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Master's Thesis

**Molecular cloning of glucosyl-
transferase from**

***Citrus reticulata* cv. Siranui**

**[(*Citrus unshiu* X *Citrus sinensis*) X *Citrus
reticulata*]**

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GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

July, 2012

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2012년 7월

**Molecular cloning of glucosyltransferase from *Citrus*
reticulata cv. Siranui
[(*Citrus unshiu* X *Citrus sinensis*) X *Citrus*
reticulate]**

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(Supervised by Professor Jae Hoon Kim)

A thesis submitted in partial fulfillment of the requirement

For the degree of Master of Science

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ABSTRACT

The glycosylation of flavonoids is an essential modification contributing to the complexity of secondary metabolites and is often the last step in the biosynthesis of natural compound, affecting water solubility and stability of flavonoids. Glucosyltransferase (GT) are member of glycosyltransferases and transfer glucose to an acceptor molecule.

In this study, ten novel GT genes were cloned and characterized from *Citrus reticulata* cv. Siranui. These genes are named as *GT150*, *GT1669*, *GT259*, *GT285*, *GT173*, *GT2282*, *GT3363*, *GT3591*, *CiGT1*, and *CiGT2*, respectively. Eight GT genes (*GT150*, *GT1669*, *GT259*, *GT285*, *GT173*, *GT2282*, *GT3363*, and *GT3591*) were amplified from first-strand cDNA that synthesized from total RNA of *C. reticulata* leaves by using Polymerase chain reaction (PCR) with gene specific primers. *CiGT1* and *CiGT2* gene were amplified by using reverse transcription (RT) - PCR with gene specific primers. The full-length GT cDNAs were cloned and expressed as glutathione S-transferase fusion proteins, and then, their activities were assayed with naringenin and quercetin, which are typical substrates for the enzyme producing flavonoid 7-O-glucoside and flavonoid 3-O- glucoside, respectively.

Based on HPLC analysis, *GT173*, *GT285*, and *GT3363* gene products showed GT activities on naringenin and quercetin, demonstrating these genes were both related flavonoid 7-O-glucoside and flavonoid 3-O- glucoside. Compared to *GT285* and *GT3363*, *GT173* showed only a detectable activity on naringenin.

GT2282 and *GT3591* were determined to glucosylate naringenin, producing its 7-O-glucoside. Interestingly, *GT259* can glucosylate quercetin but its modification was not 3-O site, suggesting the other site could be glucosylated. On the other hand, *GT150*, *GT1669*, *CiGT1*, and *CiGT2* did not transfer glucose to two flavonoid substrates in this study. For these four genes, further study need to identify their substrate.

1. INTRODUCTION

All plants contain secondary metabolites including flavonoids, alkaloids and terpenoids [Wink M, 1999]. Flavonoids have general skeletal representation with 2 aromatic rings and low molecule weight secondary metabolites. Figure 1 shows that general skeletal representation of flavonoids [Lazarus and Schmit, 2000].

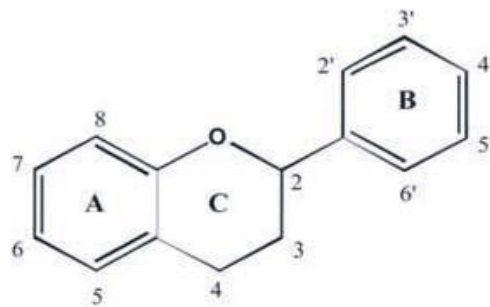


Figure 1. General skeletal of flavonoid [Lazarus and Schmit, 2000]

Flavonoids are unlike primary metabolites, which are not essential for plant survival. Generally, flavonoids do not participate in primary biochemical activities such as growth, development, and reproduction [Sarkar et al., 2007]. Nevertheless, flavonoids play an important roles acting as feeding deterrents, UV protections, pollinator attractants, growth regulators, and pigments for flower coloration [Harbone, 1976; Mori et al., 1987; Broillard and Dangles, 1993]. Flavanones serve in specific plant life cycles by imparting different taste properties, and play a key role of anti cancer in human. For example, naringenin found in grapefruit, sour oranges, and lemon that affect bitter taste of these fruit. In human, quercetin plays a role of antioxidation, anti-tumor of breast, and reduction in blood pressure (Fiugre2) [Michael W. King, 2011].

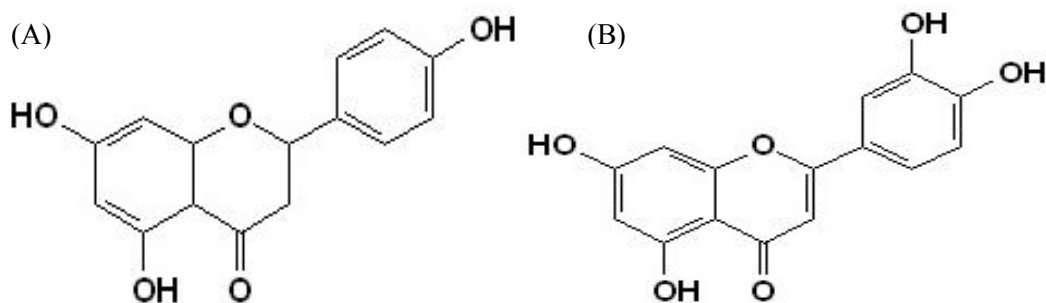


Figure 2. Structure of (A) naringenin and (B) quercetin [supplementary science]

Glycosylation refers to the enzymatic process that attaches glycoside to proteins or other organic molecules. This modification is a common modification reaction in plant metabolism and is the last step in the biosynthesis of natural compounds. Glycosylation donor molecule is often an activated nucleotide sugar. The glycosylation of flavonoids affects their water solubility that improves stability and bioavailability [Sayaka et al., 2009].

Glucosyltransferase (GT) are enzymes that transfer glucose to an acceptor molecule. GT share a conserved region called plant secondary product glucosyltransferase (PSPG) box. This box consists of 44 amino acid residue close to C-terminal region of the protein [Vogt and Jones, 2000; Joe Ross (2001); Tian et al., 2006; Hughes and Hughes, 1994]. Over 12,000 sequences encoding GT in many different organisms have collected (<http://afmb.cnrs-mrs.fr/CAZY>) [Kim et al, 2006 A; Claire et al, 2005]. These sequences classified into 77 families through biochemical studies of their gene products or through sequence homology comparison to the genes encoding enzymes of known catalytic activity [Lim, 2005]. Many flavonoid GT have been isolated from different sources and biochemically characterized. These enzymes act on different classes of flavonoids at different positions including the 3, and 7-OH groups. GT tend to be specific as to flavonoid class, sugar to be transferred, and position for substrates [Moraga et al, 2009; Patrik et al, 2003; Daniel et al, 2009; Goro et al, 2003], which give plants their characteristic feature [McIntosh, 1990; Heller and Forkmann, 1993].

Citrus is one of the most important fruit plant in the world for fruits and other products, such as juice, soap, cosmetics, etc. Citrus species are much interest because they accumulate large amounts of flavanone glycosides, whose aglycons are early intermediates in the flavonoid biosynthetic pathway [Castillo, 1991].

In this study were carried out cloning and characterization of their enzymatic activity of ten novel putative GT, *GT150*, *GT1669*, *GT259*, *GT285*, *GT173*, *GT2282*, *GT3363*, *GT3591*, *CiGT1*, and *CiGT2*, from *Citrus reticulata* cv. Siranui.

2. MATERIALS & METHODS

2.1. Plant materials

Citrus reticulata cv. Siranui [(*Citrus unshiu* X *Citrus sinensis*) X *Citrus reticulata*] was obtained from Citrus Research Station, National Institute of Horticultural & Herbal Science, Rural Development Administration, Jeju 697-943, Korea

2.2. Total RNA extraction

Four of citrus tissues were collected from the greenhouse in the Citrus Research Station, National Institute of Horticultural & Herbal Science and were kept on liquid nitrogen to carry them to the laboratory. Frozen citrus tissues were ground to a fine powder quickly and completely using a mortar and pestle with liquid nitrogen. Total RNAs were extracted from flowers, young fruits, stems, and leaves by using PureLink RNA mini kit (Invitrogen, USA). The total RNA quality and concentration were assessed using an Ultraspec 2100 Pro UV/Vis spectrophotometer (Amersham Biosciences, UK) and by formaldehyde denaturing agarose gel electrophoresis.

2.3. First-strand cDNA synthesis

First-strand cDNA from total RNA of Citrus leaves was synthesized according to the protocol of In-Fusion® SMARTer™ Directional cDNA Library Construction Kit (Clontech, USA): 2 µg of Total RNA sample, and 3' In-Fusion SMARTer CDS primer (3' CDS primer: 5'-CGGGGTACGATGAGACACCAAd(T)20VN-3' (N=A, G, C, or T; V=A, G, or C) were

incubated at 72°C for 3 min and 42°C for 2 min. After incubation for short time on ice, 5 X First-Strand Buffer, 0.1 M DTT, 10 mM dNTP Mix, SMARTer V Oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTGGTACXXXXX-3'), 10 units of RNase Inhibitor and 100 units of SMARTScript™ Reverse Transcriptase were added and incubated at 42°C for 1 hr 30 min and terminated at 68°C for 10 min. The first-strand reaction product was diluted to 15 µl with TE buffer (10 mM Tris [pH 8.0], 0.1 mM EDTA).

2.4. Primer design

Among 150 GT sequences of the *C. platymamma* Hort. ex. Tanaka (Byungkyool) genomic DNA library data in our laboratory, eight gene sequences were chosen based on the highest conserved region within the PSPG (Plant Secondary Product Glucosyltransferase) box. *CiGT1* and *CiGT2* gene sequences were predicted on the basis of EST sequences of NCBI and Citrus Genetic Resources Bank, Korea.

Primer sequences used for amplifying target gene were as follows Table 1.

Table 1. The primers of ten glucosyltransferases

Primer name	Sequence
GT150- <i>Bam</i> HI-5	GCG <u>CGG ATC CAT</u> GGA AGA CAA GAA ACT TCA CAT A
GT150- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TCA CAT GAG ATC AGC CAG GCT ATG
GT1669- <i>Bam</i> HI-5	GCG <u>CGG ATC CAT</u> GGC TCA CGG CCA CAT GAT TCC A
GT1669- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TTA GCT GCT TTG ATG GCG ACT TAA
GT259- <i>Eco</i> RI-5	CCG <u>GGA ATT CAT</u> GTC GAG CTC TAA CAC CAG AAC C
GT259- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TCA TTT CGC ATG AAG ATT TCT CAA
GT285- <i>Bam</i> HI-5	GCG <u>CGG ATC CAT</u> GCC GCC GGC CGG CGC TCA TAT T
GT285- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TCA AAC TGC TGT CAA ATT TTT TCC
GT173- <i>Bam</i> HI-5	GCG <u>CGG ATC CAT</u> GTC AGA AGC AGC CGG AAG CAC C
GT173- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TCA AGT CCT GTT GAC AAC TTC AAC
GT2282- <i>Bam</i> HI-5	GCG <u>CGG ATC CAT</u> GGC ATC CGA AGC CAG CCA GTC A
GT2282- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TCA CTT CTG AGG TTG GCC ATG GAA
GT3363- <i>Bam</i> HI-5	GCG <u>CGG ATC CAT</u> GGC TTC TGA AGG GAG ATG CCA G
GT3363- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TCA GGC GTT ATC TAT ATG TTG GTG
GT3591- <i>Bam</i> HI-5	GCG <u>CGG ATC CAT</u> GGC ATC CGA AGC CAG CGA GTT T
GT3591- <i>Xho</i> I-3	CGC CGC <u>CTC GAG</u> TTA CAT CAC TTC TGA GGT CGG CTG
CiGT1- <i>Eco</i> RI-5	<u>CGG AAT TCA TGG</u> AGA AGC AGA TCA TGT TC
CiGT1- <i>Xho</i> I-3	<u>CCC TCG AGT CAG</u> CAA TTT TGT GGT TGT GAG C
CiGT2- <i>Bam</i> HI-5	<u>CGG GAT CCA TGG</u> AGC AGC AAC AGC AAC CC
CiGT2- <i>Xho</i> I-3	<u>CCC TCG AGT TAG</u> GGT CGT TTT TCT CTA CT

Each underline was indicated enzyme site

2.5. Plasmid cloning and nucleotide sequence analysis of GT gene

PCR was performed with 1 μ l first-strand cDNA and *npfu*-special DNA polymerase (Enzynomics, Korea) under the following condition : 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 1 min, and 72°C 1 min 30 sec and finally, 72°C for 10 min. 5 μ l of each PCR products were separated on 1.0% agarose gel and stained with Safeview (Applied Biological Materials, Canada). The PCR products were extracted from agarose gel using GeneAll^R ExpinTM combo GP (Geneall, Korea).

