Doctoral Dissertation (Shinshu University)

Bioconversion of functional compounds into their glycosides by *Escherichia coli* expressing plant glycosyltransferases

September 2022

DORJJUGDER NASANJARGAL

(Student ID: 19HS112A)

Table of Contents

Abbreviations

Chapter 1 General Introduction **1**

1.1. Introduction 2

1.2. References 5

Chapter 2 Production of flavonoid 6-*C*-glucosides by bioconversion using *Escherichia coli* expressing a *C*-glucosyltransferase from wasabi (WjGT1) **7**

2.1. Abstract 8

2.2. Introduction 8

2.3. Materials and methods 10

2.3.1. Reagents 10

2.3.2. Bacterial cell culture and protein expression 10

2.3.3. Substrate bioconversion 11

2.3.4. Scale-up production of the flavonoid 6-*C*-glucosides 11

2.3.5. Purification of the products 11

2.3.6. UPLC-MS analysis 12

2.3.7. NMR analysis 12

2.4. Results 14

2.4.1. Bioconversion of flavones and flavonols to their 6-*C*-glucosides 14

2.4.2. Scale-up production of flavone and flavonol 6-*C*-glucosides 18

2.4.3. Purification of 6-*C*-glucosides and NMR analysis 21

2.4.4. Bioconversion of other substrates 21

2.5. Discussion 27

2.6. References 28

Chapter 3 Production of cinnamate and benzoate glucosides by bioconversion using *Escherichia coli* expressing a cinnamate glucosyltransferase from sweet potato (IbGT1) 30

3.1. Abstract 31

Chapter 4 Production of glucosides of flavonoids and phenolics by bioconversion using *Escherichia coli* expressing an *O*-glucosyltransferase from tobacco (NtGT2 or NtGT3)32

4.1. Abstract 33

4.2. Introduction 33

4.3. Materials and methods 35

4.3.1. Reagents 35

4.3.2. Bacterial cell culture and protein expression 35

4.3.3. Substrate bioconversion 36

4.3.4. Scale-up production of the flavonoid 7-*O*-glucosides 36

4.3.5. Purification of the products 36

4.3.6. UPLC-MS analysis 37

4.3.7. NMR analysis 37

4.4. Results 41

4.4.1. Bioconversion of flavonoids to their 7-*O*-glucosides 41

4.4.2. Bioconversion of flavonoids and phenolics to their glucosides 54

4.5. Discussion 59

4.6. References 61

Chapter 5 Production of gentiobiosides of phenolics and flavonoids by bioconversion using *Escherichia coli* expressing a novel glucosyltransferase from tobacco (NtGGT2) 63

5.1. Abstract 64

Chapter 6 General Discussion and Conclusion 65

6.1. General discussion and conclusion 66

6.2. References 68

APPENDIX **69**

LIST OF PUBLICATION **70**

ACKNOWLEDGEMENTS **72**

ABBREVIATIONS

Blast:	basic local alignment search tool
BSA:	bovine serum albumin
cDNA:	complimentary DNA, synthesized from mRNA
CGT:	*C*-glycosyltransferase
DMSO:	dimethyl sulfoxide
GGT:	glycoside glycosyltransferase
GT:	glycosyltransferase
HMBC:	heteronuclear multiple bond correlation
IPTG:	isopropyl β-D-1-thiogalactopyranoside
IS:	internal standard
K-Pi:	potassium phosphate
LB:	Luria-Bertani
MS:	mass spectrometry
2-ME:	2-mercaptoethanol
NMR:	nuclear magnetic resonance
OD:	optical density
ODS:	octadecylsilyl
PCR:	polymerase chain reaction
RT:	rhamnosyltransferase
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
UDP:	uridine diphosphate
UGT:	UDP-sugar dependent glycosyltransferase
UPLC:	ultra-performance liquid chromatography

Chapter 1

General Introduction

1.1. INTRODUCTION

Glycosylation has been widely acknowledged as one of the most important mechanisms to enhance the solubility of lipophilic compounds (Lim 2005). Furthermore, it increases stability and alters pharmacokinetics and bioactivity of aglycone (Liu et al. 2018). A special enzyme, glycosyltransferases (GTs), accelerates the glycosylating reaction by transferring sugar moiety from an activated sugar donor to a sugar acceptor (Vogt and Jones 2000). The sugar acceptor can be protein, lipids, polysaccharides, and various plants' small molecules (Lairson et al. 2008). The resulting sugar moieties improve physiological activity, specificity, and pharmacological properties of various natural compounds (Shrestha et al. 2018). Based on some criteria including amino acid sequence similarities, GTs can be classified into 110 families (CAZy database, http://www.cazy.org/) (Zhang et al. 2020). Also, glycosyltransferases are different depending on their sugar donor preference (Yang et al. 2018). For instance, the glycosyltransferases that accept nucleoside diphosphates such as uridine diphosphate (UDP)-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-rhamnose as a sugar donors belong to UDP-sugar dependent glycosyltransferases (UGTs) family (GT family 1), and are found in diverse organisms including mammals, plants, yeast, fungi, and bacteria (Bowles et al. 2005; Zhang et al. 2020).

Various kinds of metabolites such as flavonoids and phenylpropanoids usually exist in their glycosylated forms in plants. Because of enhanced water solubility, these glycosylated metabolites are easily transported into the vacuole (Pandey et al. 2014). The glycosylated metabolites have higher molecular weight and melting point which allow for improving stability as compared to the original metabolites (Kytidou et al. 2020). Depending on the number and position of the attached sugar moieties, glycosides exhibit diverse properties. The number of attached sugar moieties is usually one (monosaccharide) or two (disaccharide) such as gentiobiose, sophorose, and rutinose (Cressey and Reeve 2019). According to the position

where sugar moiety attaches to aglycone, glycosides are divided into *O*-, *N*-, *S*-, *C*- and carboxy-glycosides (Yang et al. 2018). Plants generally accumulate all of these glycosides, among them *O*-glycosides are mostly widespread (Vogt and Jones 2000). Common plant pigments that are responsible for flower color, smell, and flavor exist as *O*- and carboxy-glycosides (Sasaki et al. 2014). Flavonoids, well-known secondary metabolites, usually accumulate as *O*-glycosides and *C*-glycosides. The position of attached sugar moiety could play an important role in the bioactivity of flavonoids. For instance, flavonoid *O*-glycosides show high anti-stress and anti-HIV activities, while flavonoid *C*-glycosides demonstrate high antioxidant and anti-diabetic properties (Xiao 2017; Xiao et al. 2014).

From the beginning of the 21st century, new ideas and novel technologies have been investigated for the synthesis of diverse compounds in the bioengineering and biotechnology fields. Both chemical and enzymatic methods have been developed for synthesizing glycosides of various natural compounds. But chemical methods are not suitable for regioselective glycosylation and usually require complicated multiple steps. Compared to that, enzymatic methods are suitable for regioselective glycosylation, but they have troublesome problems such as instability of the GT enzymes and the high cost of UDP-sugars. An enzymatic glycosylating reaction coupled with synthase to recycle UDP-glucose has also been developed (Masada et al. 2007; Pei et al. 2019), but it still is not applicable for a large-scale bioconversion since it needs a proper amount of UDP-glucose and high concentration of sucrose. Microorganisms have been used as alternative hosts to produce various types of plant-originated metabolic compounds. Production of such compounds through a biotechnological process is a more efficient and environmentally sustainable way to compare with extraction methods from plant materials (Eichenberger et al. 2017). Moreover, microbial systems require cheaper materials and a simple device with a low driving cost. Production of flavonoid glycosides using budding yeast and *Escherichia coli* as hosts has been reported (Brazier-Hicks and Edwards 2013; De

Bruyn et al. 2015). Particularly, the system using *E. coli* cells administered with glucose and aglycone has been proven to be a simple and effective method for the production of specific glycosides (Ito et al. 2014; Kim et al. 2013).

In this study, I focused on the production of mono- and di-glucosides of plant metabolites such as flavonoids and cinnamates using a bioconversion system driven by the *E. coli* cells expressing plant GTs, since little had been reported for such an example. Here, the bioconversion of flavonoids, cinnamates, and phenolics to their mono- and di-glucosides using recombinant *E. coli* cells expressing plant-derived GTs were reported and the scheme was outlined in **Fig. 1-1**. In chapter 2, the production of flavonoid *C*-glucosides such as flavone and flavonol *C*-glucosides using *E. coli* expressing a *C*-glucosyltransferase (WjGT1) originating from wasabi is described. In chapter 3, a production of cinnamate glucosides using *E. coli* expressing cinnamate glucosyltransferase (IbGT1) that is isolated from sweet potato is provided. In chapter 4, the production and bioconversion process of flavonoid *O*-glucosides and phenolic glucosides through tobacco-derived *O*-glucosyltransferase expressed in *E. coli* (NtGT2 or NtGT3) is offered. In chapter 5, isolation and characterization of a phenolic glucoside 1,6-glucosyltransferase from tobacco, and the synthesis of di-glucoside compounds using this enzyme were reported. In the final chapter, chapter 6, a general discussion and conclusion are provided.

