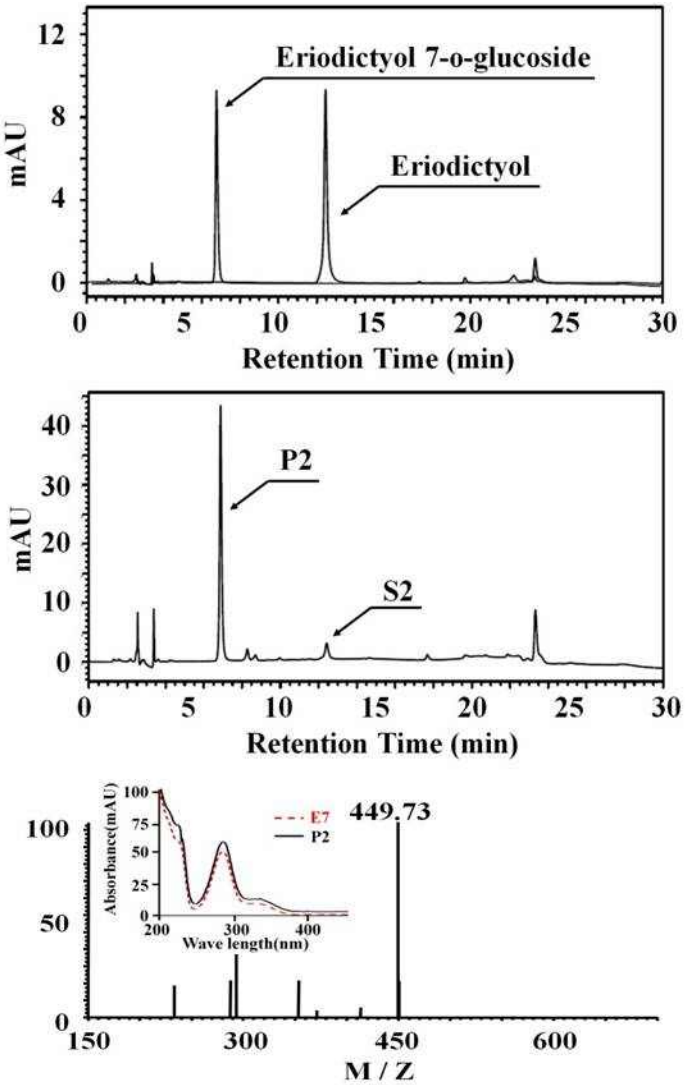


Figure 7. High-performance liquid chromatography(HPLC) of *bGT3292* Reaction Products.

Eriodictyol : Reaction product of Eriodictyol (P2) and authentic eriodictyol(S2).