The *CiGT1* and *GT259* were digested using *EcoRI* and *XhoI* enzyme (Takara, Japan), and rest of the GT were digested using *BamHI* and *XhoI* enzyme, respectively and incubated at 37°C for 2 h followed by purification with GeneAll^R ExpinTM combo GP (Geneall, Korea). The purified DNA was finally dissolved in 30 μ l of deionized H₂O. 2 μ l it was run on 1.0% agarose/EtBr gel alongside 5 μ l of 1 Kb⁺ DNA size marker (Enzynomics, Korea) at 100 V for 40 min.

The pGEX 4T-1 and pGEX 4T-3 (GE Healthcare, UK), a glutathione S-transferase (GST) gene expression system vector, were digested with *EcoRI* and *XhoI*, *BamHI* and *XhoI*, respectively. And they were ligated with digested DNA fragments at a ratio of 5:1 using DNA Ligation Kit <Mighty Mix> (Takara, Japan). The *GT1* and *GT259* PCR products were ligated into the pGEX 4T-1 plasmid. And the rest of GT PCR products were ligated into the pGEX 4T-3 plasmid. All ligation tubes were incubated at 16°C overnight. The recombinant plasmids were transformed into *E. coli* DH5 α cells by heat shock, and an aliquot was spread on a pre-warmed 90 mm LB agar plate containing 100 μ g/mL of ampicillin. The plates were incubated at 37°C overnight [Ko, 2008; Miller et al., 1999; Lin et al., 2006]. The inserted fragment sizes of the positive recombinants were analyzed by PCR amplification using the vector-specific, pGEX 5 and pGEX 3, primers. The PCR products were resolved by agarose gel electrophoresis to determine the size of each product. Sequence analysis was performed

by using an ABI 3130 XL Genetic analyzer (Applied Biosystems, Foster, USA). The obtained sequences were compared with sequence database using BLASTN and annotated on the basis of the existing annotation of non-redundant databases at the NCBI. Sequence analyses of the GT genes were performed by using GENETYX-WIN Software Ver. 5.0 (Genetyx, Tokyo, Japan) and a ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2>).

2.6. Comparison of the amino acid sequence and phylogenetic analysis

Multiple alignments of ten GT amino acid sequences were made using the program BioEdit software (Ver. 7.0.9.0, <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The phylogenetic tree of GT genes were constructed using the neighbor-joining methods [Saitou and Nei, 1987] in Molecular Evolutionary Genetics Analysis (MEGA) software (ver. 4.0, <http://www.megasoftware.net/index.html>) [Tamura et al., 2007].

2.7. Expression and biotransformation of glucosyltransferases

Full-length cDNAs encoding ten GT genes were cloned into bacterial GST expression vector pGEX4T-1 and pGEX4T-3 (Amersham Biosciences/GE Healthcare, UK) to create in frame fusions at the 5' terminus with the GST coding sequence. Inserted genes were sequenced to confirm their gene sequence and correct reading frame. To express fusion protein, BL21 (DE3) *E. coli* cells were used. Transformants were selected on LB containing ampicillin (AMP) plates and were grown overnight in 5 ml of LB medium at 37°C and 2 ml of the cultured cells were used to inoculate 20 ml of LB-AMP fresh medium. The culture was grown until absorbance at 600 nm reached 0.6~0.7. At this point, IPTG (isopropyl-1-

thio- β -D-galactopyranoside) was added at a final concentration of 0.1mM, and the transformants was grown for 5 hours at 25°C. The cells were harvested and washed with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 2 mM KH₂PO₄, pH 7.4), resuspended in the lysis buffer (1X PBS, 1mM EDTA, 1mM DTT, 1mM PMSF, 1% TritonX-100) and lysed by sonication (5 sec pulse, 30 sec resting for 1 min, on ice) [Kim et al., 2006B; Noguchi et al., 2008, Hong et al., 2007]. After sonication, the samples were centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant and pellet were analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) for solubility of the fusion protein.

For the biotransformation assays, the cells were harvested and resuspended in half volume of 50 mM potassium phosphate buffer (pH 7.0) containing 1 % glucose. Substrates (Quercetin and Naringenin) were added 30 μ M at each culture and the sample was incubated at 28°C for 12 hr. The supernatant was collected and was extracted twice with ethyl acetate. Then, the ethyl acetate extract was evaporated completely and the pellet was dissolved in methanol [Kim, 2005; Kim, 2007; Kim, 2006C; Lin, 2006].

2.8. Analysis of flavonoids with high performance liquid chromatography (HPLC)

The reaction products were analyzed using a HPLC (Ultimate 3000, dionex, Idstein, Germany) equipped with a photo diode array (PDA) detector and a Luna C18(2) column (5 μ m particle size, 4.6 mm X 250 mm, Phenomenex, Torrance, CA, USA). For analytical scale, the mobile phase consisted of 50 mM potassium phosphate buffer (pH 3.0) and was programmed as follows: 20 % acetonitrile at 0 min, 40 % acetonitrile at 10 min, 70 % acetonitrile at 20 min, 70 % acetonitrile at 25 min, 100 % D.W at 30 min, 100 % D.W at 35 min, 20 % acetonitrile at 40 min. The flow rate was 1 ml/min and UV detection was performed at 270 nm.

3. Results

3.1. Total RNA extraction of GT

Total RNAs were extracted from flowers, young fruits, stems, and leaves as previously described. The concentration and purity of the total RNA was determined by measuring the absorbance at 260nm (A_{260}) and 280nm (A_{280}) in a spectrophotometer (Table 2). Integrity was determined running the total RNA (3 μ g) in a formaldehyde gel stained with ethidium bromide. Formaldehyde gel electrophoresis of total RNA showed Figure 3.

Table 2. Absorbance measurement of total RNA samples.

	Concentration (μ g/ μ l)	260/280 (O/D)
Flower	1.005	2.01
Leaf	2.647	1.96
Stem	0.401	2.01
Fruit	2.46	2.01

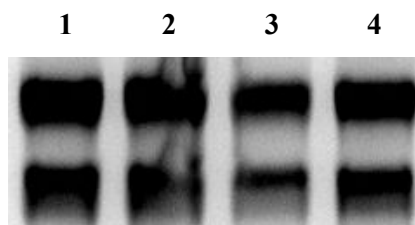


Figure 3. Formaldehyde gel electrophoresis of *C. reticulata* total RNA. Total RNA isolated from flower, leaf, stem and fruit using PureLink RNA mini kit (Invitrogen).

lane 1, Flower; lane 2, Leaf; lane 3, Stem; lane 4, Fruit.

3.2. cDNA cloning and phylogenetic analysis of GT

cDNAs were synthesized from the total RNA of citrus leaf and PCR was carried out with cDNA as template and specific primers. PCR products were separated on agarose gel. DNA band of expected size was observed in the gel after staining with ethidium bromide (Figure 4). DNA in the band was extracted from the gel and ligated into the pGEX expression vector. Colony containing target gene was identified by colony PCR with pGEX primers. Purified plasmids were used for analysis of gene sequence.

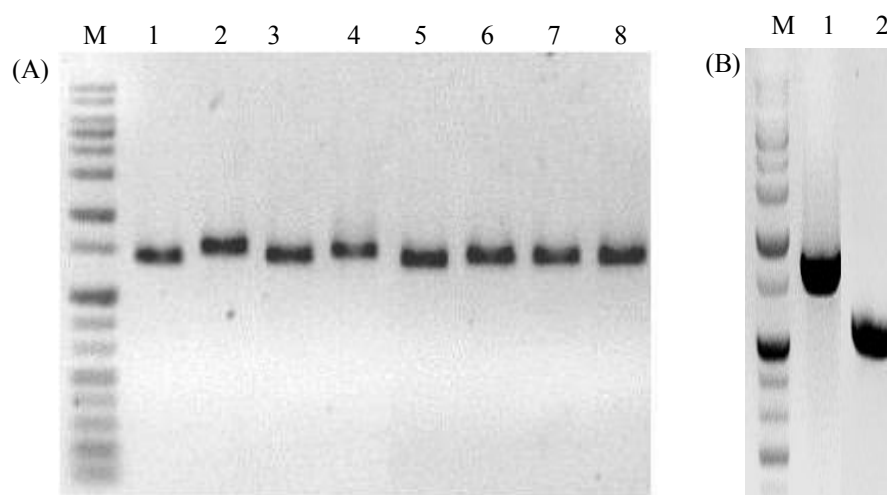


Figure 4. The results of agarose gel electrophoresis of PCR products. (A) lane 1; *GT150*, lane 2; *GT1669*, lane 3; *GT259*, lane 4; *GT285*, lane 5; *GT173*, lane 6; *GT2282*, lane 7; *GT3363*, and lane 8; *GT3591* (B) lane 1; *CiGT1* and lane 2; *CiGT2*.

Each of the genes concerning nucleic acid and amino acid were described table 3 and Supplementary data

Table 3. Each GT gene of nucleic acid and amino acid size, respectively.

Gene name	nucleic acid	amino acid
GT150	1377bp	458aa
GT1669	1428bp	475aa
GT259	1410bp	469aa
GT285	1422bp	473aa
GT173	1365bp	454aa
GT2282	1467bp	488aa
GT3363	1491bp	496aa
GT3591	1473bp	490aa
CiGT1	1431bp	477aa
CiGT2	951bp	317aa

Figure 5 shows the Clustal W alignment of the deduced amino acid sequences of glucosyltransferase family. Among the sequences, the highest amino acid sequence similarity (80%) was observed between *GT2282* and *GT3591*. The greatest level of dissimilarity (13%) was observed between *GT150* and *CiGT2*.