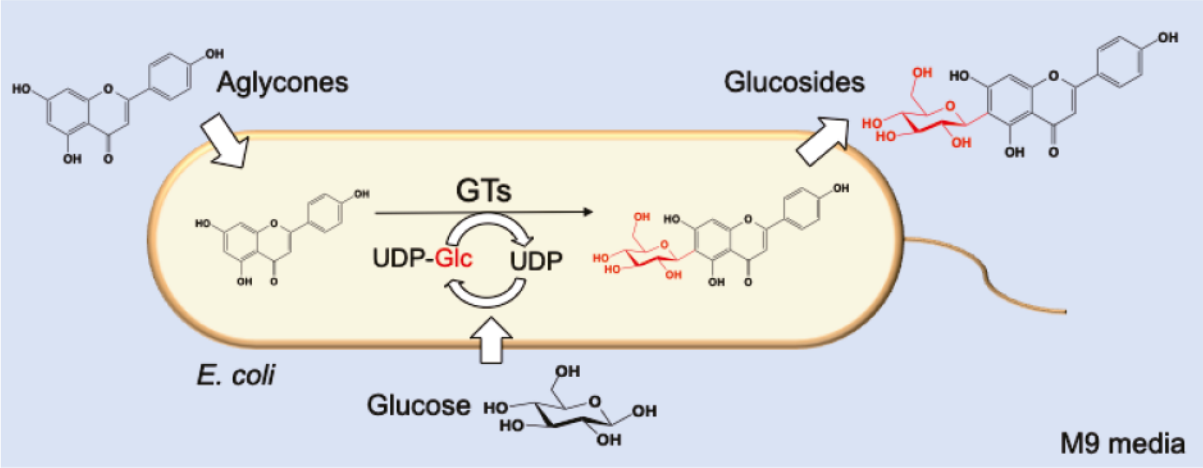


Figure 1-1 Scheme of study

E. coli cells expressing plant-derived glycosyltransferases (GTs) convert various compounds into their glucosides in M9 minimal medium containing glucose

1.2. REFERENCES

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

Brazier-Hicks M, Edwards R (2013) Metabolic engineering of the flavone-*C*-glycoside pathway using polyprotein technology. Metab Eng 16:11-20

Cressey P, Reeve J (2019) Metabolism of cyanogenic glycosides: A review. Food Chem Toxicol 125:225-232

De Bruyn F, Van Brempt M, Maertens J, Van Bellegem W, Duchi D, De Mey M (2015) Metabolic engineering of *Escherichia coli* into a versatile glycosylation platform: production of bio-active quercetin glycosides. Microb Cell Fact 14:138

Eichenberger M, Lehka BJ, Folly C, Fischer D, Martens S, Simón E, Naesby M (2017) Metabolic engineering of *Saccharomyces cerevisiae* for de novo production of dihydrochalcones with known antioxidant, antidiabetic, and sweet tasting properties. Metab Eng 39:80-89

Ito T, Fujimoto S, Shimosaka M, Taguchi G (2014) Production of *C*-glucosides of flavonoids and related compounds by *Escherichia coli* expressing buckwheat *C*-glucosyltransferase. Plant Biotechnol 31:519-524

Kim HJ, Kim BG, Ahn JH (2013) Regioselective synthesis of flavonoid bisglycosides using *Escherichia coli* harboring two glycosyltransferases. Appl Microbiol Biotechnol 97:5275-5282

Kytidou K, Artola M, Overkleeft HS, Aerts JM (2020) Plant glycosides and glycosidases: a treasure-trove for therapeutics. Front Plant Sci 11:357

Lairson L, Henrissat B, Davies G, Withers S (2008) Glycosyltransferases: structures, functions, and mechanisms. Annu Rev Biochem 77:521-555

Lim E-K (2005) Plant glycosyltransferases: their potential as novel biocatalysts. Chemistry 11(19):5486-5494

Liu X, Li X-B, Jiang J, Liu Z-N, Qiao B, Li F-F, Cheng J-S, Sun X, Yuan Y-J, Qiao J (2018) Convergent engineering of syntrophic *Escherichia coli* coculture for efficient production of glycosides. Metab Eng 47:243-253

Masada S, Kawase Y, Nagatoshi M, Oguchi Y, Terasaka K, Mizukami H (2007) An efficient chemoenzymatic production of small molecule glucosides with in situ UDP-glucose recycling. FEBS Lett 581:2562-2566

Pandey RP, Parajuli P, Koirala N, Lee JH, Park YI, Sohng JK (2014) Glucosylation of isoflavonoids in engineered *Escherichia coli.* Mol Cells 37:172-177

Pei J, Sun Q, Gu N, Zhao L, Fang X, Tang F, Cao F (2019) Production of isoorientin and isovitexin from luteolin and apigenin using coupled catalysis of glycosyltransferase and sucrose synthase. Appl Biochem Biotechnol 190:601-615

Sasaki N, Nishizaki Y, Ozeki Y, Miyahara T (2014) The role of acyl-glucose in anthocyanin modifications. Molecules 19:18747-18766

Shrestha A, Pandey RP, Dhakal D, Parajuli P, Sohng JK (2018) Biosynthesis of flavone *C*-glucosides in engineered *Escherichia coli.* Appl Microbiol Biotechnol 102:1251-1267

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

Xiao J (2017) Dietary flavonoid aglycones and their glycosides: which show better biological significance? Crit Rev Food Sci Nutr 57:1874-1905

Xiao J, Muzashvili TS, Georgiev MI (2014) Advances in the biotechnological glycosylation of valuable flavonoids. Biotechnol Adv 32:1145-1156

Yang B, Liu H, Yang J, Gupta VK, Jiang Y (2018) New insights on bioactivities and biosynthesis of flavonoid glycosides. Trends Food Sci Technol 79:116-124

Zhang P, Zhang Z, Zhang L, Wang J, Wu C (2020) Glycosyltransferase GT1 family: phylogenetic distribution, substrates coverage, and representative structural features. Comput Struct Biotechnol J 18:1383-1390

Chapter 2

Production of flavonoid 6-*C*-glucosides by bioconversion using *Escherichia coli* expressing a *C*-glucosyltransferase from wasabi (WjGT1)

2.1. ABSTRACT

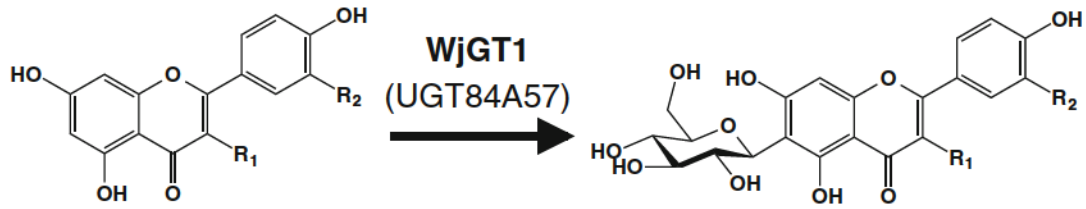
I attempted to produce flavonoid 6-*C*-glucosides using *Escherichia coli* expressing a *C*-glucosyltransferase (WjGT1) from wasabi in this chapter. *E. coli* expressing WjGT1 (Ec-WjGT1 system) converted flavones (apigenin and luteolin) and flavonols (quercetin and kaempferol) into their 6-*C*-glucosides in M9 minimal media with glucose. The final products increased after the sequential administration of apigenin, kaempferol, and quercetin at a concentration of 20–50 μM every 15–60 min. The system produced isovitexin, kaempferol 6-*C*-glucoside, and quercetin 6-*C*-glucoside at an 89–99% conversion rate. The system also converted a flavanone (naringenin) into its *C*-glucoside with a 60% conversion rate in 6 h.

2.2. INTRODUCTION

Flavonoids are natural products produced in plants. These compounds are predominantly accumulated in plants as *O*-glycosides but also be found as *C*-glycosides and further acylated or glycosylated compounds (Williams 2006). *C*-glycosides are characterized by the presence of a carbon-carbon bond between the sugar and the aglycone, which makes them more stable during enzymatic or acidic hydrolysis than other glycosides (Jay et al. 2006). They often demonstrate higher pharmacokinetic activities than their *O*-glycosides and aglycones (Xiao et al. 2014). Flavone *C*-glycosides have been found in many plant species. A well-known flavone *C*-glucoside is isovitexin (an apigenin 6-*C*-glucoside). Isovitexin is an important ingredient of medical plants because of its biological benefits including hypotensive and neuroprotective activities (Choi et al. 2014; Xiao et al. 2016). In contrast, flavonol *C*-glycosides are rare and most of them have been reported in Ulmaceae plants (Iwashina 2000; Jay et al. 2006). Flavonol *C*-glycosides have been shown to exert various bioactivities including anti-cancer and osteoblast growth-promoting activities (Hamidullah et al. 2015).

Bioprocesses are more effective in producing flavonoid glycosides than chemical synthesis as they can modify the compounds easily and regiospecifically (Talhi and Silva 2012). Several methods for producing flavonoid glycosides using microorganisms, such as budding yeast and *Escherichia coli*, have been reported (Brazier-Hicks and Edwards 2013; De Bruyn et al. 2015). Both yeast and *E. coli* have been used to produce flavonoid *C*-glycosides (Brazier-Hicks and Edwards 2013; Ito et al. 2014). The production of mono-*C*-glucosides and di-*C*-glucosides of flavone have been reported (Brazier-Hicks and Edwards 2013; Ito et al. 2014; Ito et al. 2017; Shrestha et al. 2018; Sun et al. 2020; Vanegas et al. 2018), but flavonol *C*-glucosides have not been produced yet.

In this chapter, I attempt to produce flavonoid 6-*C*-glucosides using *E. coli* expressing *C*-glucosyltransferase (WjGT1) from wasabi (*Eutrema japonicum*) that reacts to the 6-position of both flavones and flavonols (Mashima et al. 2019) (**Fig. 2-1**). A recombinant *E. coli* cells (Ec-WjGT1) produced a large-scale of flavone 6-*C*-glucosides and flavonol 6-*C*-glucosides through a sequential administration of aglycones.



Flavones and Flavonols		**6-*C*-Glucosides**	
R_1=H, R_2=H	apigenin	R_1=H, R_2=H	isovitexin
R_1=H, R_2=OH	luteolin	R_1=H, R_2=OH	isoorientin
R_1=OH, R_2=H	kaempferol	R_1=OH, R_2=H	kaempferol 6-*C*-glucoside
R_1=OH, R_2=OH	quercetin	R_1=OH, R_2=OH	quercetin 6-*C*-glucoside

Figure 2-1 Structures of the substrates and products in the Ec-WjGT1 system
The enzyme catalyzes 6-*C*-glucosylation of both flavones and flavonols

2.3. MATERIALS AND METHODS

2.3.1. Reagents

Substrates and standard compounds were obtained as follows; apigenin (Ark Pharm, Arlington Heights, IL, USA); luteolin (Indofine Chemical, Hillsborough, NJ, USA); kaempferol and resveratrol (Tokyo Chemical Industry, Tokyo, Japan); quercetin (Cayman Chemicals, Ann Arbor, MI, USA); naringenin (AmBeed, Arlington Heights, IL, USA); isovitexin (Extrasynthèse, Genay, France); isoorientin (Toronto Research Chemicals, Toronto, ON, Canada). Other chemicals, solvents, and media were purchased from Sigma-Aldrich (St. Louis, MO, USA), Nacalai Tesque (Kyoto, Japan), Tokyo Chemical Industries, Kanto Chemical (Tokyo, Japan), or Wako Pure Chemical Industries (Osaka, Japan).