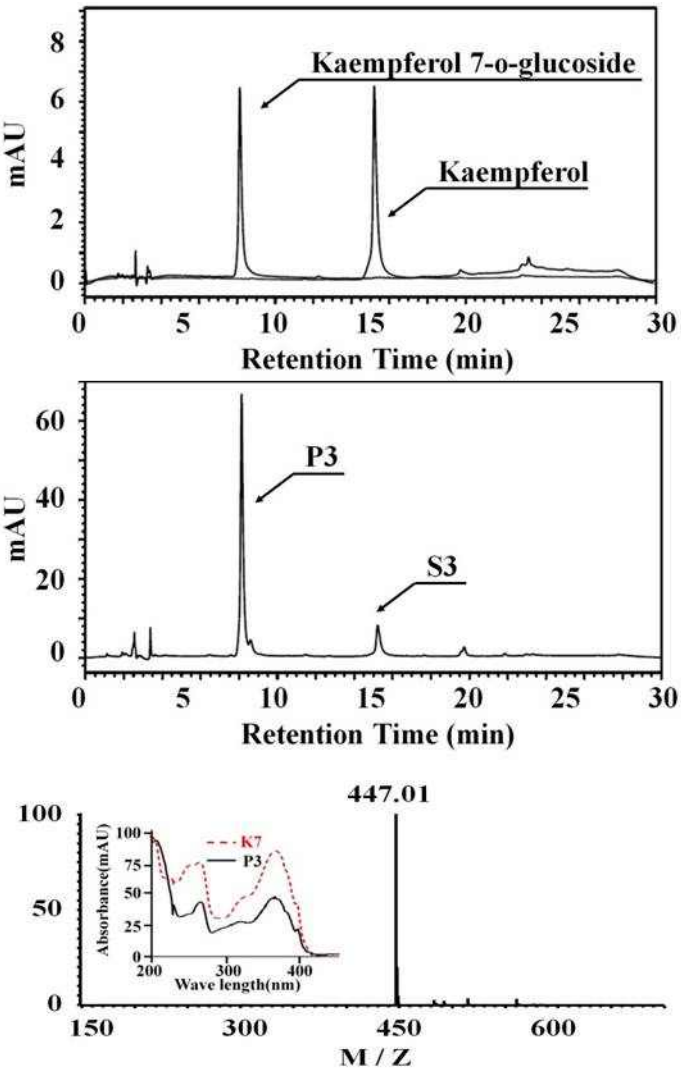


Figure 8. High-performance liquid chromatography(HPLC) of *bGT3292* Reaction Products.

Kaempferol : Reaction product of kaempferol (P3) and authentic kaempferol (S3).

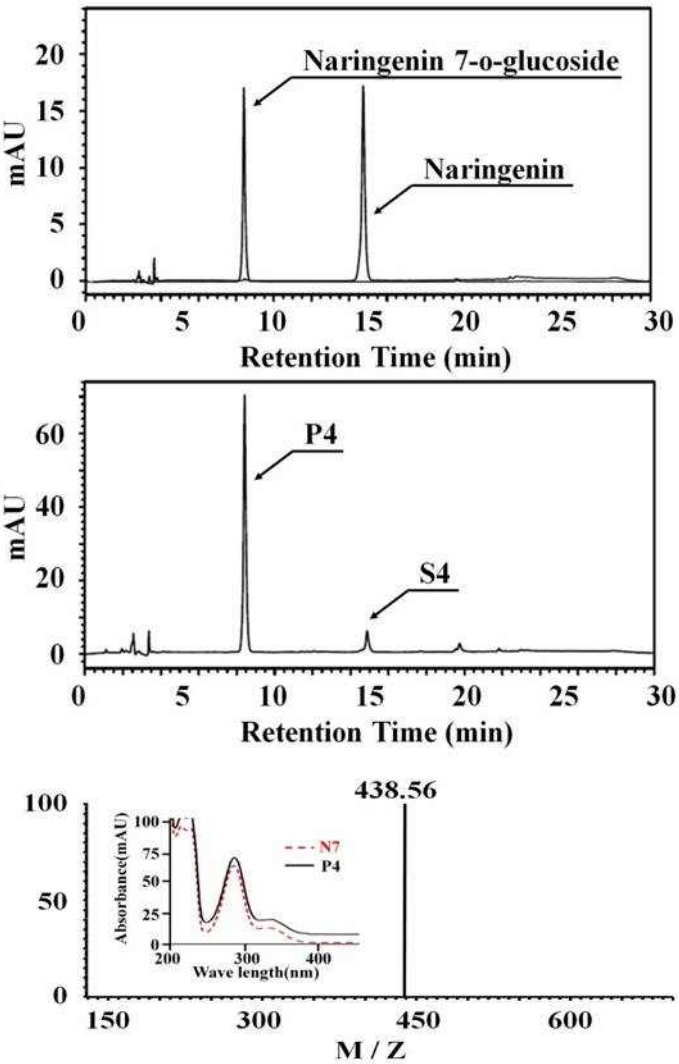


Figure 9. High-performance liquid chromatography(HPLC) of *bGT3292* Reaction Products.

Naringenin : Reaction product of naringein (P4) and authentic naringenin (S4).