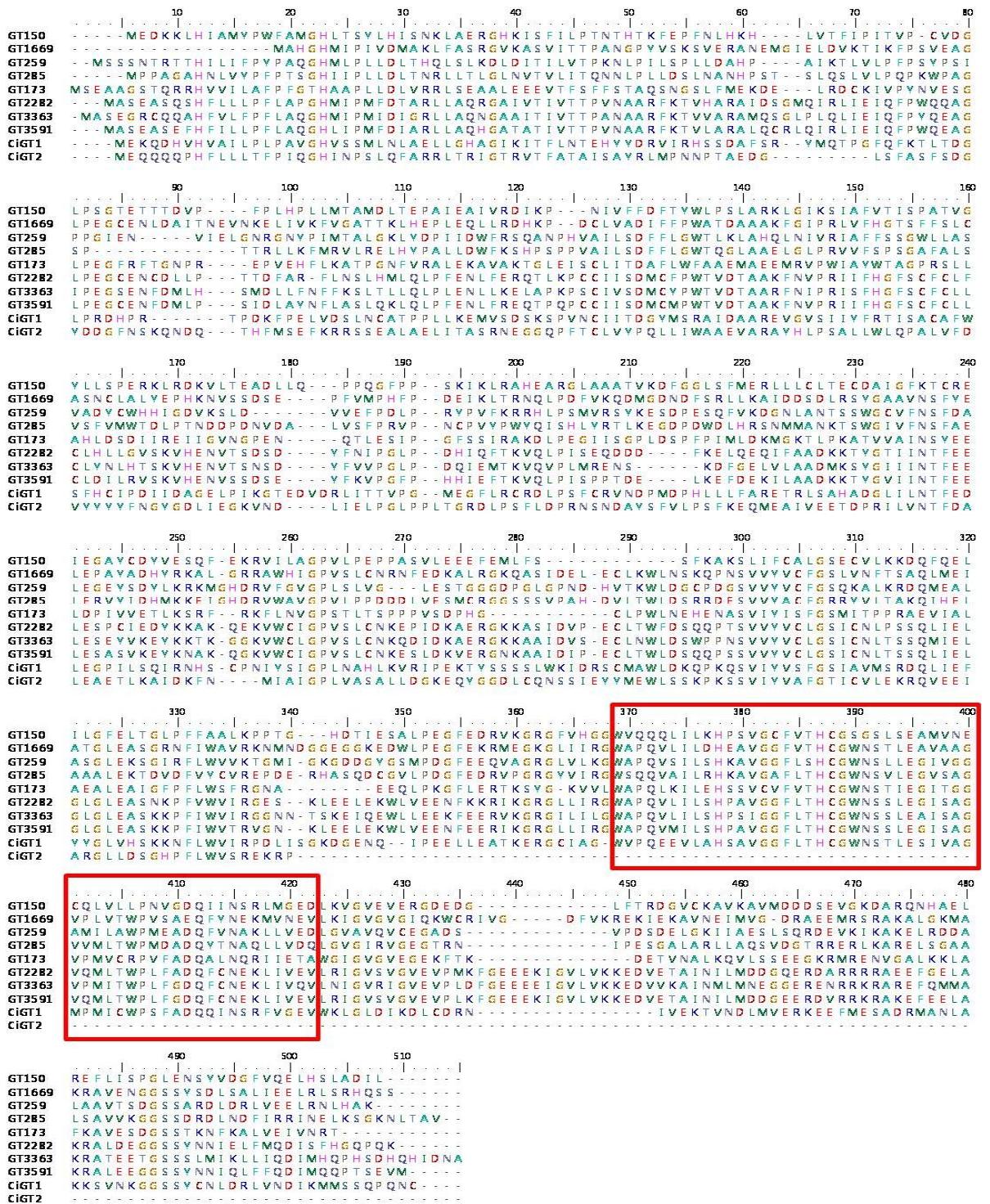


Figure 5. Alignments of the deduced amino acid sequences of *GT* genes isolated from Citrus. The box indicates the conserved region among plant secondary product glucosyltransferase (PSPG) box.

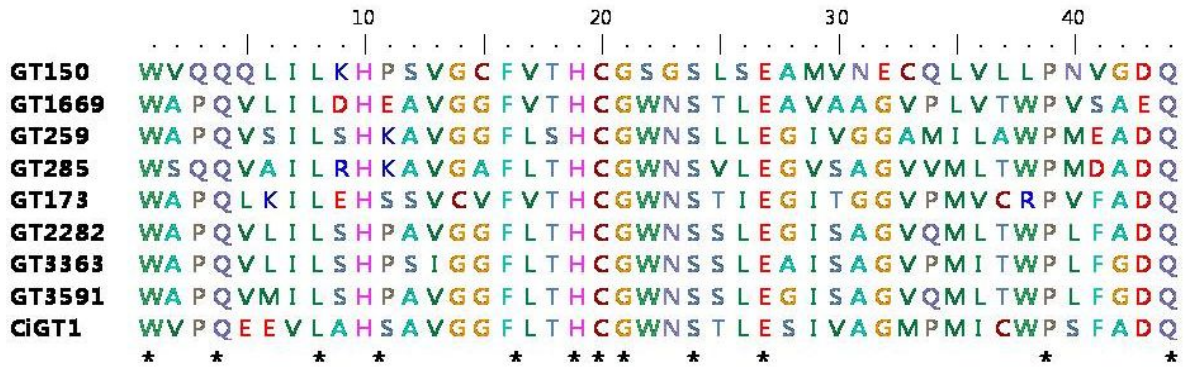


Figure 6. PSPG-box consensus sequence of Citrus glucosyltransferases. Highly conserved amino acids were indicated star mark.

PSPG-box of the derived amino acid sequences of the nine putative glucosyltransferase clones from *C. reticulata* cv. Siranui were shown in Figure 6. The *CiGT2* did not have PSPG box.

The phylogenetic tree of nine Citrus GT based on PSPG box is shown in Figure 7. The minimum value of similarity between group I and group II was about 59%, group II and group III was about 45%, and group I and III was about 43%. In Group I, the level of similarity between *CiGT1* and *GT173*, except *CiGT2* that did not contain PSPG box, was over 61%. A higher degree of similarity of group II was 65% and low degree of similarity of group II was 40%. A level of similarity in group III was range from 65% to 86%.

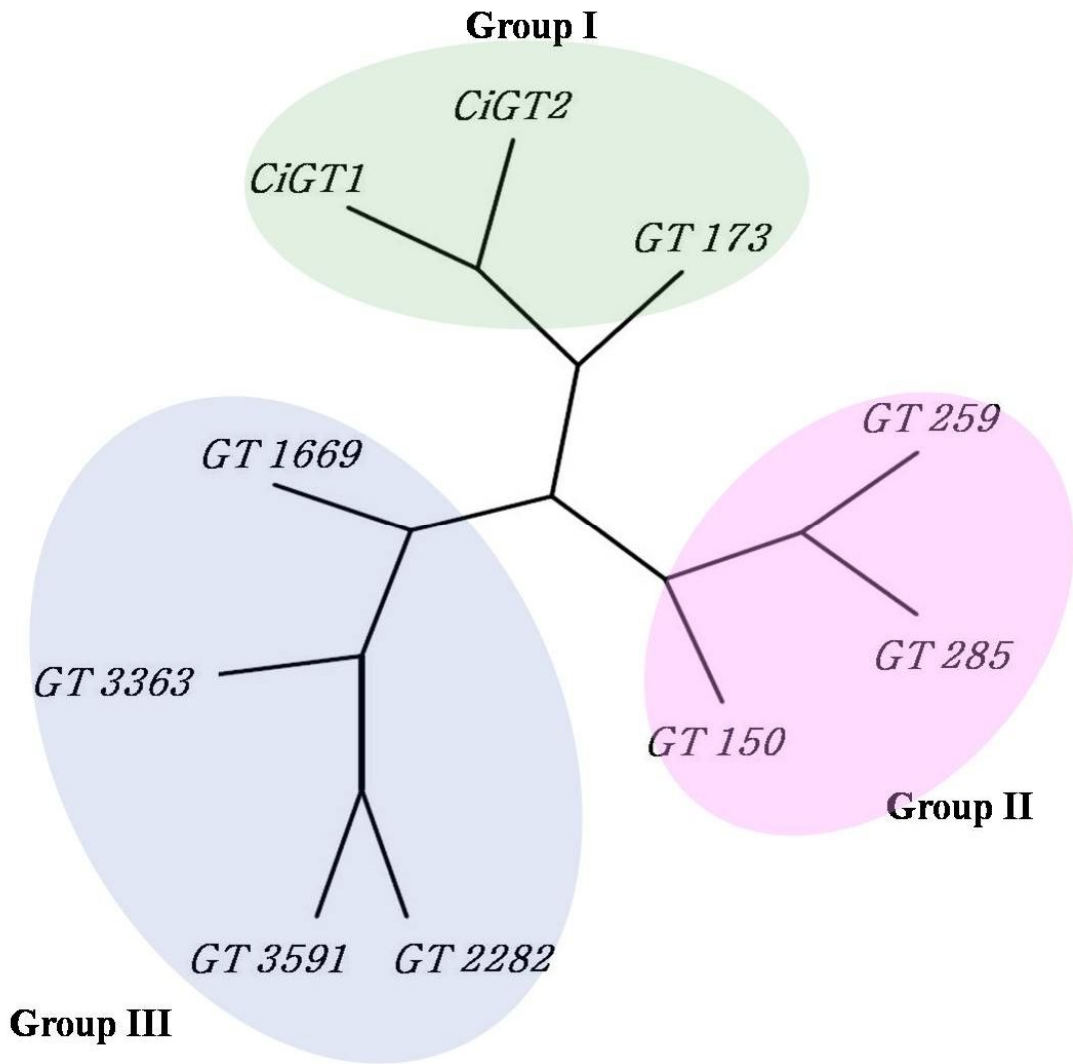


Figure 7. Phylogenetic analysis of family *GT*

3.3. Expression of the GST-GT fusion protein

E. coli (DE3) rosetta2 cells were transformed with pGEX 4T-1/*CiGT1* plasmid and induced with IPTG. The cells produced a protein of around 78kDa (GST-*CiGT1* fusion protein) that was absent in non-induced cells. Expression of *CiGT1* fusion protein was induced at different IPTG concentration and 0.1mM IPTG concentration was the best concentration (Figure 8). As shown in Figure 9, fusion protein expression was tested at different temperature and time. When induced at 25°C for 5 h, the soluble protein reached the highest level.

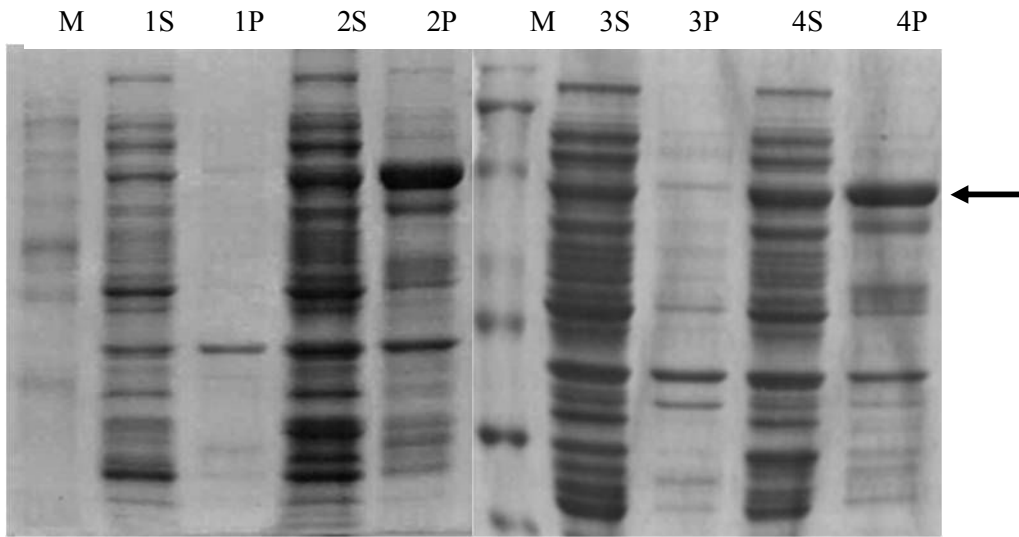


Figure 8. Expression of *CiGT1* fusion protein at different IPTG concentration. M, Protein Marker; 1, Uninduced lysate; 2, Induced lysate, 0.1mM IPTG. 3, Uninduced lysate; 4, Induced lysate, 1mM IPTG. S : Supernatnat, P : Pellet. The arrow indicates the expected protein band in 78kDa