2.3.2. Bacterial cell culture and protein expression

A 6-*C*-glycosyltransferase WjGT1 (UGT84A57, GenBank Accession No. LC465149) was isolated from wasabi cauline leaves (*Eutrema japonicum*), then subcloned into pDEST15 (Life Technologies, Tokyo, Japan) for introduction to *E. coli* Rosetta 2(DE3) (Merck, Darmstadt, Germany) (Mashima et al. 2019). Recombinant *E. coli* cells were grown in 5 mL LB broth containing ampicillin (50 mg/L) and chloramphenicol (34 mg/L) at 37 °C and 150 rpm overnight. A 1 mL of an overnight culture was transferred into 100 mL fresh LB broth with the same concentration of antibiotics and incubated at the same condition until OD_{600} reached 0.5–0.6. The culture was then induced with 0.4 mM IPTG and incubated at 22 °C and 150 rpm overnight for expression of WjGT1 protein.

2.3.3. Substrate bioconversion

The overnight culture was centrifugated at 3,000 ×*g* for 10 min and the cell pellet was resuspended in M9 minimal media containing 2% (w/v) glucose (see Appendix) (Sambrook and Russell 2001) at a cell density was OD_{600}=2.5–3.5 for bioconversion reaction. Each cell suspension was supplemented with 20–50 μM concentrations of apigenin, luteolin, kaempferol, quercetin, naringenin, and resveratrol then incubated at 30 °C and 150 rpm for substrate conversion. An aliquot of the culture medium was sampled at a certain time and centrifuged at 10,000 ×*g* for 2 min to remove bacterial cells. Then an equal volume of methanol was added to the supernatant to precipitate unnecessary content and the sample was centrifuged again with the same speed. The supernatant was filtrated through a 0.2 μm membrane filter (Millex LG, Merck) for UPLC-MS analysis.

2.3.4. Scale-up production of the flavonoid 6-*C*-glucosides

A total of 440–500 mL cell cultures were sequentially administered 20 μM of apigenin, 40 μM of kaempferol, and 50 μM of quercetin as dimethyl sulfoxide (DMSO) solution every 15–60 min in six to twenty cycles. The culture was then incubated at 30 °C overnight. Samples were collected from cell cultures before and after every administration and analyzed using UPLC-MS.

2.3.5. Purification of the products

After the overnight bioconversion, the cultures were centrifuged at 10, 200 ×*g* for 10 min and the supernatants were applied to an ODS column (28 mm i.d. × 230 mm, Wako-gel 50C18; Wako Pure Chemical Industry) prewashed with 100% and equilibrated with 10% methanol. Each compound was then eluted using a linear gradient of 150 mL of 10–50% methanol, and

the fraction containing the final product was then concentrated by rotary evaporator, followed by centrifugal concentration.

2.3.6. UPLC-MS analysis

UPLC-MS analysis was performed using an ACQUITY UPLC SQD system (Waters, Milford, MA, USA) equipped with an ODS column (2.1 mm i.d. × 50 mm, 1.7 µm; ACQUITY UPLC BEH C18 Column, Waters), an electron-spray ionization probe (negative mode), and a diode array detector as described by Mashima et al. (2019). For flavone glucosides, flavonol glucosides, and stilbene glucoside, the column was eluted with 20% solvent B (methanol supplemented with 0.1% formic acid) in A (water supplemented with 0.1% formic acid) for 30 s, followed by a gradient from 20–60% solvent B for 2 min, 60 % solvent B in A for 2 min, and 20% solvent B in A for 2 min at a flow rate of 0.25 mL min^{-1} and temperature of 40 °C (**LC-Method A**). For flavanone glucoside, the column was eluted with 5% solvent B in A for 30 s, followed by a gradient of 5–20% solvent B for 3 min, 20% solvent B in A for 30 s, a gradient of 20–60% solvent B for 2 min, 60% solvent B in A for 2 min 30 s, and 5% solvent B in A for 1 min 30 s at the same rate and temperature (**LC-Method B**). The eluent was monitored at 340 (apigenin and luteolin), 360 (kaempferol and quercetin), 290 (naringenin), and 310 nm (resveratrol).

2.3.7. NMR analysis

All purified products were dissolved in dimethyl sulfoxide (DMSO)-d_6 for an analysis and NMR spectra were recorded on a Bruker Avance Neo 400 spectrometer (Bruker BioSpin, Yokohama, Japan) at 400 MHz and was compared with the reported NMR spectra of related compounds. The NMR assignment were shown in **Table 2-1** (Maatooq et al. 1997; Markham et al. 1978).

Table 2-1 NMR assignment of the purified compounds produced by bioconversion (400 MHz, dimethyl sulfoxide-d$_6$ (DMSO-*d*$_6$))

	Isovitexin			Kaempferol 6-*C*-glucoside			Quercetin 6-*C*-glucoside		
	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)
Flavonoid									
2	-	-	163.8	-	-	147.0	-	-	148.2
3	6.79	*s*	103.2	-	-	136.0	-	-	136.1
4	-	-	182.4	-	-	176.6	-	-	176.5
5	-	-	161.1	-	-	160.3	-	-	160.3
6	-	-	109.4	-	-	108.7	-	-	108.6
7	-	-	164.0	-	-	163.7	-	-	163.7
8	6.53	*s*	94.1	6.49	*s*	93.6	6.47	*s*	93.5
9	-	-	156.7	-	-	155.6	-	-	155.5
10	-	-	103.8	-	-	103.2	-	-	103.1
1′	-	-	121.6	-	-	122.1	-	-	122.4
2′	7.94	*d* (8.8)	128.9	8.06	*d* (9.0)	130.0	7.68	*d* (2.2)	115.4
3′	6.94	*d* (8.8)	116.5	6.94	*d* (9.0)	115.9	-	-	145.6
4′	-	-	161.7	-	-	159.7	-	-	147.0
5′	6.94	*d* (8.8)	116.5	6.94	*d* (9.0)	115.9	6.89	*d* (8.5)	116.1
6′	7.94	*d* (8.8)	128.9	8.06	*d* (9.0)	130.0	7.55	*dd* (8.5, 2.2)	120.4
Glucose moiety									
1″	4.60	*d* (9.8)	73.5	4.61	*d* (9.8)	73.6	4.61	*d* (9.8)	73.5
2″	4.06	*dd* (9.1, 9.1)	70.7	4.07	*dd* (9.1, 9.1)	70.7	4.07	*dd* (9.0, 9.0)	70.7
3″	3.13-3.20	*m*	79.4	3.13-3.20	*m*	79.5	3.13-3.21	*m*	79.5
4″	3.13-3.20	*m*	71.1	3.13-3.20	*m*	71.1	3.13-3.21	*m*	71.1
5″	3.13-3.20	*m*	82.1	3.13-3.20	*m*	82.1	3.13-3.21	*m*	82.1
6″	3.70	*d* (10.7)	62.0	3.70	*d* (10.6)	62.0	3.70	*d* (10.7)	62.0
	3.42	*dd* (11.7, 5.8)		3.42	*dd* (11.7, 5.8)		3.42	*dd* (11.7, 5.7)	

2.4. RESULTS

2.4.1. Bioconversion of flavones and flavonols to their 6-*C*-glucosides

Apigenin and luteolin belong to the flavone subgroup and are natural substrates of WjGT1; moreover, apigenin 6-*C*-glucoside (isovitexin) and luteolin 6-*C*-glucoside (isoorientin) are highly accumulated in wasabi leaves. Therefore, I tested *E. coli* expressing WjGT1 (Ec-WjGT1) for conversion of apigenin and luteolin into isovitexin and isoorientin, respectively. The recombinant protein was expressed in *E. coli*, and SDS-PAGE analysis of the purified enzyme showed in **Fig. 2-2**. The Ec-WjGT1 culture was resuspended in M9 minimal media containing 2% (w/v) glucose, then 10 mL culture was supplemented with apigenin or luteolin at final concentration was 50 μM. The bioconversion reaction was performed for 2 h, and samples were collected from the medium for UPLC analysis. The analysis result showed that conversion of these substrates started immediately after their addition and the added substrates were completely converted to isovitexin or isoorientin in 1 h. The final products were compared and confirmed by their standard compounds (**Fig. 2-3**).

The Ec-WjGT1system was also examined to produce flavonol *C*-glucosides which have not yet been produced by the *E. coli* system. A well-known flavonols including kaempferol and quercetin were selected as substrates in this experiment. The same amount of cell culture was individually supplemented with 50 μM concentrations of kaempferol and quercetin, and all substrates were converted to their glucosides in 1 h (**Fig. 2-4 A–B, D–E**). The products presented with a $[M-H]^-$ ion at *m/z* 447 and 463, which corresponds with the glucosides of kaempferol and quercetin, respectively. They are likely *C*-glucosides, as judged by $[M-H-120]^-$ ion at *m/z* 327 and 343, respectively, which are a typical fragmentation of *C*-glucosides (de Villiers et al. 2016) (**Fig. 2-4 C, F**).