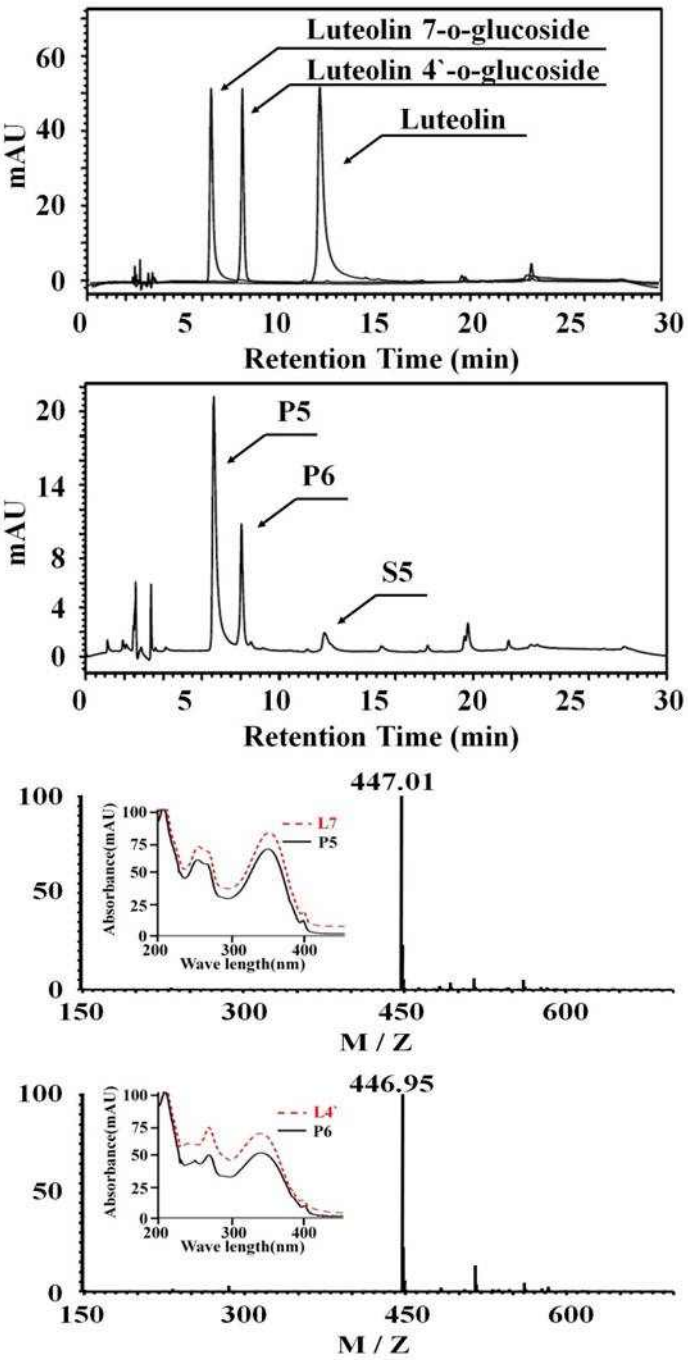


Figure 10.High-performance liquid chromatography(HPLC) of *bGT3292* Reaction Products.

Luteolin : Reaction product of luteolin (P5, P6) and authentic luteolin (S5).