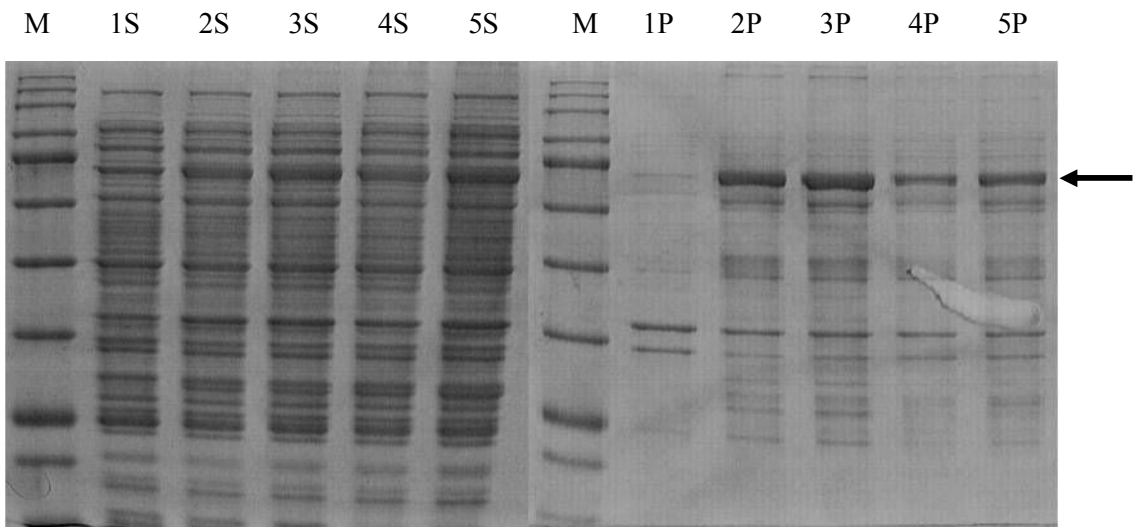


Figure 9. Expression of *CiGT1* fusion protein at different temperature and induction time. M, protein marker; lane 2-8, Uninduced *E.coli* lysate ; lane 3-9, Induced at 25°C for 4hr; lane 4-10; Induced at 25°C for 5hr ; lane 5-11, Induced at 18°C for 10hr; lane 6-12, Induced at 18°C for 12hr ; S : Supernatnat, P : Pellet. The arrow indicates the expected protein band in

78kDa

GT150, *1669*, *285*, *173*, *2282*, *3363*, *3591*, and *CiGT2* were inserted into pGEX 4T-3 expression vector for protein expression and *GT259*, *CiGT1* were inserted into pGEX 4T-1. Recombinant protein expression resulted in the production of fusion proteins of the mass described below (Table 4).

Table 4. Predicted recombinant proteins weight of GT genes

Gene name	protein mass	molecular weight
<i>GT150</i>	50.3kDa	76kDa
<i>GT1669</i>	52.2kDa	78kDa
<i>GT259</i>	51.5kDa	77kDa
<i>GT285</i>	52kDa	78kDa
<i>GT173</i>	49.9kDa	75kDa
<i>GT2282</i>	53.6kDa	79kDa
<i>GT3363</i>	54.5kDa	80kDa
<i>GT3591</i>	59.1kDa	84kDa
<i>GT1</i>	52.4kDa	78kDa
<i>GT2</i>	34.8kDa	60kDa

3.4. Biotransformation of naringenin and quercetin with GT

The *E. coli* transformants expressing GT were grown and incubated with each of naringenin and quercetin, as substrates in order to investigate the substrate specificity of the enzyme. Flavonoid compounds used in this experiment could enter into *E. coli* and then, be modified by the expressed protein. Figure 10 showed the protein expression pattern. The box indicated *GT285* and the arrow indicated expected protein band

GT285 was tested with naringenin and quercetin as substrates (Figure 11). The cells expressing *GT285* produced new products from both of naringenin and quercetin. New products were determined as naringenin 7-O-glucoside and quercetin 3-O-glucoside, respectively. *GT285* transformed all of the quercetin used as substrate to quercetin 3-O-glucoside and showed about half activity for naringenin, compared to quercetin. This result indicated that *GT285* more effectively transferred a glucose molecule to the 3-hydroxyl group than 7-hydroxy group.

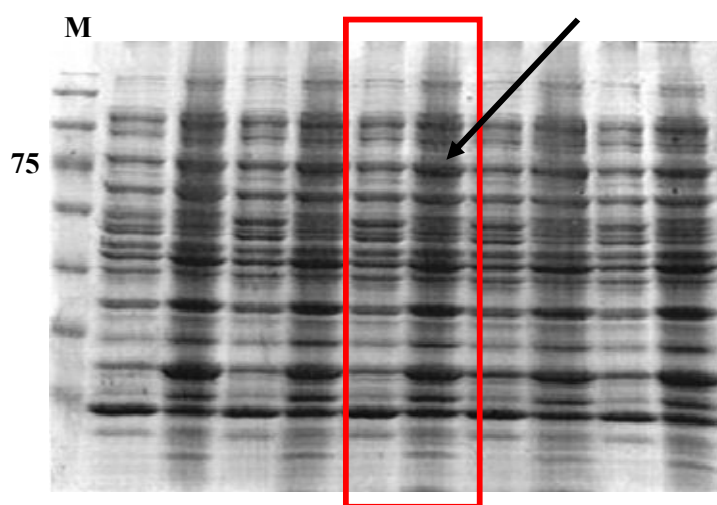


Figure 10. Expression of *GT285* recombinant protein. M, Molecular weight marker. The box indicated *GT285* and the arrow indicates expected protein band

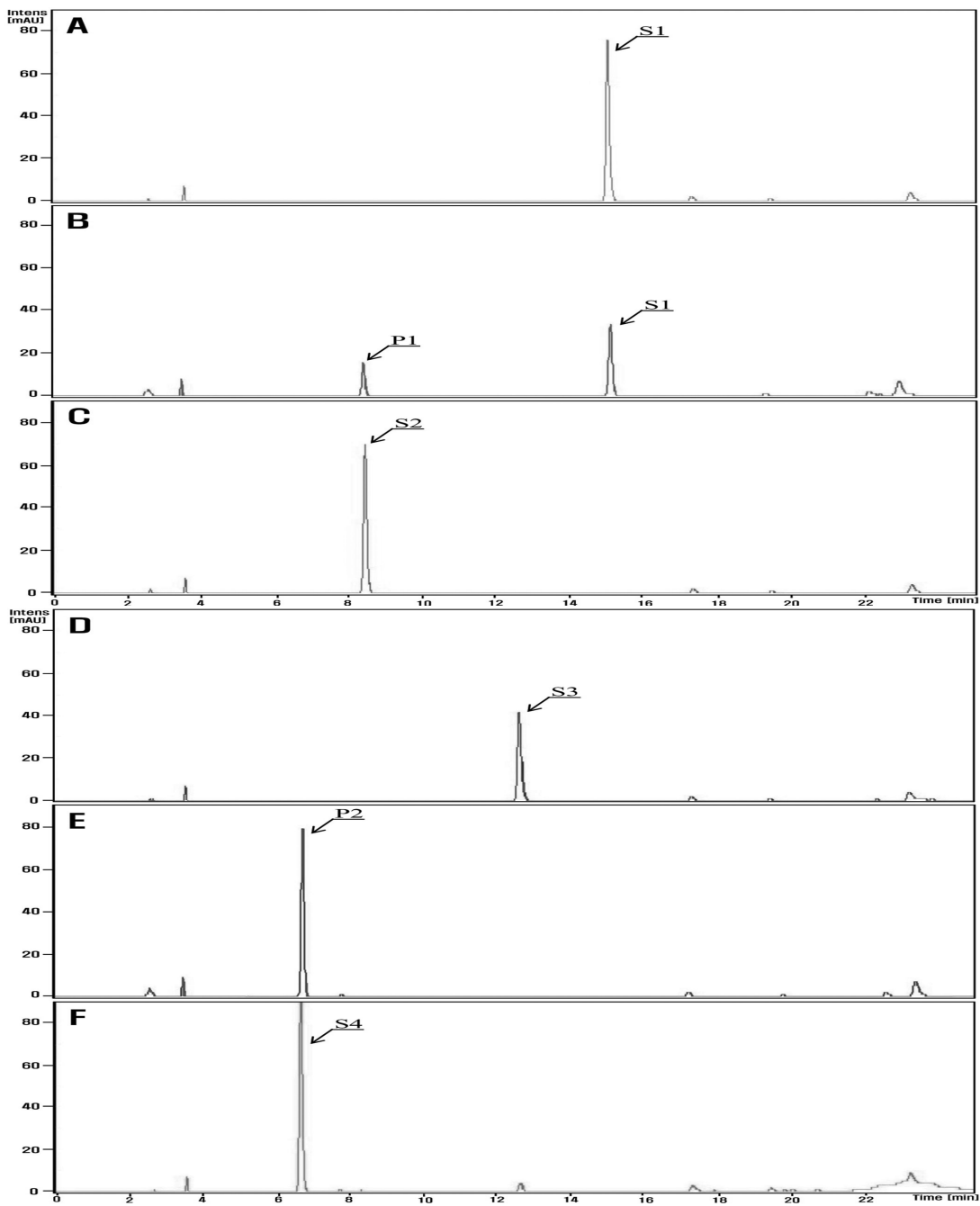


Figure 11. HPLC analysis of *GT285* with naringenin and quercetin (A) HPLC analysis of standard of naringenin (S1), (B) Reaction product of naringenin (P1), (C) Standard of naringenin 7-O-glucoside (S2), (D) Standard of quercetin (S3), (E) Reaction product of quercetin (P2), (F) Standard of quercetin 3-O-glucoside (S4)

In case of *GT173*, this protein showed sufficient transformation activity on quercetin, but only a detectable activity on naringenin (Figure 13). This result indicated that *GT173* are more specific for quercetin than naringenin, compared to *GT285*.

Figure 12 showed the protein expression pattern. The box indicated *GT173* and the arrow indicated expected protein band

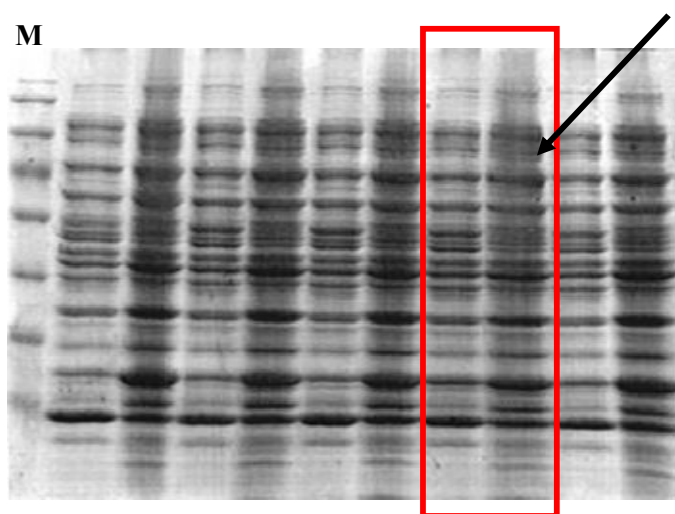


Figure 12. Expression of *GT173* recombinant protein. M, Molecular weight marker. The box indicated *GT173* and the arrow indicated expected protein band