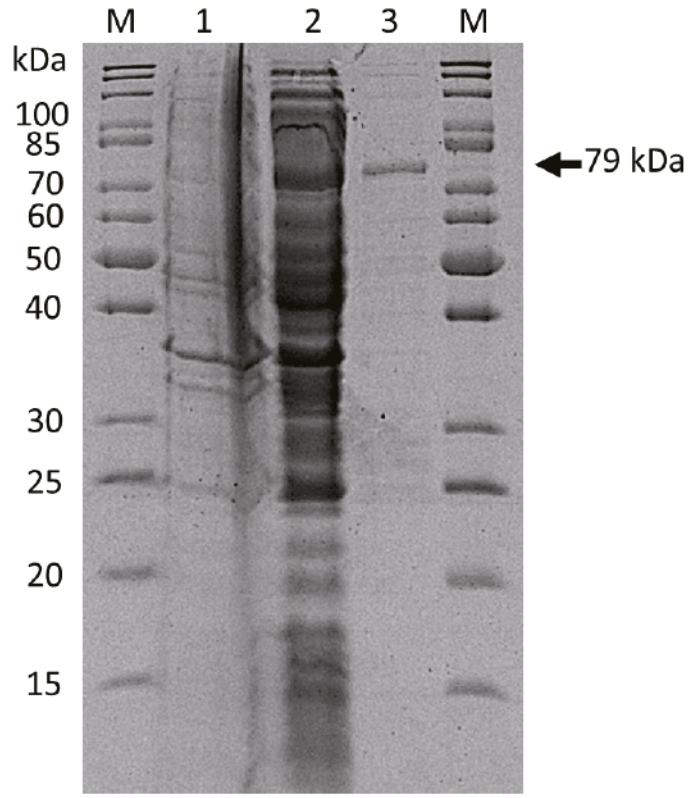


Figure 2-2 SDS-PAGE analysis of the recombinant WjGT1 protein expressed in *E. coli*. Proteins were separated on a 12.5% SDS-PAGE. Lane M, Size marker (PageRuler unstained protein ladder, ThermoFisher Scientific); lane 1 and 2, insoluble (**1**) and soluble (**2**) fraction of the cell-free extract; 3, recombinant WjGT1 protein purified with the GST tag. Arrowhead indicates the size of the recombinant WjGT1 protein.

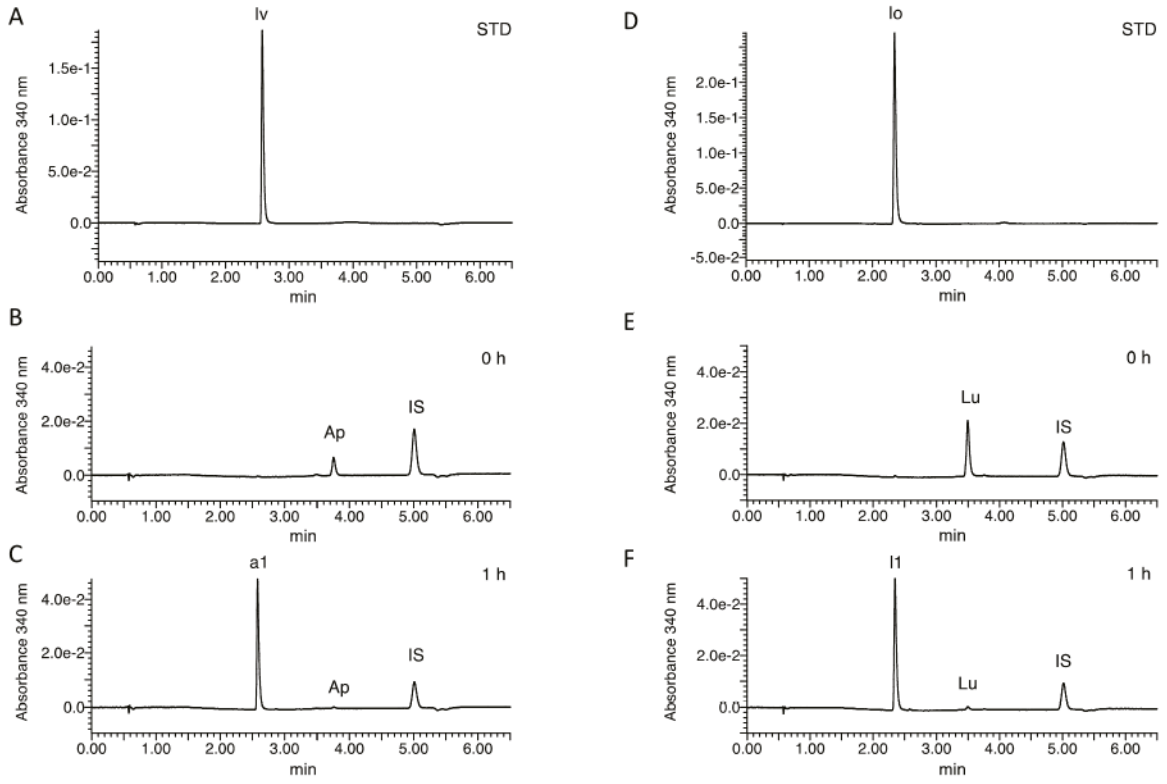


Figure 2-3 Bioconversion of flavones by the Ec-WjGT1 system

Each panel shows the UPLC-MS results of apigenin (A–C) and luteolin (D–F), with the standard compounds of isovitexin (A) and isoorientin (D), bioconversion at 0 h (B, E) and 1 h (C, F). Peak identification: Iv, isovitexin; Ap, apigenin; Io, isoorientin; Lu, luteolin; a1 and l1, product peaks of apigenin and luteolin conversion; IS, internal standard (chrysin).

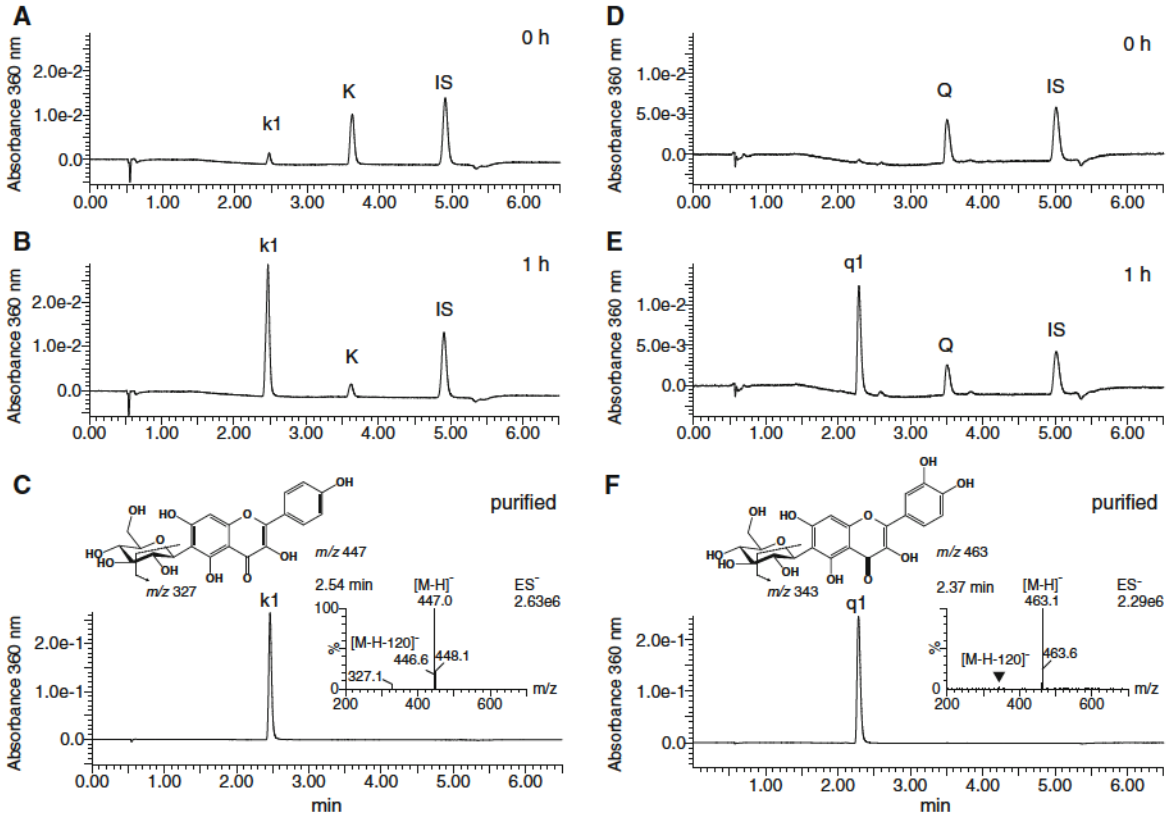


Figure 2-4 Bioconversion of flavonols (kaempferol and quercetin) to their 6-*C*-glucosides by the Ec-WjGT1 system

Each panel shows the UPLC-MS results of kaempferol (A–C) and quercetin (D–F) bioconversion at 0 h (A, D) and 1 h (B, E), and the purified products of the bioconversion following sequential administration of the substrates and their MS-spectrum (C, F). Peak identification: K, kaempferol; Q, quercetin; k1 (kaempferol 6-*C*-glucoside) and q1 (quercetin 6-*C*-glucoside), product peaks of kaempferol and quercetin conversion; IS, internal standard (chrysin).

2.4.2. Scale-up production of flavone and flavonol 6-*C*-glucosides

The production of flavonoid *C*-glucosides is getting more attention because of their high biological activity and stability. A microbial system is a simple and easy way to produce specific glucosides. Therefore, the Ec-WjGT1 was scaled up to test an efficient bioconversion of flavonoid *C*-glucosides. First, different concentrations of apigenin such as 10 μM, 20 μM, 50 μM, 100 μM, and 200 μM were examined for bioconversion reaction. Somehow, more than 20 μM concentrations of the substrate immediately precipitated in the M9 medium and could not be completely converted to its glucoside. Thus, low concentrations of substrates including 20 μM apigenin, 40 μM kaempferol, and 50 μM quercetin were selected for the experiment. A requirement of glucose concentration was also examined to optimize the reaction condition. The cell culture was suspended in M9 media containing 0%, 0.2%, 0.5%, and 2% of glucose for conversion of 20 μM apigenin. When 2% (w/v) of glucose was contained in the medium, the conversion reaction finished in 30 min, which was faster than those in other concentrations (**Fig. 2-5**). Hence glucose concentration was fixed at 2% (w/v) for the scale-up reaction to maintain bioconversion efficiency.

In scale-up reaction, apigenin, kaempferol, and quercetin were supplied to cell culture every 15 min, 30 min, and 60 min, and the total cycles were twenty, six, and seven, respectively (**Fig. 2-6**). A total of 53 mg of quercetin, 52 mg of apigenin, and 30 mg of kaempferol were sequentially administered to 500 mL, 480 mL, and 440 mL cell culture, respectively, and bioconversion process was performed at 30 °C and 150 rpm overnight. The bioconversion efficiency of isovitexin, kaempferol *C*-glucoside, and quercetin *C*-glucoside were 99%, 98.5%, and 89%, and the production yields were approximately 171.8, 105.1, and 144.9 mg/L, respectively.