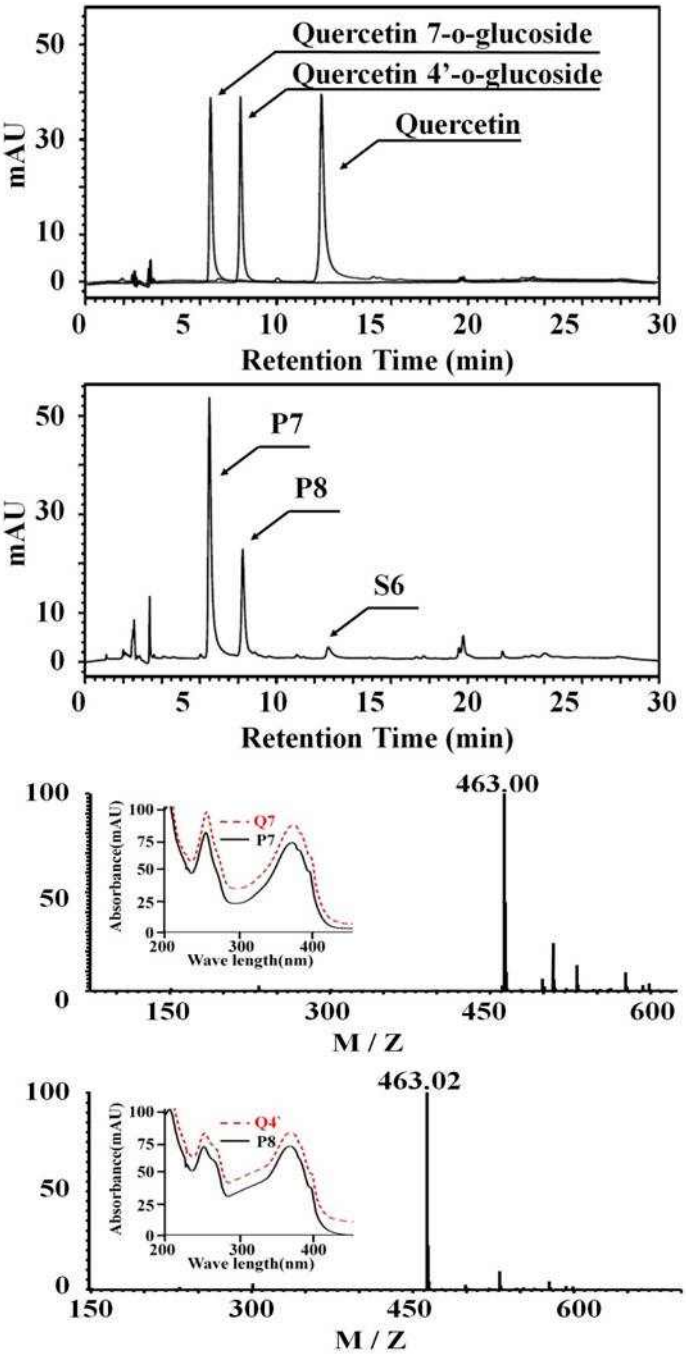


Figure 11. High-performance liquid chromatography(HPLC) of *bGT3292* Reaction Products.

Quercetin : Reaction product of quercetin (P7, P8) and authentic quercetin (S6).

Conversion rates were determined by analyzing the amount of reaction product with the peak area from HPLC analysis (Table 1). Conversion rate of *bGT3292* is 70 to 90 % in all substrates. Narigenin-7-OH was found to be the most effective modification site by *bGT3292* (93.5 ± 2.86 %). Glucosylation of quercetin was occurred in two sites, each conversion rates were 71.8% (7-OH) and 23.0% (4`-OH), respectively. Luteolin also had two glucosylation sites and conversion rates for two sites were similar with those of quercetin.

Similar to B ring of the luteolin and quercetin, Eriodictyol also has 4` - OH, 3` – OH. But eriodictyol-4` - OH did not modified by *bGT3292*. Eriodictyol is classified into flavanone of which back bone structure is different from those of luteolin and quercetin that contained 2, 3-double bond. These result suggest 2, 3-double bond plays crucial roles for 4` - OH modification activity of *bGT3292*. We also performed NMR analysis and conform quercetin-7-O-glucoside produced by *bGT3292* (Table2).

Table 1. Conversion rates of flavonoids by recombinant glycosyltransferase *bGT3292*.