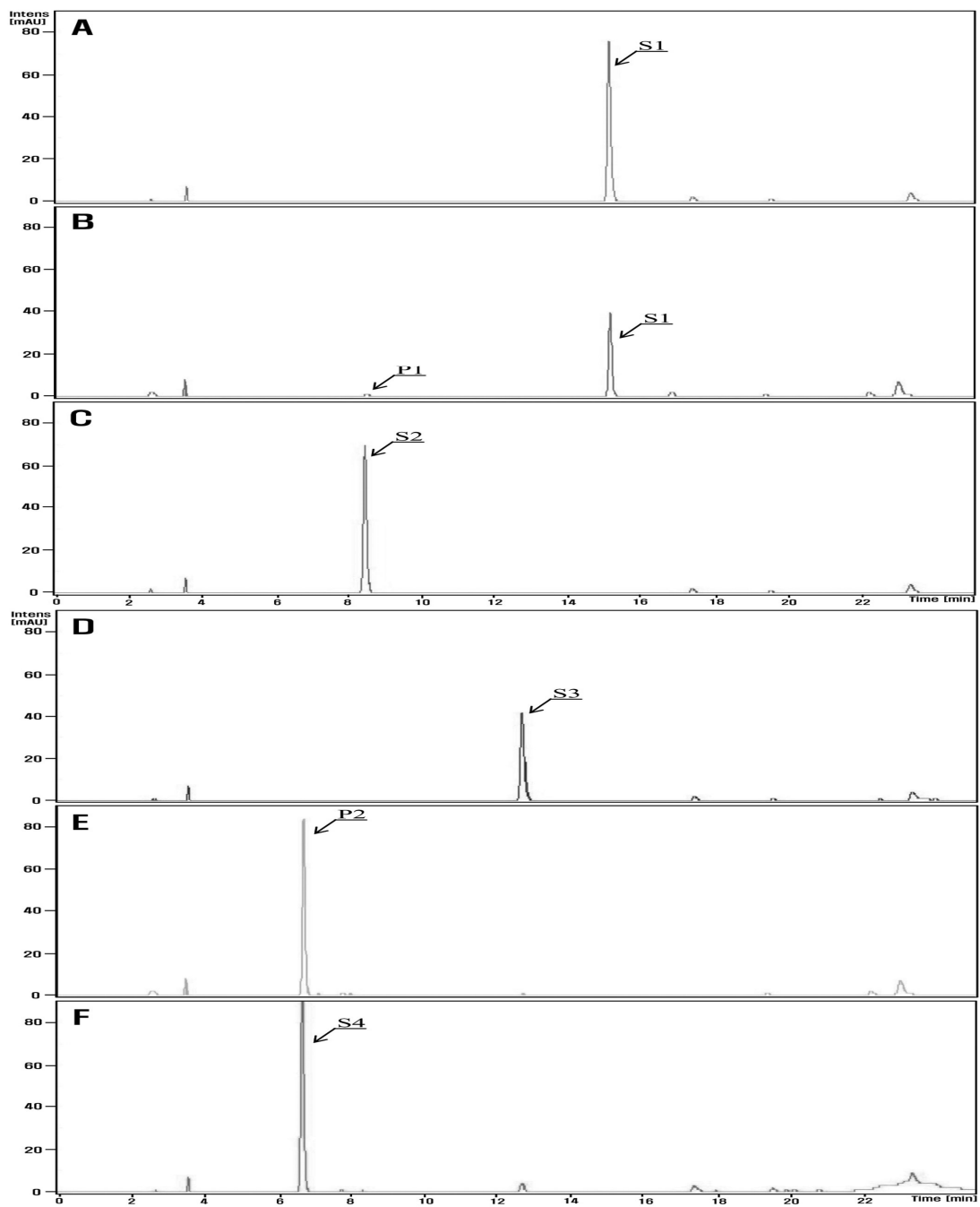


Figure 13. HPLC analysis of *GT173* with naringenin and quercetin (A) HPLC analysis of standard of naringenin (S1), (B) Reaction product of naringenin (P1), (C) Standard of naringenin 7-O-glucoside (S2), (D) Standard of quercetin (S3), (E) Reaction product of quercetin (P2), (F) Standard of quercetin 3-O-glucoside (S4)

GT3363 showed almost equally sufficient activity on both naringenin and quercetin (Figure 15) demonstrating this protein was related to produce naringenin 7-O-glucoside and quercetin 3-O-glucoside. Figure 14 showed the protein expression pattern. The box indicated *GT3363* and the arrow indicated expected protein band

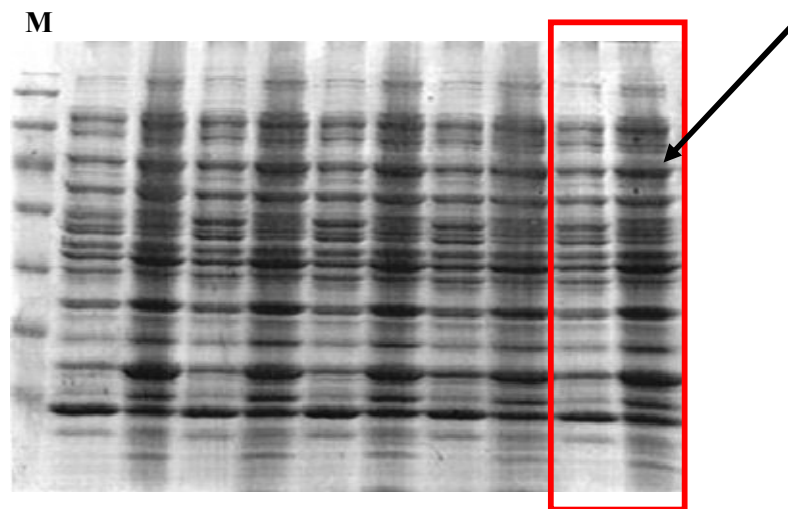


Figure 14. Expression of *GT3363* recombinant protein. M, Molecular weight marker. The box indicated *GT3363* and the arrow indicated expected protein band

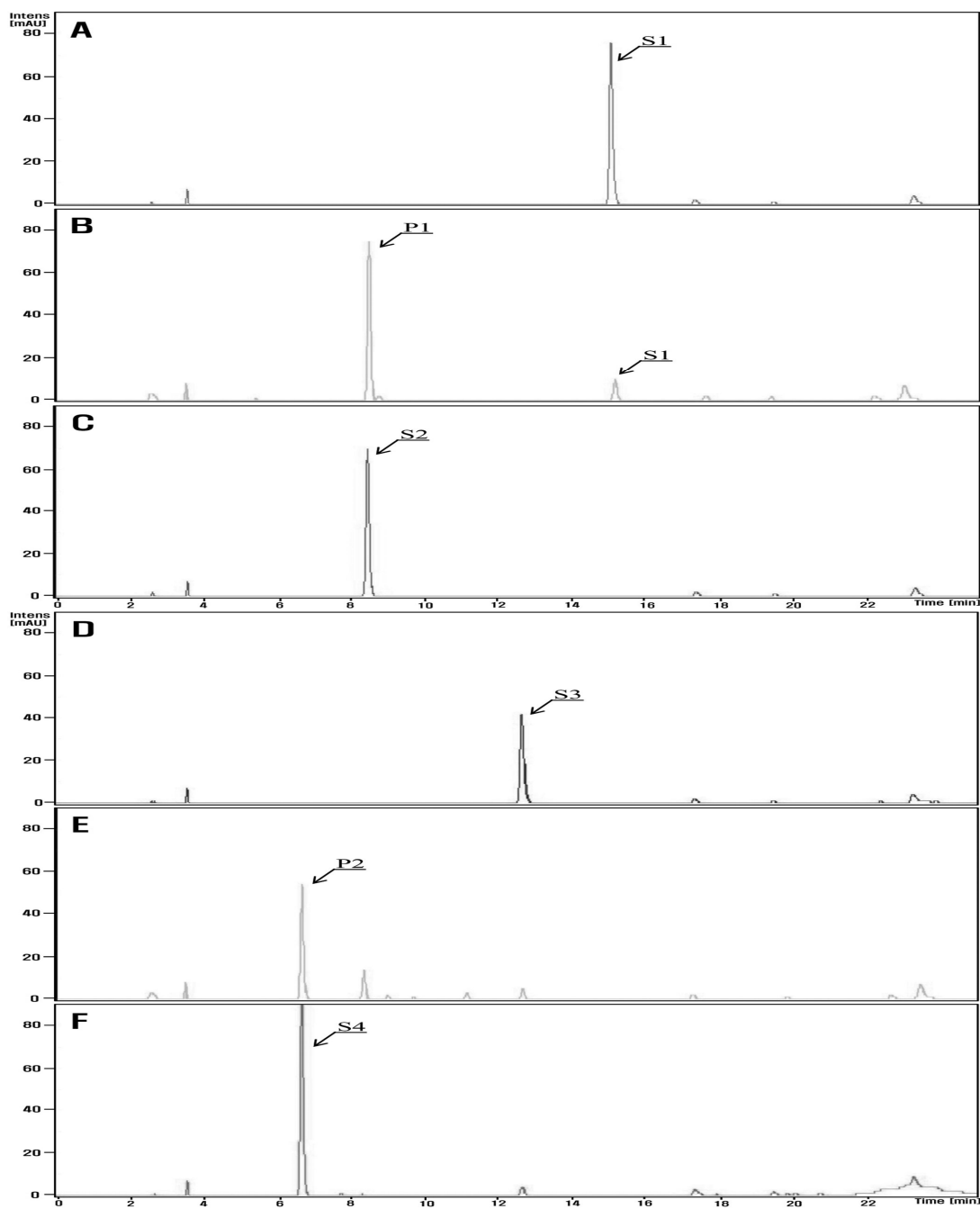


Figure 15. HPLC analysis of *GT3363* with naringenin and quercetin (A) HPLC analysis of standard of naringenin (S1), (B) Reaction product of naringenin (P1), (C) Standard of naringenin 7-O-glucoside (S2), (D) Standard of quercetin (S3), (E) Reaction product of quercetin (P2), (F) Standard of quercetin 3-O-glucoside (S4)

In case of *GT2282*, reaction product showed only a detectable activity on naringenin, but not quercetin. It is possible for this enzyme to have the other substrates modified. Further study needs to identify the correct substrate of this enzyme. Figure 16 showed the protein expression pattern. The box indicated *GT2282* and the arrow indicated expected protein band

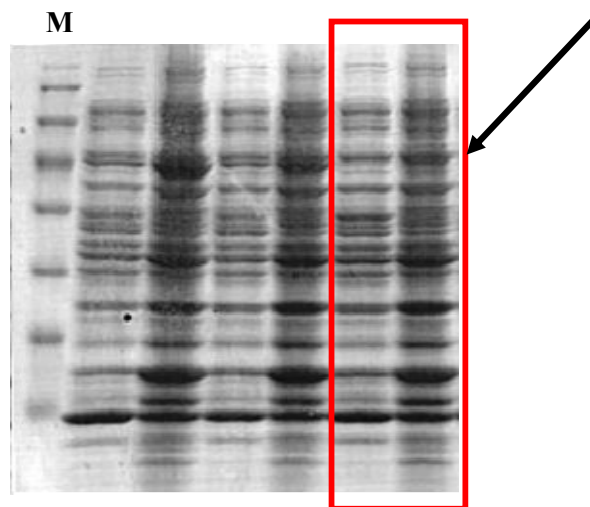


Figure 16. Expression of *GT2282* recombinant protein. M, Molecular weight marker. The box indicated *GT2282* and the arrow indicated expected protein band