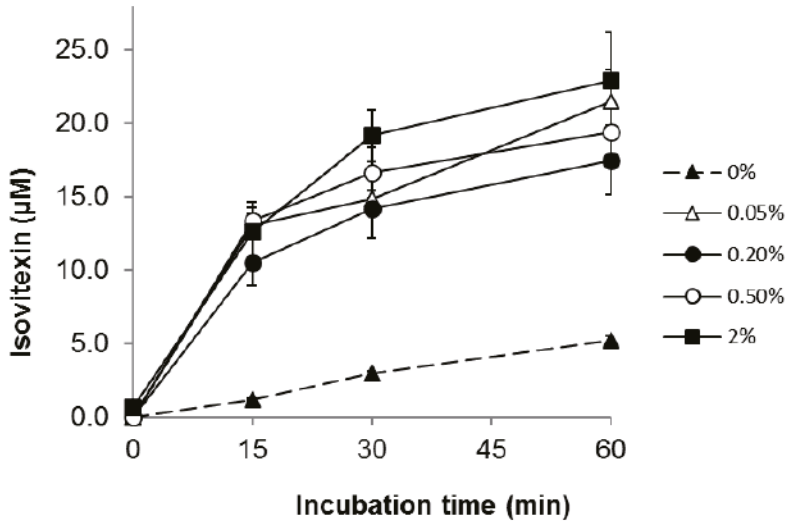


Figure 2-5 Effect of glucose concentration on bioconversion of apigenin by the Ec-WjGT1 system

Bioconversion was performed in M9 minimal media containing 0%, 0.05%, 0.2%, 0.5%, and 2% (w/v) of glucose with the addition of 20 μM apigenin, and the culture media was sampled during 60 min and analyzed by UPLC. The graph shows a concentration of isovitexin in the media of each condition.

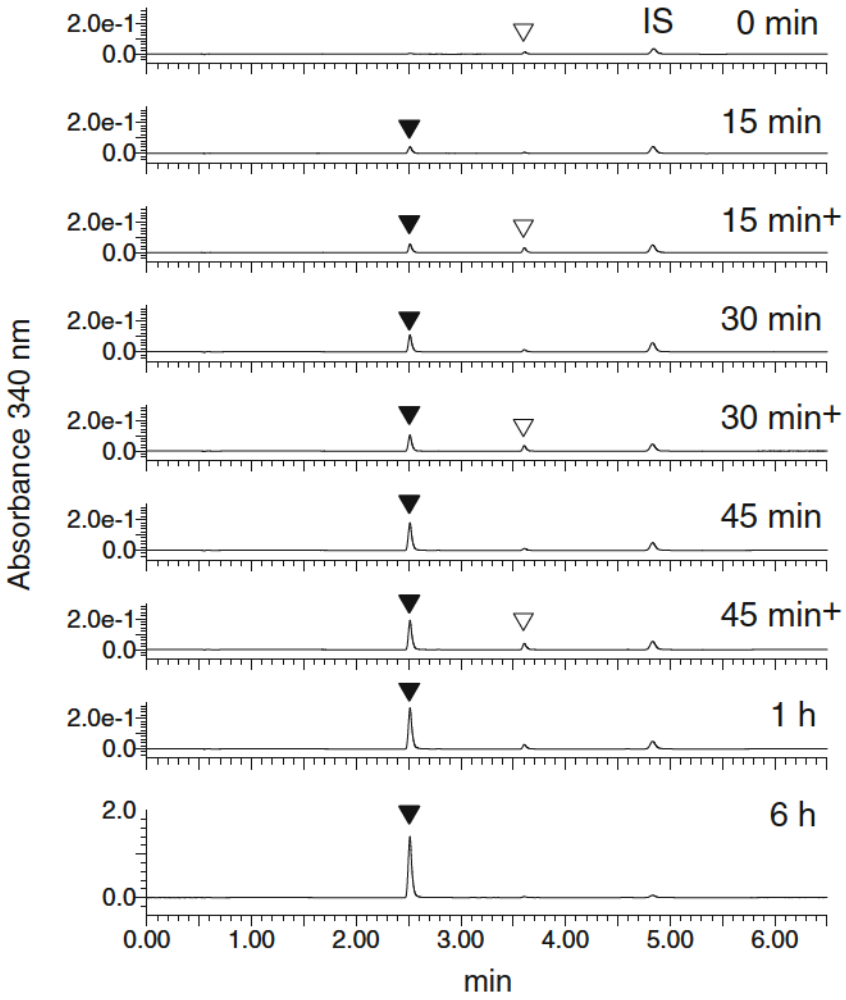


Figure 2-6 The production of isovitexin by the Ec-WjGT1 system

Apigenin (20 μM) was administered sequentially to the media every 15 min resulting in a total of 20 times introduction. The results of the first four administrations, until 1 h, and the final product after 6 h are shown. The "+" indicates the addition of apigenin. Closed and open triangles indicate the product (isovitexin) and the addition of the substrate (apigenin), respectively. IS indicates the internal standard (chrysin) which was added to the sample before UPLC.

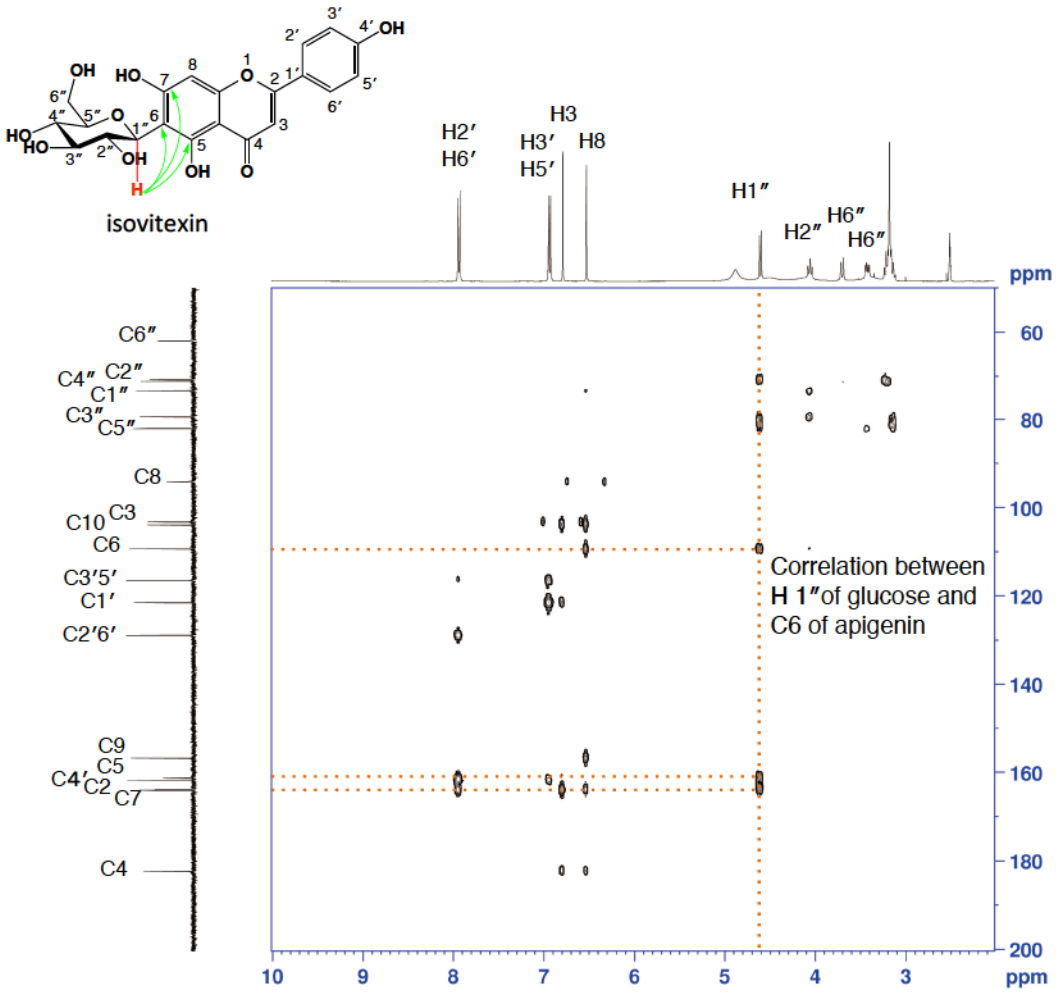
2.4.3. Purification of 6-*C*-glucosides and NMR analysis

The products were purified using an ODS column then concentrated by evaporation. Approximately 25 mg of each crystallized product was dissolved (DMSO)-d_6 for NMR analysis. The analysis showed that signals from the H-6 were not observed in ^{1}H-NMR; moreover, correlations between H-1″ (glucose moiety, δ 4.60, 4.61 and 4.61) and C-6 (109.4, 108.6 and 108.7) were observed in heteronuclear multiple bond correlation (HMBC) analysis of the converted products of apigenin, quercetin, and kaempferol, respectively. This result indicates that they are 6-*C*-glucosides of flavone and flavonol (**Table 2-1** and **Fig. 2-7**).