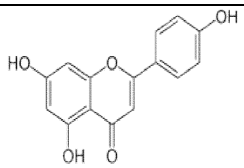
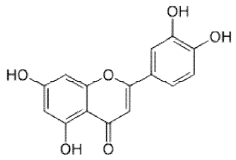
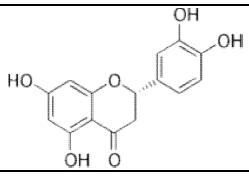
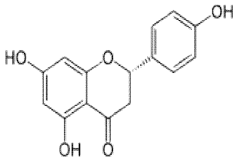
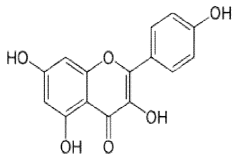
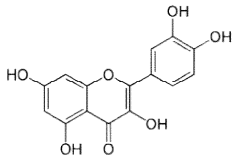
	Substrate	Structural formula	conversion rate(%)
Flavone	**Apigenin**		83.3 ± 2.90
	Luteolin		71.6 ± 5.42 21.1 ± 1.76
Flavanone	**Eriodictyol**		78.9 ± 3.21
	Naringenin		93.5 ± 2.86
Flavonol	**Kaempferol**		87.6 ± 6.04
	Quercetin		71.8 ± 3.39 23.0 ± 2.66

Table 2. NMR data of product of Quercetin using *bGT3292*

13C					
Sample		Reference			
Sample in CD3OD	Sample in DMSO-d6	Quercimeritrin 7-O-glc) in DMSO-d6	Saxifragin (5-O-glc) in Pyridine-d5	Isoquercitrin (3-O-glc) in CD3OD	Quercetin in CD3OD
177.6	175.6	176.0	179.0	179.7	176.0
164.6	162.3	162.7	166.1	166.7	164.1
162.3	160.0	160.4	162.9	163.2	160.9
157.8	155.4	155.7	158.0	158.9	156.3
149.1	147.5	148.1	157.7	150.1	147.8
148.9	147.2	147.6	151.0	148.0	146.9
146.4	144.7	145.2	146.9	146.0	145.2
137.8	135.7	136.2	136.0	135.9	135.9
124.1	121.5	121.7	123.0	123.0	122.0
122.0	119.7	120.0	122.5	122.1	120.1
116.4	115.2	115.6	118.1	117.9	115.7
116.3	115.0	115.4	116.5	116.2	115.1
106.4	104.3	104.7	105.6	105.3	103.1
101.8	99.5	99.9	105.4	100.2	
100.3	98.4	98.7	100.1	96.2	98.2
95.7	93.9	94.2	94.8	94.9	93.4
78.5	76.8	77.2	78.0	77.2	
78.0	76.0	76.4	75.8	75.1	
74.9	72.8	73.1	73.7	73.2	
71.4	69.2	69.6	70.1	70.0	
62.6	60.3	60.6	62.2	61.9	
58.5	52.5				
18.5	48.2				

4. DISCUSSION

One of the glycosyltransferase in the citrus platymamma, hort.ex .tanak, designated as *bGT3292*, was identified. 76 GTs sequence was gained from the genomic data of byeonggyul and tested each GT on various substrates. Flavonoids have pharmacological activity that can be useful in health care. Depending on substrate structure, GTs showed different modification activity. On the phylogenetic tree, genes classified in clusters based on amino sequence similarity and showed similar activity. Orthologous group(OG) is defined as group of gene from common ancestor. OG1(Group D) include UGT73s, UGT89s and UGT90s that were repoted as flavonol 7-O-glucosyltransferase, 7-rhamnosyltransferase. This group has activity on brassinosteroid and cytokinin [7].

Glucose is the most common substrate in the plant. Sugar donor activity is different between each phylogeny. In each phylogeny, just some amino acid is changed to another, resuling in the change of enzyme activity. UGT88D enzymes are F7GATs (flavonoid 7-O-glucuronosyltransferase), Arg residue plays a critical role in those enzymes [8]. Although some genes are belonged to same group, modification of critical residue can change a enzyme activity.

Both *bGT3292* from Citrus platymamma and CsUGT2 from Citurs sinensis are high similar in their sequences. However the CsUGT2 activity is not reported, yet. [5]. Many kinds of flavonoids have been identified to have regio-specificity, sugar-donor activity and phamarcological activity. From chamomile, apigenin derivatives such as apigenin-O-glucoside(Ap-7-Glc), Ap-7-(6’’-malonyl-Glc), Ap-7-(6’’-acetyl-Glc), are discovered. Because of acylated glucoside of instability, stability study was performed along temperature, pH and Rapid extraction method [9]. Biotransfromation method may be used for mass production of acylated glucoside.