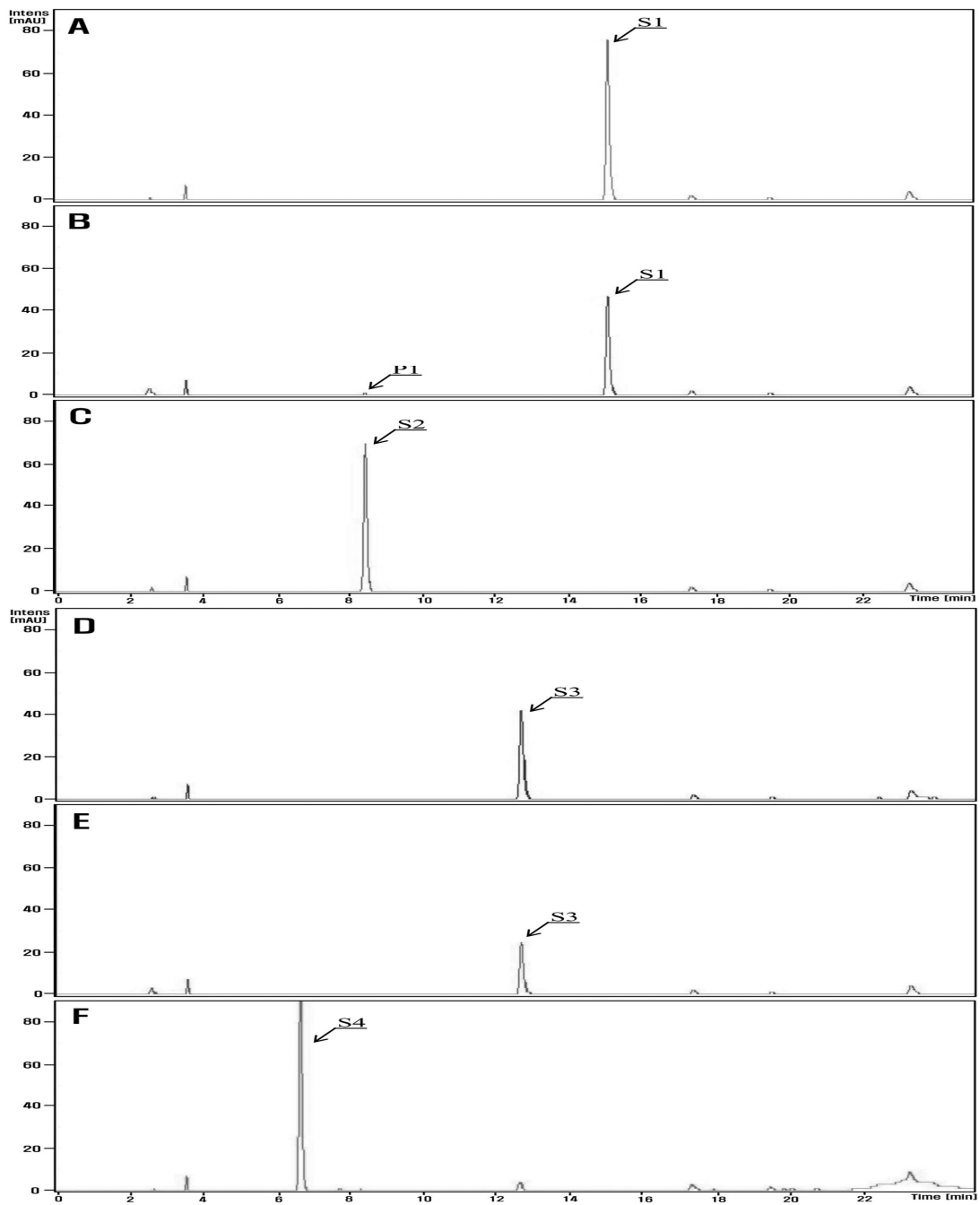


Figure 17. HPLC analysis of *GT2282* with naringenin and quercetin (A) HPLC analysis of standard of naringenin (S1), (B) Reaction product of naringenin (P1), (C) Standard of naringenin 7-O-glucoside (S2), (D) Standard of quercetin (S3), (E) Reaction product of quercetin , (F) Standard of quercetin 3-O-glucoside (S4)

Similar to *GT2282* and *GT3591* were only determined to glucosylate naringenin, producing its 7-O-glucoside. In fact, *GT2282* and *GT3591* were classified as the same group (Group III, Figure 6). These two genes were showed as very close genes because they have about 80% similarities between amino acid sequences in full-length and above 95% similarities in PSPG box region. Figure 18 showed the protein expression pattern. The box indicated *GT3591* and the arrow indicated expected protein band

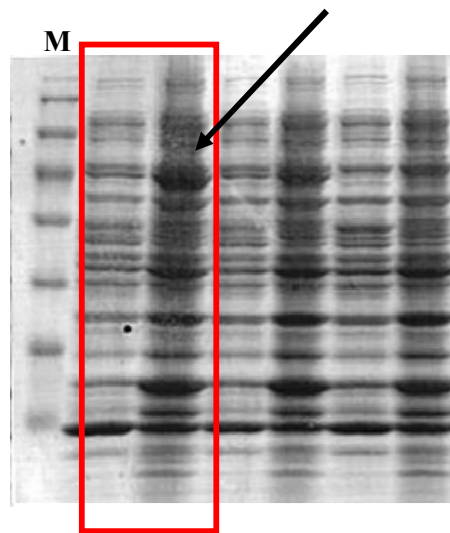


Figure 18. Expression of *GT3591* recombinant protein. M, Molecular weight marker. The box indicated *GT3591* and the arrow indicated expected protein band

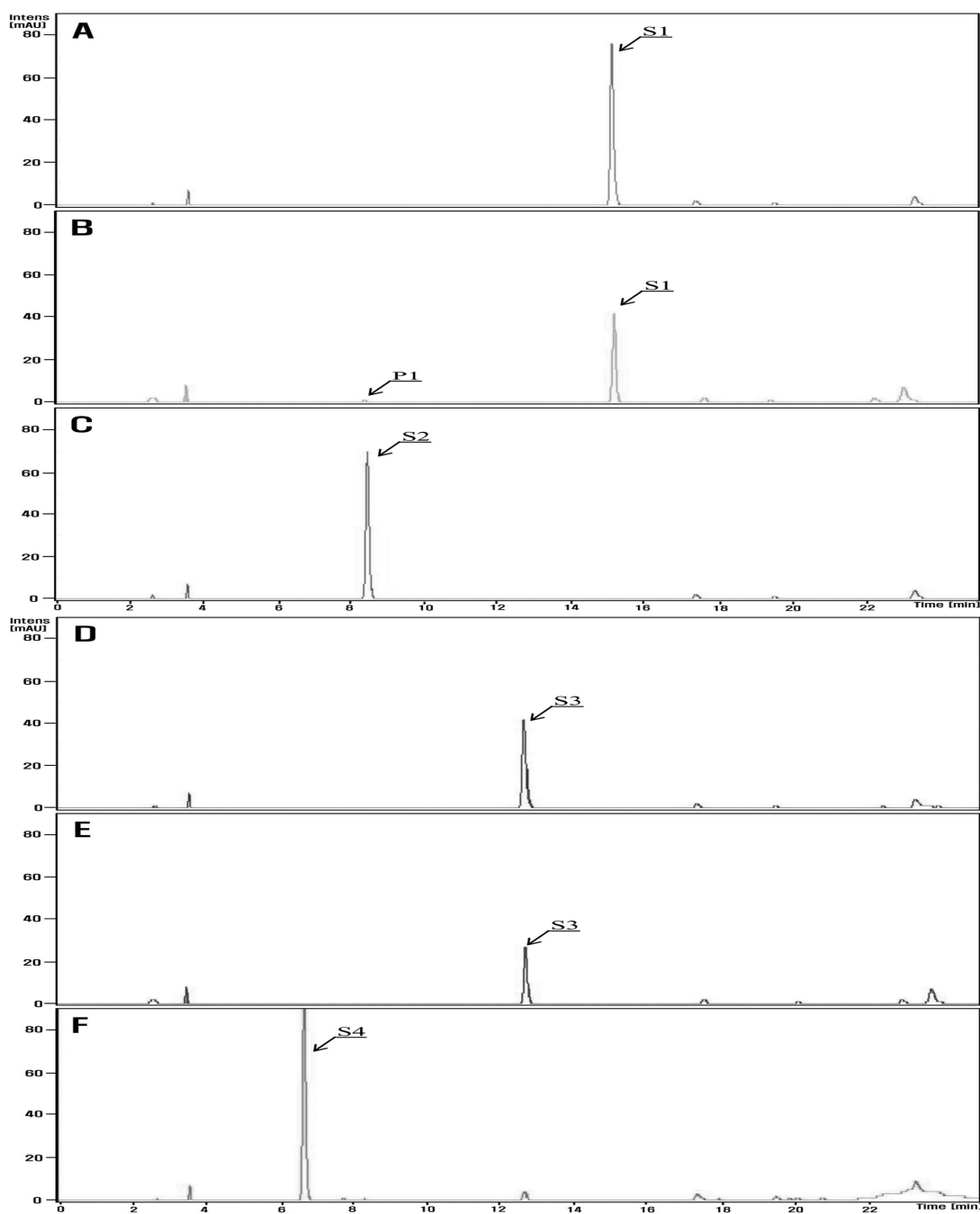


Figure 19. HPLC analysis of *GT3591* with naringenin and quercetin (A) HPLC analysis of standard of naringenin (S1), (B) Reaction product of naringenin (P1), (C) Standard of naringenin 7-O-glucoside (S2), (D) Standard of quercetin (S3), (E) Reaction product of quercetin , (F) Standard of quercetin 3-O-glucoside (S4)

Amino acid homology between *GT259* and *GT285* were about 65% in PSPG box (Group II) and about 38% in full length sequence. Different from the *GT285* that showed activities on both substrate, however *GT259* was only determined to glucosylate quercetin. But interestingly, *GT259* was not modification 3-O-site, suggesting the other site could be glucosylated (Figure 21). These results suggest that these two gene product share some common substrates, but that have different preference on modification site. Figure 20 showed the protein expression pattern. The box indicated *GT259* and the arrow indicated expected protein band

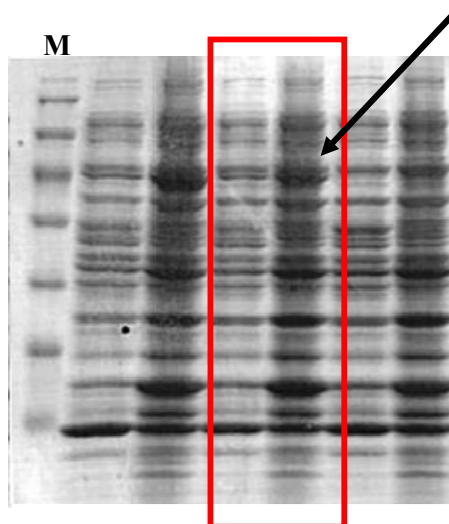


Figure 20. Expression of *GT259* recombinant protein. M, Molecular weight marker. The box indicated *GT259* and the arrow indicated expected protein band