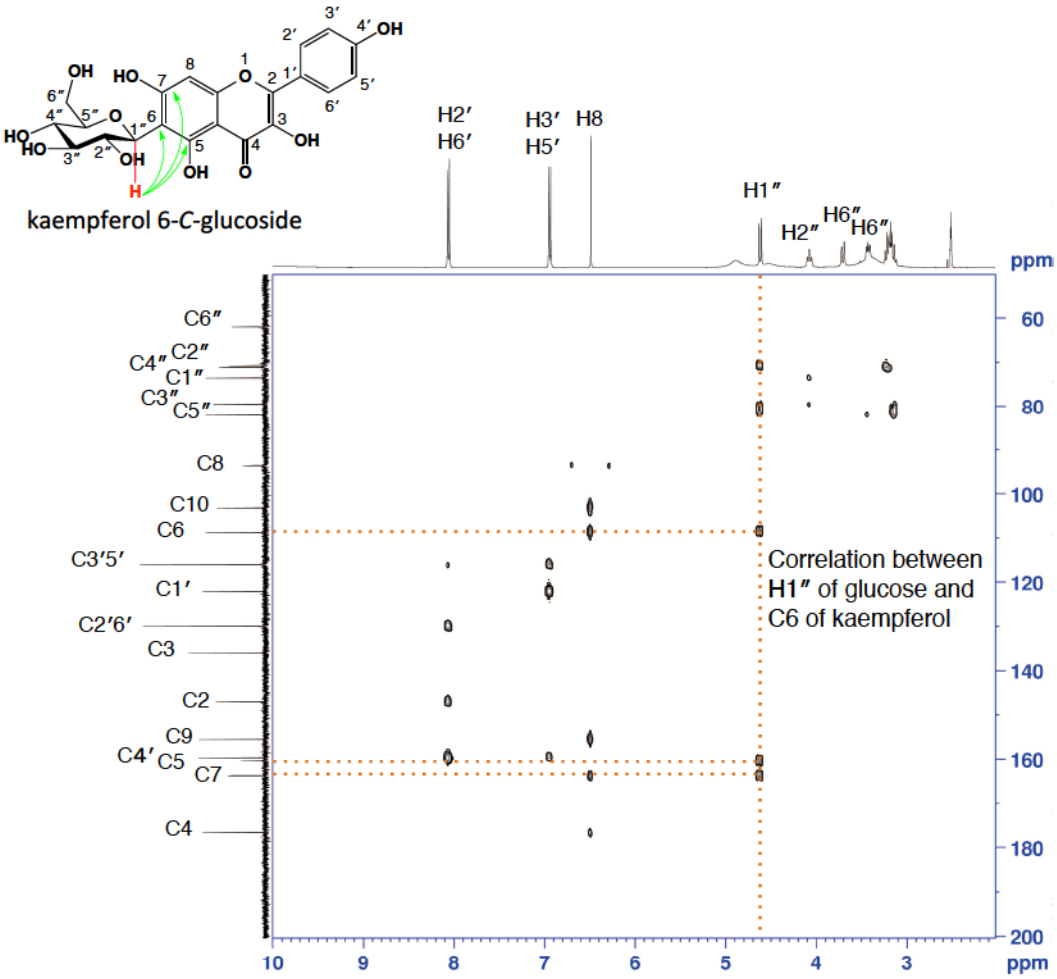
2.4.4. Bioconversion of other substrates

Naringenin (a flavanone) and resveratrol (a stilbene) were examined to understand an acceptance of the substrate in the Ec-WjGT1 system. Both substrates were administered once to 10 mL cell culture at a final concentration of 50 μM and incubated for 6 h. Two peaks of product were observed by the UPLC analysis when naringenin was used, and the LC-MS analysis showed that the main product is presumed to be a naringenin 6-*C*-glucoside. Interestingly, the MS spectrum of a minor product was much similar to that of the main product. These results proved that both products are naringenin *C*-glucosides (**Fig. 2-8**), somehow, naringenin could be glycosylated at the other position such as 3′ or 8-position next to a phenolic hydroxyl group. The conversion efficiency of naringenin over a 6 h period was 60%. The Ec-WjGT1 glucosylated only a small amount of resveratrol at a rate of 14% conversion. The signal of mass spectrum presented with a $[M-H]^-$ ion at *m/z* 389, suggesting that the product could be resveratrol glucoside (**Fig. 2-9**), but the structure could not be determined because of the less product.

A


isovitexin
H2′
H6′
H3
H3′
H5′
H8
H1″
H2″
H6″
H6″
ppm
C6″
C4″
C2″
C1″
C3″
C5″
C8
C10
C3
C6
C3′5′
C1′
C2′6′
C9
C5
C4′
C2
C7
C4
Correlation between
H 1″of glucose and
C6 of apigenin
60
80
100
120
140
160
180
200
10
9
8
7
6
5
4
3
ppm

B


kaempferol 6-C-glucoside
H2′
H6′
H3′
H5′
H8
H1″
H2″
H6″
H6″
ppm
C6″
C4″
C2″
C1″
C3″
C5″
C8
C10
C6
C3′5′
C1′
C2′6′
C3
C2
C9
C4′
C5
C7
C4
Correlation between
H1″ of glucose and
C6 of kaempferol
60
80
100
120
140
160
180
200
10
9
8
7
6
5
4
3
ppm

C

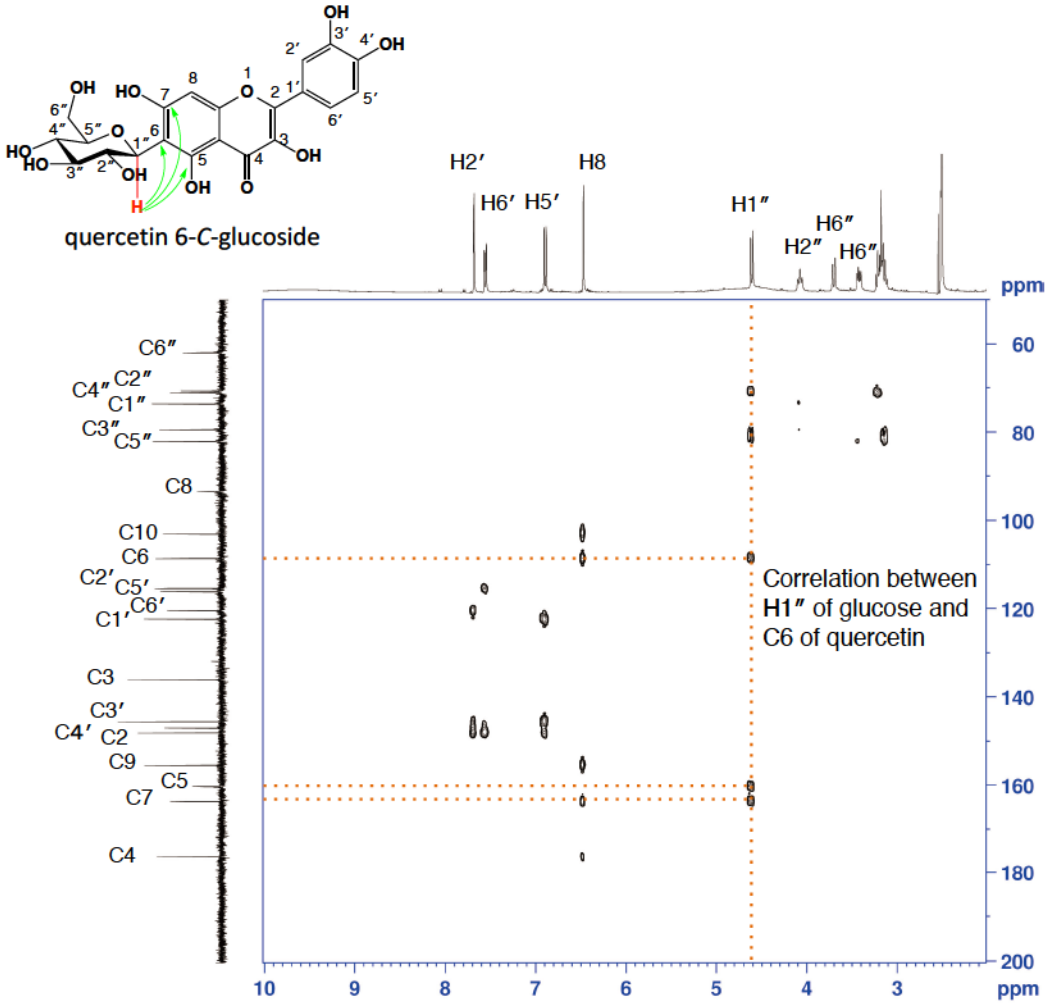


Figure 2-7 Heteronuclear multiple bond correlation (HMBC) analysis of the purified compounds produced by bioconversion process

The purified products were dissolved in DMSO-*d6* and analyzed by NMR. Each panel shows the result of HMBC analysis of isovitexin (A), kaempferol 6-*C*-glucoside (B), and quercetin 6-*C*-glucoside (C), respectively. Green arrows in chemical structure indicate the HMBC correlation between H-1″ of glucose and carbon of flavonoid.

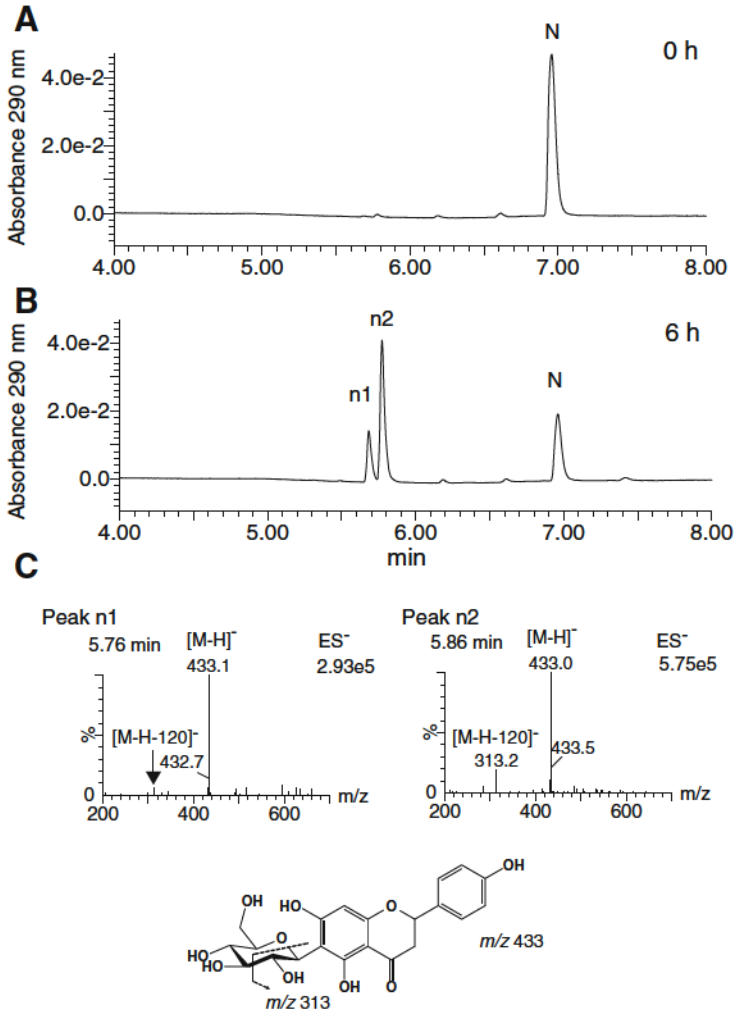


Figure 2-8 Bioconversion of naringenin by the Ec-WjGT1 system

Each panel shows the UPLC-MS result for naringenin bioconversion at 0 h (A) and 6 h (B), and the product MS spectra (C). The MS analyses of the product display $[M-H]^-$ ion at *m/z* 433 and $[M-H-120]^-$ ion at *m/z* 313, which are typical fragmentations of *C*-glucoside. Peak identification: N, naringenin; n1 and n2, product.