Flavonoid glycosyltransferase AtGT1 from A.thaliana is classified as UGT73B2 (Group D). Different from *bGT3292*, *AtGT-1* preferentially generate 3-O-glucoside and 7-O-glucoside[10]. Quercetin 5-O-glucoside is not reported in the plant, but the silkworm can synthesize quercetin 5-o-glucoside that effect to UV-protection [11].

Regioselective glucosylation was appeared in the quercetin. The quercetin is able to be synthesized four different monoglucoside at 3 –OH, 7 –OH, 3`-OH, 4` -OH. In the previous study to produce glucoside efficiently, whole – cell Biocatalyst Assay was performed using 29 UGT enzymes. Among these enzyme, UGT73C5 and UGT74F1 using substrate as quercetin have a similar activity with *bGT3292* that produces two monoglucoside, 7 –OH, 4` -OH [12]. The UGT73C5 and *bGT3292* is belong to Group D, but UGT74F1 is included in Group L. Although *bGT3292*and UGT73C5 is belong to same group and have high sequence similarity, 4`-OH activity was higher than 7-OH activity in the UGT73C5, In case of *bGT3292*, 7-OH activity was 3 times higher than 4`-OH activity . UGT74F1 and *bGT3292* is low sequence similarity, but substrate specificity was similar. These results indicate that sequcence similarity does not always explain correct enzyme activity. Therefore, we need to test enzyme activity to characterize glucosyltransferase. In our study, Eriodictyol, luteolin, quercetin have a two OH group in the B – ring, but Eriodictyol 4`-glucoside was not produced. With comparison of substrate structures, we can estimates what is crucial structure. 2 -3 double bond of C – ring of substrates might be crucial structure for producing 4`-glucoside

5. CONCLUSION

bGT3292 라고 명명된 glucosyltransferase 를 클로닝하고 동정하였다. 병귤로부터 유래된 *bGT3292* 는 GroupD 에 속하는 것으로 보이고 *C.sinensis* 와 *C.clementina* 에도 Amino acid sequence 가 유사한 GT 가 존재하나 활성에 대한 연구는 보고되지 않았다. bGT 3292, 1884, 1888, 629-3 과 같은 여러가지 GT 를 대장균에서 발현을 후 활성을 비교하였고, bGT3292 가 다른 GT 들에 비해 기질특이성이 뛰어나고 conversion rate 가 높은 것을 확인하였다.

식물을 비롯해서 다양한 생물군에서 glucosyltransferase 가 발견되고 있으며 효소활성을 나타내는 기작에 대한 연구를 비롯해서 다양한 응용분야에 대한 연구가 진행되고 있다. 식물에서는 종분화가 될수록 특이적인 glycosyltransferase 가 발견되고 있으며, 식물에서 활성을 찾아볼 수 없는 효소가 다른 생물군에서 발견되기도 한다[8][11]. Citrus 속에서는 C.sinensis 와 C.clementina 에서의 GT 에 대한 연구가 많이 진행되어 있으며, 유사한 서열을 갖더라도 다른 생리활성을 갖기 때문에 다른 종의 glucoysltransferase 에 대한 연구가 필요한 것으로 보인다.

본 연구를 통하여 *bGT3292* 는 flavonoid7-O-glucosyltransferase 로 밝혀냈으며 luteolin 과 quercetin 에 대해서는 4`-OH 에도 활성을 나타내는 것으로 보인다. Apigenin, kaempferol, Luteolin, naringenin 은 B-ring 에 –OH 를 갖지 않는 것이 4`-OH 에 활성을 나타내지 않는 원인으로 추측되며, eriodictyol 의 경우 luteolin, quercetin 과 약간의 구조적인 차이를 보인다. 이러한 입체구조의 차이가 4`-OH 에 효소활성을 나타내지 않는 현상의 주요한 원인일 것이라고 예상된다.

bGT3292 를 비롯하여 다양한 glucosyltransferase 의 활성에 대한 연구는 glucosyltransferase 의 활성에 대한 기작을 분석하고 연구하는데 도움이 될 것이다.