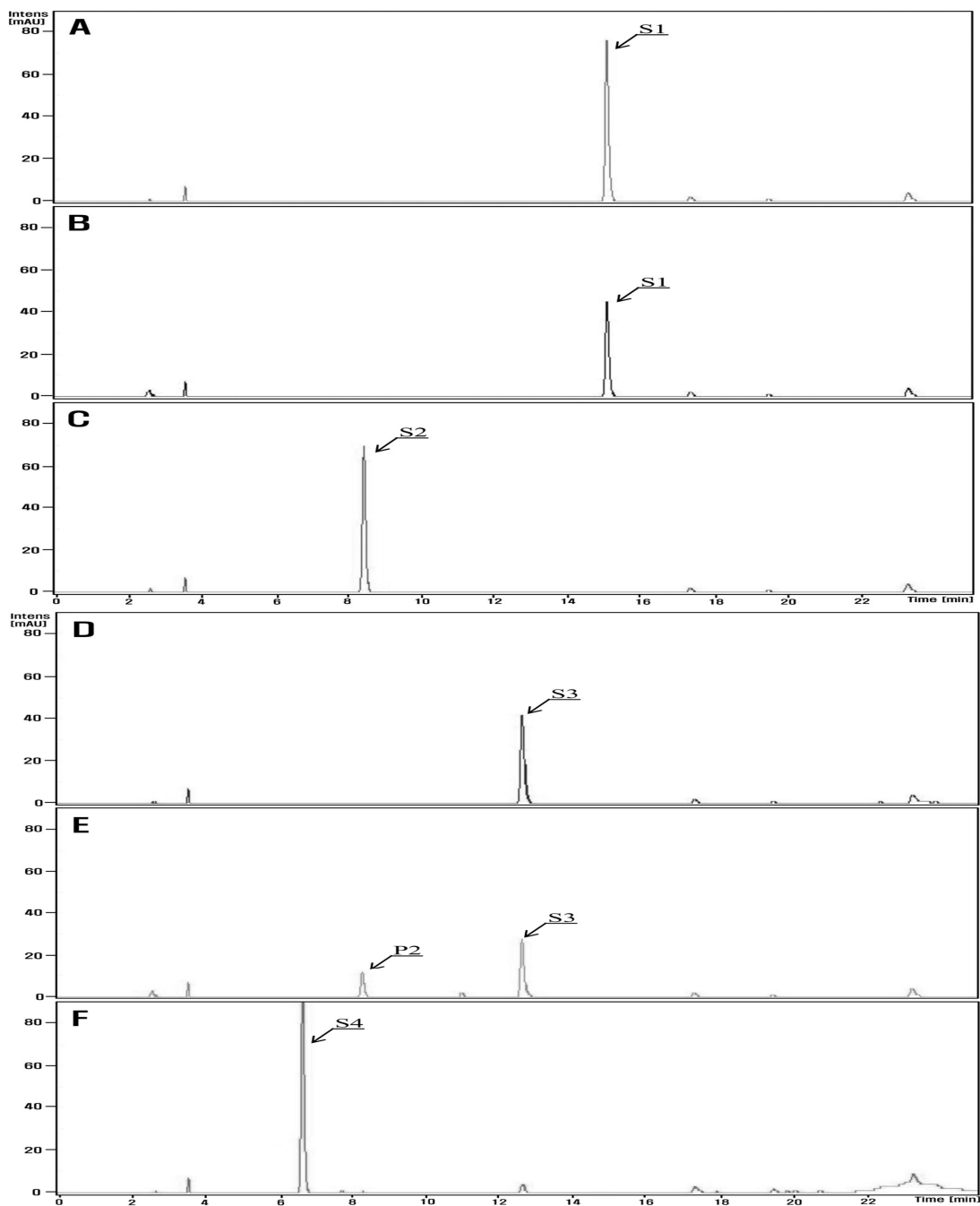


Figure 21. HPLC analysis of *GT259* with naringenin and quercetin (A) HPLC analysis of standard of naringenin (S1), (B) Reaction product of naringenin, (C) Standard of naringenin 7-O-glucoside (S2), (D) Standard of quercetin (S3), (E) Reaction product of quercetin (P2), (F) Standard of quercetin 3-O-glucoside (S4)

CiGT1, *CiGT2*, *GT150*, and *GT1669* were tested with naringenin and quercetin as substrates. These four gene product did not show any activities on both naringenin and quercetin (data not shown). *CiGT1*, *CiGT2*, and *GT150* were involved in Group I, suggesting this group may have the other substrate in plant. In this study, did not clearly confirm the extent of protein expression and protein solubility. Further study needs to identify real substrates for these genes.

4. CONCLUSION

In this study, a novel ten full-length glucosyltransferase (GT) genes, *GT150*, *GT1669*, *GT259*, *GT285*, *GT173*, *GT2282*, *GT3363*, *GT3591*, *CiGT1*, and *CiGT2* were isolated from *Citrus reticulata* cv. Siranui. Eight genes of the GT family were searched from the *C. platymamma* Hort. ex. Tanaka (Byungkyool) genomic DNA library database. *CiGT1* and *CiGT2* genes were based on the EST sequences of NCBI. The partial glucosyltransferase nucleotide sequences were obtained from the searches in the EST databases of the Citrus Genetic Resources Bank and used as query.

A phylogenetic tree based on PSPG box of the family of glucosyltransferases from *C. reticulata* cv. Siranui showed that *CiGT1*, *CiGT2*, and *GT 173* consist of group I, *GT259*, *285*, and *150*, group II, and *GT1669*, *3363*, *3591*, and *228*, group III.

To confirmed activity of GT, transformation assay were performed with naringenin and quercetin as substrates. Three of genes (*GT173*, *285*, and *3363*) were found to have function as both flavonoid 7-O-glucosyltransferase and flavonoid 3-O-glucosyltransferase. Compared to *GT285* and *GT3363*, *GT173* showed only a detectable activity on naringenin. *GT2282* and *GT3591* were determined to glucosylate naringenin, producing its 7-O-glucoside.

Interestingly, *GT259* could glucosylate quercetin but its modification was not 3-O site, suggesting the other site could be glucosylated. On the other hand, *GT150*, *GT1669*, *CiGT1*, and *CiGT2* did not transfer glucose to two flavonoid substrates in this study. For these four genes, further study need to identify their substrate.

This research will increase our insight for the mechanism of glucosylation of flavonoid and contribute the understanding of glucosylation in Citrus.

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SUPPLEMENTARY DATA

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      10      20      30      40      50      60      70      80
MEDKKLHIAM YPWFAMGHLT SYLHISNKLA ERGHKISFIL PTNHTKFEP FNLHKHLVTF IPITVPCVDG LPSGTETTTD

      90     100     110     120     130     140     150     160
VPFPLHPLLM TAMDLTEPAI EAIVRDIKPN IVFFDFTYWL PSLARKLGIK SIAFVTISPA TVGYLLSPER KLRDKVLTEA

      170     180     190     200     210     220     230     240
DLLQPPQGF  PSKIKLRAHE  ARGLAAATVK  DFGGLSFMER  LLLCLTECDA  IGFKTCREIE  GAYCDYVESQ  FEKRVILAGP

      250     260     270     280     290     300     310     320
VLPEPPASVL EEEFEMLFSS FKAksLIFCA LGSECVLKKD QFQELILGFE LTGLPFFAAL KPPTGHDITIE SALPEGFEDR

      330     340     350     360     370     380     390     400
VKGRGFVHGG WYQQQLILKH PSVGCFTVHC GSGSLSEAMV NECQLVLLPN VGDQIINSRL MGEDLKVGVE VERGEDDGLF

      410     420     430     440     450     460     470     480
TRDGVCKAVK AVMDDDSEVG KDARQNHAEL REFLISPGLE NSYVDGFVQE LHSLADIL*

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Supplementary Fig 1. The cDNA sequence and deduced amino sequence of *GT150* gene isolated from *Citrus reticulata* cv. Siranui.

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      10      20      30      40      50      60      70      80
MAHGHIPIV DMAKLFASRG VKASVITTPA NGPYVKSVE RANEMGIELD VKTIKFPSVE AGLPEGCENL DAITNEVNSE

      90     100     110     120     130     140     150     160
LIVKVFVATT KLHEPLEQLL RDHKPDCLVA DIFFPWATDA AAKFGIPRLV FHGTSFFSLC ASNCLALYEP HKNVSSDSEP

      170     180     190     200     210     220     230     240
FVMPHFPEI KLTRNQLPDF VKQDMGDNDF SRLKAIIDS DLRSYGAAYN SFYELEPAYA DHYRKALGRR AWHIGPVSLC

      250     260     270     280     290     300     310     320
NRNFEDKALR GKQASIDELE CLKWLNSKQP NSVYVYCFGS LVNFTSAQLM EIATGLEASG RNFIWAVRKN MNDGGEKGKE

      330     340     350     360     370     380     390     400
DWLPEGFVKR MEGKGLIIRG WAPQVLILDH EAVGGFVTHC GWNSTLEAVA AGVPLVTPV SAEQFYNEKM VNEVLKIGVG

      410     420     430     440     450     460     470     480
VGIQKWCRIV GDFVKREKIE KAVNEIMVGD RAEEMRSRAK ALGKMAKRAY ENGGSSYSDL SALIEELRLS RHQSS*

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Supplementary Fig 2. The cDNA sequence and deduced amino sequence of *GT1669* gene isolated from *Citrus reticulata* cv. Siranui.

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      10      20      30      40      50      60      70      80
MSSSNTRTTH ILIFYPYPAQG HMLPLLDLTH QLSLKDLDT ILVTPKNLPI LSPLLDAHPA IKTLVLPFPS YPSIPPGIEN

      90     100     110     120     130     140     150     160
VIELGNRGNY PIMTALGKLY DPIIDWFRSQ ANPHVAILSD FFLGWTCLKA HQLNIVRIAF FSSGWLLASV ADYCWHHIGD

      170     180     190     200     210     220     230     240
VKSLDVVEFP DLPRYPVFKR RHLPSMVRYSY KESDPESQFV KDGNLANTSS WGCVFNSFDA LEGEYS DYLK RKMGHDRVFG

      250     260     270     280     290     300     310     320
VGPLSLVGLE STGGGDPGLG PNDHVTKWLD GCPDGSVYVY CFGSQKALKR DQMEALASGL EKSGIRFLWV VKTGMIGKGD

      330     340     350     360     370     380     390     400
DGYGSM PDGF EEQVAGRGLV LKGWAPQVSI LSHKAVGGFL SHCGWNSLLE GIVGGAMILA WPMEADQFVN AKLLVEDLGV

      410     420     430     440     450     460     470     480
AVQVCEGADS VPDSDDELGKI IAESLSQRDE VKIKAKELRD DALAAVTSDG SSARDLDRLV EELRNLHAK*

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Supplementary Fig 3. The cDNA sequence and deduced amino sequence of *GT259* gene isolated from *Citrus reticulata* cv. Siranui.

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      10      20      30      40      50      60      70      80
MSSSNTRTTH ILIFYPYPAQG HMLPLLDLTH QLSLKDLDT ILVTPKNLPI LSPLLDAHPA IKTLVLPFPS YPSIPPGIEN

      90     100     110     120     130     140     150     160
VIELGNRGNY PIMTALGKLY DPIIDWFRSQ ANPHVAILSD FFLGWTCLKA HQLNIVRIAF FSSGWLLASV ADYCWHHIGD

      170     180     190     200     210     220     230     240
VKSLDVVEFP DLPRYPVFKR RHLPSMVRYSY KESDPESQFV KDGNLANTSS WGCVFNSFDA LEGEYS DYLK RKMGHDRVFG

      250     260     270     280     290     300     310     320
VGPLSLVGLE STGGGDPGLG PNDHVTKWLD GCPDGSVYVY CFGSQKALKR DQMEALASGL EKSGIRFLWV VKTGMIGKGD

      330     340     350     360     370     380     390     400
DGYGSM PDGF EEQVAGRGLV LKGWAPQVSI LSHKAVGGFL SHCGWNSLLE GIVGGAMILA WPMEADQFVN AKLLVEDLGV

      410     420     430     440     450     460     470     480
AVQVCEGADS VPDSDDELGKI IAESLSQRDE VKIKAKELRD DALAAVTSDG SSARDLDRLV EELRNLHAK*

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Supplementary Fig 4. The cDNA sequence and deduced amino sequence of *GT285* gene isolated from *Citrus reticulata* cv. Siranui.