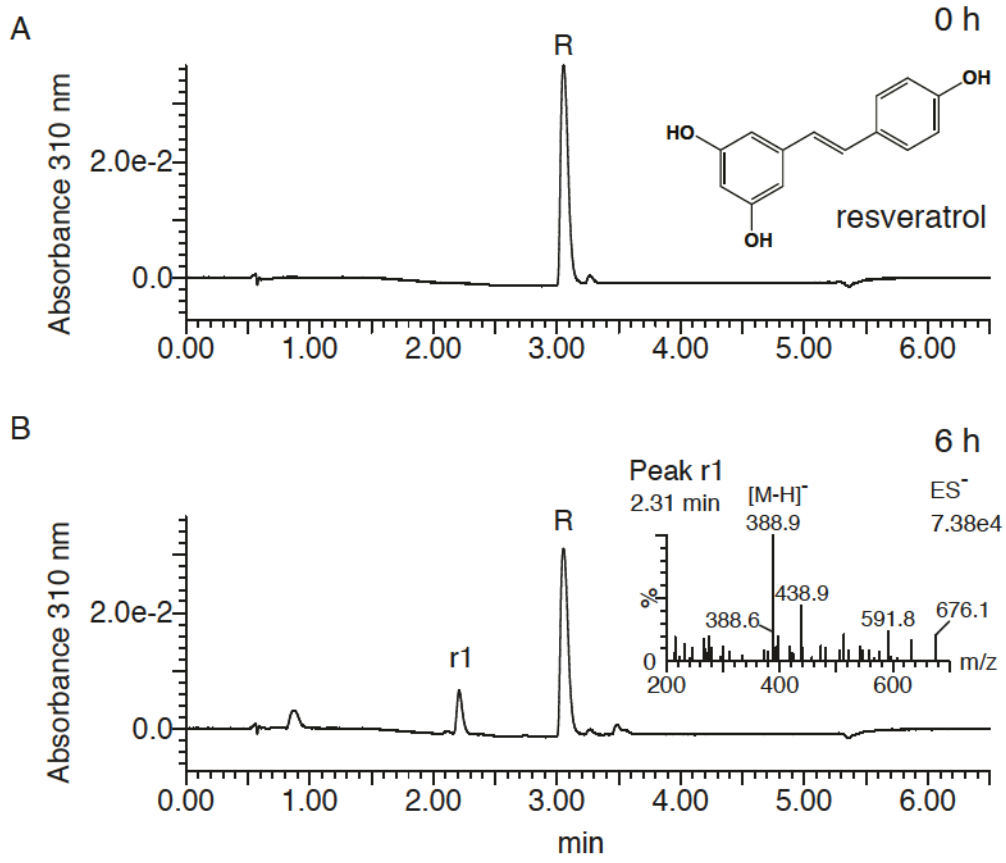


Figure 2-9 Bioconversion of resveratrol by the Ec-WjGT1 system

The UPLC-MS result of resveratrol bioconversion at 0 h (A) and 6 h (B) and the product MS spectrum. Peak identification: R, resveratrol; r1, product.

2.5. DISCUSSION

Plant flavonoids serve a variety of biological functions, and their glycosides are of particular interest as this modification often alters their underlying properties. Various flavonoid glycosides have been produced using recombinant microbial systems augmented with GTs originating from both plants and microorganisms, but most of these systems produce *O*-glycosides. The production of flavonoid *C*-glycosides is limited because of the limited number of *C*-glycosyltransferases (CGTs) currently characterized. Bioconversion of flavonoids into their *C*-glycosides has been performed using rice OsCGT or buckwheat FeCGTa (Brazier-Hicks and Edwards 2013; Ito et al. 2014); however, these glycosides were mixtures of 6-*C*- and 8-*C*-flavonoid glucosides due to the reactivity of the enzymes. In addition, bioconversion using citrus FcCGT successfully produced 6,8-di-*C*-glycosyl flavonoids (Ito et al. 2017). Recently, the de novo synthesis of these compounds from tyrosine was reported in *E. coli* augmented with a CGT isolated from bamboo, which also produces a mixture of *C*-glucosides (Sun et al. 2020).

In recent years, the production of only the 6-*C*-glucosides of flavone by either *E. coli* or yeast has been reported (Shrestha et al. 2018; Vanegas et al. 2018) with relying on GtUF6CGT derived from gentian (Sasaki et al. 2015). Improved productivity of *C*-glucosides has also been achieved by combining cellobiose phosphorylase and controlling the concentration of acetic acid (Pei et al. 2018). However, flavonol *C*-glycosides are not produced by these biological processes. In this chapter, both flavonol 6-*C*-glucosides and flavone 6-*C*-glucosides were produced easily and quickly using the Ec-WjGT1 system which is *E. coli* expressing WjGT1 enzyme from wasabi (Mashima et al. 2019). This enzyme shows a broader substrate specificity for aglycones than GtUF6CGT (Sasaki et al. 2015), which made the conversion of multiple substrates possible. The conversion rate also reached 89–99%, depending on the substrate, after sequential administration of low concentrations of substrate,

which was similar to the previous study describing the use of substrates with low solubility in these systems (Ito et al. 2014). Furthermore, the Ec-WjGT1 system converted flavanone (naringenin) into its *C*-glucosides, suggesting that it can be used for the bioconversion of a wide range of substrates. Recently, the structures of several CGTs have been reported, and their substrate recognition has also been elucidated (He et al. 2019; Zhang et al. 2020). Hence, it may be possible to expand the application of the Ec-WjGT1 system for the production of various *C*-glucosides using the genetically modified enzyme with altered substrate specificity.

2.6. REFERENCES

Brazier-Hicks M, Edwards R (2013) Metabolic engineering of the flavone-*C*-glycoside pathway using polyprotein technology. Metab Eng 16:11-20

Choi JS, Islam MN, Ali MY, Kim EJ, Kim YM, Jung HA (2014) Effects of *C*-glycosylation on anti-diabetic, anti-Alzheimer's disease and anti-inflammatory potential of apigenin. Food Chem Toxicol 64:27-33

De Bruyn F, Van Brempt M, Maertens J, Van Bellegem W, Duchi D, De Mey M (2015) Metabolic engineering of *Escherichia coli* into a versatile glycosylation platform: production of bio-active quercetin glycosides. Microb Cell Fact 14:138

de Villiers A, Venter P, Pasch H (2016) Recent advances and trends in the liquid-chromatography–mass spectrometry analysis of flavonoids. J Chromatogr A 1430:16-78

Hamidullah, Kumar R, Saini KS, Kumar A, Kumar S, Ramakrishna E, Maurya R, Konwar R, Chattopadhyay N (2015) Quercetin-6-*C*-*β*-D-glucopyranoside, natural analog of quercetin exhibits anti-prostate cancer activity by inhibiting Akt-mTOR pathway via aryl hydrocarbon receptor. Biochimie 119:68-79

He JB, Zhao P, Hu ZM, Liu S, Kuang Y, Zhang M, Li B, Yun CH, Qiao X, Ye M (2019) Molecular and structural characterization of a promiscuous *C*-glycosyltransferase from *Trollius chinensis*. Angew Chem Int Ed 58:11513-11520

Ito T, Fujimoto S, Shimosaka M, Taguchi G (2014) Production of *C*-glucosides of flavonoids and related compounds by *Escherichia coli* expressing buckwheat *C*-glucosyltransferase. Plant Biotechnol 31:519-524

Ito T, Fujimoto S, Suito F, Shimosaka M, Taguchi G (2017) *C*-glycosyltransferases catalyzing the formation of di-*C*-glucosyl flavonoids in citrus plants. Plant J 91:187-198

Iwashina T (2000) The structure and distribution of the flavonoids in plants. J Plant Res 113:287

Jay M, Viricel MR, Gonnet JF (2006) *C*-glycosylflavonoids. In: Andersen ØM, Markham KR (eds) Flavonoids: chemistry, biochemistry and application. CRC Press, Boca Raton, FL, pp 858-903

Maatooq GT, El-Sharkawy SH, Afifi M, Rosazza JP (1997) *C*-*p*-hydroxybenzoylglycoflavones from *Citrullus colocynthis.* Phytochemistry 44:187-190

Markham K, Ternai B, Stanley R, Geiger H, Mabry T (1978) Carbon-13 NMR studies of flavonoids—III: naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron 34:1389-1397

Mashima K, Hatano M, Suzuki H, Shimosaka M, Taguchi G (2019) Identification and characterization of apigenin 6-*C*-glucosyltransferase involved in biosynthesis of isosaponarin in wasabi (*Eutrema japonicum*). Plant Cell Physiol 60:2733-2743

Pei J, Sun Q, Zhao L, Shi H, Tang F, Cao F (2018) Efficient biotransformation of luteolin to isoorientin through adjusting induction strategy, controlling acetic acid, and increasing UDP-glucose supply in *Escherichia coli*. J Agric Food Chem 67:331-340

Sambrook J, Russell DW (2001) Molecular cloning a laboratory manual. (3rd ed., Vol 3), Cold spring harbor laboratory press, NY A2.2

Sasaki N, Nishizaki Y, Yamada E, Tatsuzawa F, Nakatsuka T, Takahashi H, Nishihara M (2015) Identification of the glucosyltransferase that mediates direct flavone *C*-glucosylation in *Gentiana triflora*. FEBS Lett 589:182-187