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요약문

병귤은 국내에서 재배되고 있는 재래귤이지만 대중에게는 생소한 품종으로서 연구 및 개발이 잘 되지 않았다. 이러한 병귤을 연구개발하는 방향으로 병귤의 genome 으로부터 기능성 유전자를 발굴하게 되었다. 귤에는 플라보노이드 성분이 다량으로 포함되어 있고, Flavonoid 는 항균, 항암, 항바이러스 등 많은 생리활성을 갖는다고 알려져 있다. 이번 실험에서는 귤에 많이 들어있는 플라보노이드 중 6 가지인 apigenin, eriodictyol, kaempfeol, luteolin, naringenin, quercetin 을 기질로서 사용하였다.

당전이효소(glucosyltransferase, GT)는 Flavonoid 와 같은 이차대사산물에 당을 전달하는 기능을 한다. Flavonoid 에 당이 붙어있지 않은 상태를 아글리콘(aglycone)이라고 하는데 당전이효소에 의해서 당이 붙은 배당체(glycoside)로 전환한다. 당전이효소는 여러 가지 종류가 존재하는데 이러한 효소들은 각기 다른 활성을 나타낸다. 당전이효소는 위치특이성(regio-specificity)과 기질 공여체 특이성(substrate donor specificity)를 갖는다. Flavonoid 의 한 종류인 quercetin 은 다섯 곳에 -OH 잔기를 갖는다. 각각의-OH 잔기마다 활성을 나타낼 수 있는 당전이 효소가 다르다. 그리고 활성을 나타낸 위치에 따라서 그 flavonoid -glucoside 의 생리활성이 다르게 나타난다. 기질 공여체 특이성은 당전이효소의 종류에 따라서 Flavonoid 에 결합시키는 당의 종류가 다르다는 것을 나타낸다.

Glucosylation 이 일어날 때 Glucose 가 어떤 위치에 일어나는가에 따라서 항산화효과가 증가되거나 감소가 될 수 있다는 보고가 있다. Glucosylation 으로 인해 -OH 그룹이 보호됨으로써 Auto-oxidation 이 일어나지 않게 되어서 Flavonoid 가 갖고 있는 항산화효과가 필요하지 않는 곳에서 일어나는 것을 방지하게 된다.

기질특이성을 검정하기 위해 HPLC 와 LC-MS 를 진행하였다. Standard, product, MS data 와 spectrum 결과를 비교하여서 기질특이성 검정을 수행하였다. 활성테스트에 사용된 Aglycone 의 피크가 줄어들고 product peak 가 생성되는 것을 볼 수 있었다. Major peak 로서 생성되는 생성물이 flavonoid7-glucoside 가 맞으나 minor peak 로 나타나는 생성물로서 flavonoid 4`-glucoside 라는 것을 확인하였다.

기존에 UGT73C5 와 UGT74F1 는 Quercetin 에 대해서 7 –OH 와 4`-OH 에 활성이 있다는 보고가 있다. 하지만 UGT73C5 는 *bGT3292* 와는 다르게 4`-OH 에 활성이 더 높은 것으로 보고되었다. UGT74F1(Group L)의 경우 *bGT3292*(Group D)와 amino acid sequence 가 다른데도 불구하고 비슷한 활성을 나타내었다. 이러한 결과는 서열상으로 similarity 가 다르더라도 비슷한 활성을 나타낼 수 있기 때문에 직접 activity test 를 해봐야 한다는 것을 의미한다. 다양한 Glucosyltransferase 의 활성에 대한 연구는 glucosyltrasferase 의 활성에 대한 기작을 분석하고 연구하는데 도움이 될 것이라 생각된다.

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