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10      20      30      40      50      60      70      80
MSEAA GSTQR RHHVILAFP GTHAAPLLDL VRRLSEAALE EEVTFSSFST AQSNGSLFME KDELRDCKIV PYNVESGLPE

90      100     110     120     130     140     150     160
GFRFTGNPRE PVEHFLKATP GNFRVRALEKA VAKTGLEISC LITDAFLWFA AEMAEE MRVP WIA YW TAGPR SLLAHLDSDI

170     180     190     200     210     220     230     240
IREIIGVNGP ENQTLESIPG FSSIRAKDLP EGIISGPLDS PFPIMLDKMG KTLPKATVVA INSYEELDPI VVETLKSFRF

250     260     270     280     290     300     310     320
KFLNVGPSTL TSPPPVSDPH GCLPWLNEHE NASVIYISFG SMITPPRAEV IALAEALEAI GFPFLWSFRG NAEELPKGF

330     340     350     360     370     380     390     400
LERTKSYGKV VLWAPQLKIL EHSSVCVFVT HCGWNSTIEG ITGGVPMVCR PVFADQALNQ RIIETAWGIG VGVGEKFTK

410     420     430     440     450     460     470     480
DETVNALKQV LSSEEGKRM R ENVGALKKLA FKAVESDGSS TKNFKALVEI VNRT*

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Supplementary Fig 5. The cDNA sequence and deduced amino sequence of *GT173* gene isolated from *Citrus reticulata* cv. Siranui.

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10      20      30      40      50      60      70      80
MASEASQSHF LLLPFLAPGH MIPMFDTARL LAQRGAIVTI VTPVNAARF KTVHARAIDS GMIQIRLIEIQ FPWQOAGLPE

90      100     110     120     130     140     150     160
GCENCDLLPT TDFARFLNSL HMLQLPFENL FERQTLKPC C IISDMCFPWT VDTAAKFNVP RIIFHGFSCF CLFCLHLLGV

170     180     190     200     210     220     230     240
SKVHENVTS DSDYFNIPGLP DHIQFTKYQL PISEQDDDFK ELQEQIFAAD KKT YGTIINT FEELESPCIE DYKKAKQEKV

250     260     270     280     290     300     310     320
WCIGPVSLCN KEPIDKAERG KKASIDVPEC LTFWDSQQPT SVVYVCLGSI CNLPSSQLIE LGLGLEASNK PFVWVIRGES

330     340     350     360     370     380     390     400
KLEEELEKWL V EENFKKRIKG RGLLIRGWAP QVLILSHPAV GGFLTHCGWN SSLEGISAGV QMLTWPLFAD QFCNEKLIVE

410     420     430     440     450     460     470     480
VLRIGVSVGV E VPMKFGEEE KIGVLYKKED VETAINILMD DGQERDARRR RAE EFGELAK RALDEGGSSY NNIELFMDI

490     500     510     520     530     540     550     560
SFHGQPQK*

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Supplementary Fig 6. The cDNA sequence and deduced amino sequence of *GT2282* gene isolated from *Citrus reticulata* cv. Siranui.

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      10      20      30      40      50      60      70      80
MASEGRCQQA HFVLFPFLLAQ GHMIPMIDIG RLLAONGAAI TIVTTPANAA RFKTVVARAM QSGPLQLIE IOFPYQEAGI

      90     100     110     120     130     140     150     160
PEGSENFDM L HSMDLLFNFF KSLTLLQLPL ENLLKELAPK PSCIVSDMCY PWTVDTAARF NIPRISFHGF SCFCLLCLYN

      170     180     190     200     210     220     230     240
LHTSKVHENV TSNSDYFVVP GLPDQIEMTK VQVPLMRENS KDFGELVLAA DMKSYGIIIN TFEELSEYV KEYKKTGGK

      250     260     270     280     290     300     310     320
VWCLGPVSLC NKQDIDKAER GKAAIDVSE CLNWLDWPP NSVYVYVCLGS ICNLTSSQMI ELGLGLEASK KPFIVVIRGG

      330     340     350     360     370     380     390     400
NNTSKEIQEW LLEEKFEERV KGRGILILGW APQVLILSHP SIGGFLTHCG WNSLEAISA GVPMTWPLF GDQFCNEKLI

      410     420     430     440     450     460     470     480
VQVLNIGVRI GVEVPLDFGE EEEIGVLYKK EDVVKAINML MNEGGERENR RKRAREFQMM AKRATEETGS SSLMIKLLIQ

      490     500     510     520     530     540     550     560
DIMHQPHSDH QHIDNA*

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Supplementary Fig 7. The cDNA sequence and deduced amino sequence of *GT3363* gene isolated from *Citrus reticulata* cv. Siranui.

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      10      20      30      40      50      60      70      80
MASEASEFHF ILLPFLAQGH LIPMFDIARL LAQHGATATI VTTPYNAARF KTVLARALQC RLQIRLIEIQ FPWQEAGLPE

      90     100     110     120     130     140     150     160
GCENFDM LPS IDLAYNFLAS LQKLQLPFEN LFREQTPQPC CIISDMCMPW TVDTAAKFNV PRIIFHGFS FCLLCILDILR

      170     180     190     200     210     220     230     240
VSKVHENVSS DSEYFKVPGF PHHIEFTKVQ LPISPPTDEL KEFDEKILAA DKKTYGVIIN TFEELASAV KEYKNAKQGGK

      250     260     270     280     290     300     310     320
VWCIGPVSLC NKESLDKVER GNKAAIDIFE CLTWLDSQQP SSVYVYVCLGS ICNLTSSQLI ELGLGLEASK KPFIVVTRVG

      330     340     350     360     370     380     390     400
NKLEELEKWL VEENFEERIK GRGLLIRGWA PQVMILSHPA VGGFLTHCGW NSSLEGISAG VQMLTWPLFG DQFCNEKLIV

      410     420     430     440     450     460     470     480
EVLRIQVSVG VEVPLKFGEE EKIGVLYKKE DVETAINILM DDGEERDVRR KRAKEFEELA KRALEEGGSS YNNIQLFFQD

      490     500     510     520     530     540     550     560
IMQOPTSEVM *

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Supplementary Fig 8. The cDNA sequence and deduced amino sequence of *GT3591* gene isolated from *Citrus reticulata* cv. Siranui.

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      10      20      30      40      50      60      70      80
MEKQDHWVHA ILPLPAVGHV SSMLNLAELL GHAGIKITFL NTEHYDRVI RHSSDAFSRY MQTPGFQFKT LTDGLPRDHP

      90     100     110     120     130     140     150     160
RTPDKFPELV DSLNCATPPL LKEMVSDSKS PYNCIITDGY MSRAIDAARE VGYSTIIFRT ISACAFWSFH CIPDIIDAGE

      170     180     190     200     210     220     230     240
LPIKGTEDVD RLITTVPGME GFLRCRDLPV FCRVNDPMDP HLLLFARETR LSAHADGLIL NTFEDLEGPI LSQIRNHSCP

      250     260     270     280     290     300     310     320
NIYSIGPLNA HLKVRPEKT YSSSSLWKID RSCMAWLDKQ PKQSVIYVSF GSIAVMSRDQ LIEFYGLYH SKKNFLWVIR

      330     340     350     360     370     380     390     400
PDLISGKDG E NQIPEELLEA TKERGCIAGW VPQEEVLAHS AVGGFLTHCG WNSTLESIVA GMPMICWPSF ADQQINSRFV

      410     420     430     440     450     460     470     480
GEVWKLGLDI KDLCDRNIVE KTVNDLMVER KEEFMESADR MANLAKKSYN KGGSSYCNLD RLVNDIKMMS SQPQNC*

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Supplementary Fig 9. The cDNA sequence and deduced amino sequence of *CiGT1* gene isolated from *Citrus reticulata* cv. Siranui.

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      10      20      30      40      50      60      70      80
MEQQQPHFL LLTFPIQGH I NPSLQFARRL TRIGTRVTF A TAISAYRLMP NNPTAEDGLS FASFSGDYDD GFNSKQNDQT

      90     100     110     120     130     140     150     160
HFMSEFKRRS SEALAELITA SRNEGGQPFT CLVYPQLLIW AAEVARAYHL PSALLWLQPA LVFDVYYYYF NGYGLIEGK

      170     180     190     200     210     220     230     240
VNDLIELPGL PPLTGRDLPS FLDPRNSDA YSFVLP SFKE QMEAI VEETD PRILVNTFDA LEAETLK AID KFN MIAIGPL

      250     260     270     280     290     300     310     320
VASALLDGKE QYGGDLQNS SIEYYMEWLS SKPKSSVIYV AFGTICVLEK RQVEEIARGL LDSGHPFLWV SREKRP*

```

Supplementary Fig 10. The cDNA sequence and deduced amino sequence of *CiGT2* gene isolated from *Citrus reticulata* cv. Siranui.

요약문

플라보노이드의 글라이코실레이션은 이차 대사물질의 복잡성에 관여하는 필수적인 변환과정이고 천연물 생합성의 마지막 단계에서 발생하며, 플라보노이드의 안정성과 수용성에 영향을 끼친다. 글루코실트랜스퍼라아제는 글리코실트랜스퍼라아제의 한 멤버이고 글루코스를 수용체에 운반한다.

본 연구에서는, 한라봉으로부터 10개의 글루코실트랜스퍼라아제 유전자, *GT150*, *GT1669*, *GT259*, *GT285*, *GT173*, *GT2282*, *GT3363*, *GT3591*, *CiGT1*, and *CiGT2* 를 동정하였다. 한라봉에서 추출한 total RNA로부터 싱글 cDNA를 합성 후, 유전자 특이 프라이머와 PCR을 이용하여 8개의 유전자 (*GT150*, *GT1669*, *GT259*, *GT285*, *GT173*, *GT2282*, *GT3363*, and *GT3591*)를 분리하였다. *CiGT1*과 *CiGT2*는 reverse transcription (RT)-PCR을 이용하여 분리하였다. 완전장 GT cDNA는 클로닝 하였고, GST 재조합 단백질을 합성하여 발현시켰으며, 이 재조합 단백질들의 효소활성은 플라보노이드 7-O-글리코사이드와 플라보노이드 3-O-글리코사이드를 생산하는 전형적인 기질인 naringenin과 quercetin을 이용하여 분석하였다. HPLC분석에 의하면, *GT173*, *285*와 *3363*은 naringenin과 quercetin에서 활성을 나타내는 것을 확인할 수 있었고, 각각의 반응 산물의 구조는 7-O-glucoside와 3-O-glucoside와 관련이 있다는 것을

확인하였다. *GT285*는 *GT3363*과 *GT173*에 비해 naringenin의 활성이 약하게 나타났고, *GT3591*과 *GT2282*는 naringenin-7-O-glucoside와 관련이 있는 것을 확인하였다. 본 연구 결과에서 *GT259*는 흥미롭게도 quercetin을 기질로 하였을 때 3-O-glucoside가 아닌 다른 생산물을 생성하는 것을 확인할 수 있었다. 반면에, *GT150*, *1669*, *CiGT1*, 그리고 *CiGT2*는 naringenin과 quercetin 기질에 활성이 없음을 확인하였다. 이러한 결과들은 한라봉에서 분리한 각각의 GT 유전자들이 기질 특이성을 갖는다는 것을 보여주는 결과이다. 본 연구 결과를 기초로 추후에는 활성을 보이지 않은 *GT150*, *1669*, *CiGT1*, 그리고 *CiGT2*에 대해 kaempferol, anthocyanin 그리고 hesperidin과 같은 기질을 이용하여 플라보노이드 분석을 할 예정이다. 이러한 결과들은 앞으로 당합성 관련 유용 유전자의 유전정보 확보를 통한 생리 및 물질대사 기능 분석등의 결과 도출과 나아가 감귤 분자육종 및 고품질 감귤 생산의 기반을 확립하는데 큰 도움이 될 것이다.

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먼저, 포기하지 않는다면 원하는 바를 얻을 수 있다는 것을 몸소 보여주신 김재훈 교수님께 깊은 감사의 뜻을 표합니다. 교수님께서 가르쳐주신 모든 가르침을 잊지 않고 앞으로의 생활에 큰 밑거름으로 사용하겠습니다. 진심으로 감사합니다.

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지면을 통해서 일일이 언급을 하지 못했지만 그 동안 저를 아끼고 사랑해주신 모든 분들께 다시 한번 진심으로 감사 드립니다.

감사합니다.