Shrestha A, Pandey RP, Dhakal D, Parajuli P, Sohng JK (2018) Biosynthesis of flavone *C*-glucosides in engineered *Escherichia coli*. Appl Microbiol Biotechnol 102:1251-1267

Sun Y, Chen Z, Yang J, Mutanda I, Li S, Zhang Q, Zhang Y, Zhang Y, Wang Y (2020) Pathway-specific enzymes from bamboo and crop leaves biosynthesize anti-nociceptive *C*-glycosylated flavones. Commun Biol 3:1-11

Talhi O, Silva A (2012) Advances in *C*-glycosylflavonoid research. Curr Org Chem 16:859-896

Vanegas KG, Larsen AB, Eichenberger M, Fischer D, Mortensen UH, Naesby M (2018) Indirect and direct routes to *C*-glycosylated flavones in *Saccharomyces cerevisiae*. Microb Cell Fac 17:107

Williams CA (2006) Flavone and flavonol *O*-glycosides. In: Andersen ØM, Markham KR (eds) Flavonoids: chemistry, biochemistry and application. CRC Press, Boca Rato, FL, pp 749-824

Xiao J, Capanoglu E, Jassbi AR, Miron A (2016) Advance on the flavonoid *C*-glycosides and health benefits. Crit Rev Food Sci Nutr 56:S29-S45

Xiao J, Muzashvili TS, Georgiev MI (2014) Advances in the biotechnological glycosylation of valuable flavonoids. Biotechnol Adv 32:1145-1156

Zhang M, Li F-D, Li K, Wang Z-L, Wang Y-X, He J-B, Su H-F, Zhang Z-Y, Chi C-B, Shi X-M (2020) Functional characterization and structural basis of an efficient di-*C*-glycosyltransferase from *Glycyrrhiza glabra*. J Am Chem Soc 142:3506-3512

Chapter 3

Production of cinnamate and benzoate glucosides by bioconversion using *Escherichia coli* expressing a cinnamate glucosyltransferase from sweet potato (IbGT1)

3.1. ABSTRACT

In this chapter, I studied the production of glucose esters using *Escherichia coli* expressing a cinnamate glucosyltransferase (IbGT1) from sweet potato. The recombinant IbGT1 was found to react toward not only several kinds of cinnamates but also some benzoates, such as *p*-hydroxybenzoic acid and benzoic acid. *E. coli* cells expressing IbGT1 (Ec-IbGT1 system) converted 200 μM cinnamates (*t*-cinnamic acid, *p*-coumaric acid, ferulic acid, and *o*-coumaric acid) into their glucose esters mostly within 3 h. The conversion rates of caffeic acid and sinapic acid were lower than other substrates tested, though enzyme activities toward these compounds were comparable to others. The system also converted *p*-hydroxybenzoic acid into its glucose ester. A scaled-up production combined with sequential administration of sinapic acid and *p*-hydroxybenzoic acid yielded their glucose esters with conversion rates of 69% and 98%, respectively.

Chapter 4

Production of glucosides of flavonoids and phenolics by bioconversion using *Escherichia coli* expressing an *O*-glucosyltransferase from tobacco (NtGT2 or NtGT3)

4.1. ABSTRACT

In this chapter, production of flavonoid *O*-glucosides and phenolic glucosides using *E. coli* cells expressing respective NtGT2 (Ec-NtGT2) and NtGT3 (Ec-NtGT3) from tobacco was studied. Ec-NtGT2 system converted flavonols (quercetin and kaempferol, 50 μM), flavones (apigenin and luteolin, 20 μM), and flavanone (naringenin, 50 μM) into their 7-*O*-glucosides in 1 h with conversion rates of 67–98%. In scaled-up production, the system yielded 59 mg/L of apigenin 7-*O*-glucoside, 52 mg/L of luteolin 7-*O*-glucoside, 118 mg/L of quercetin 7-*O*-glucoside, 90 mg/L of kaempferol 7-*O*-glucoside, and 166 mg/L of naringenin 7-*O*-glucoside through sequential administration of substrates in 4–9 h. The conversion rates of apigenin, luteolin, quercetin, kaempferol, and naringenin were 97%, 72%, 77%, 98%, and 96%, respectively. Similarly, Ec-NtGT3 system converted flavonols (quercetin and kaempferol, 50 μM) and phenolic compounds (1-naphthol, 2-naphthol, and *p*-nitrophenol, 100 μM) into flavonol 3-*O*-glucosides and phenolic glucosides, respectively with conversion rates of 75–96%.

4.2. INTRODUCTION

Flavonoids are natural phytochemicals with various health benefits, including anti-allergenic, anti-cancer, and anti-cardiovascular disease properties (Harborne and Williams 2000). These compounds typically accumulate in plants mostly as *O*-glycosides, although *C*-glycosides also accumulate (Williams 2006). More than 2000 types of flavonoid *O*-glycosides have been identified and are commonly used in pigments, cosmetics, and natural medicines (Wang et al. 2019; Williams 2006). Flavonoids in plants are typically glycosylated at the 3- or 7- hydroxyl group, and rarely at the 4′- hydroxyl group (Yang et al. 2018).

Enzymatic production using plant UGT enzymes is an efficient method for the regiospecific synthesis of flavonoid glycosides, as the flavonoid structure has many hydroxyl

groups that can be glycosylated. Furthermore, microbial systems expressing these UGTs are economic and convenient because they supply an endogenous source of costly and unstable cofactors. The production of some *O*-glycosides has been studied in microbial systems (De Bruyn et al. 2015; Ji et al. 2020; Pandey et al. 2013). However, most of the compounds produced are flavonoid 3-*O*-glycosides, whereas the production of a few flavonoid 7-*O*-glycosides has been evaluated (Kim et al. 2015). The production system for flavonoid 7-*O*-glucosides still has some difficulties with regard to final product efficiency and production period (He et al. 2008; Koirala et al. 2019; Penso et al. 2014; Thuan et al. 2018; Zhu et al. 2020). One reason for this is the reactivity of the enzyme. Therefore, Zhu et al. improved the enzyme reactivity through point mutation of amino acid residues to increase the product efficiency, but the production period was not shortened (Zhu et al. 2020).

In this chapter, the production of flavonoid *O*-glucosides and phenolic glucosides using *E. coli* cells expressing either of two glucosyltransferases isolated from tobacco (*Nicotiana tabacum*), NtGT2 (UGT75L3) denoted as Ec-NtGT2 and NtGT3 (UGT71A11) denoted as Ec-NtGT3 was investigated. The NtGT2 catalyzes a glucosylation of the 7-hydroxyl group of flavonoids, although it was extremely unstable after protein extraction (Taguchi et al. 2003b). In contrast, the NtGT3 was more stable and catalyzes a glucosylation of broad substrates, including 3-hydroxyl group of flavonoids, 7-hydroxyl group of coumarins, and naphthols (Taguchi et al. 2003a). Here, I presented a production of flavonoid 7-*O*-glucosides and 3-*O*-glucosides using Ec-NtGT2 and Ec-NtGT3, respectively (**Fig. 4-1**). The Ec-NtGT3 system was also examined to transform phenolic compounds (naphthols and *p*-nitrophenol) into their glucosides.

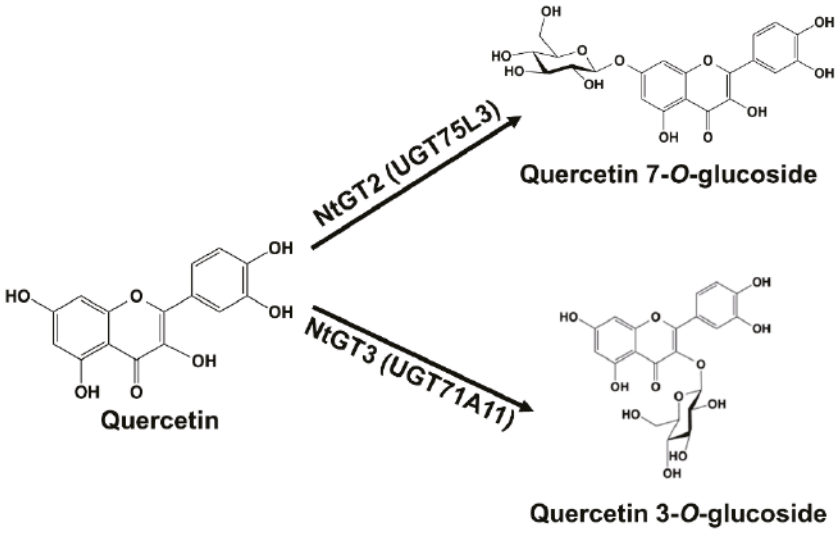


Figure 4-1 Overview of NtGT2 and NtGT3 reactions.

NtGT2 and NtGT3 catalyze 7-*O*- and 3-*O*-glucosylation of flavonoid, respectively.

4.3. MATERIALS AND METHODS

4.3.1. Reagents

Substrates and standard compounds were obtained as follows; apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside, naringenin 7-*O*-glucoside (Extrasynthèse); 1-naphthol, 2-naphthol, and *p*-nitrophenol (Wako Pure Chemical Industry); *p*-nitrophenol glucoside (Nacalai Tesque). Kaempferol 7-*O*-glucoside and quercetin 7-*O*-glucoside were enzymatically synthesized in our laboratory as previous method (Taguchi et al. 2003b). 1-Naphthol glucoside and 2-naphthol glucoside were synthesized from 1-naphthol and 2-naphthol by the method previously described (Taguchi et al. 2003a). Others were listed in Chapter 2.

4.3.2. Bacterial cell culture and protein expression

E. coli BL21(DE3) cells expressing NtGT2 (UGT75L3, GenBank Accession No. AB072919) or NtGT3 (UGT71A11, GenBank Accession No. AB072918) cloned into the pET-28c (+) vector (pET-NtGT2 (Taguchi et al. 2003b) or (pET-NtGT3 (Taguchi et al. 2003a)) were used

in this work. Bacterial cell culture and protein expression were performed as described in Chapter 2, using LB broth containing 50 mg/L kanamycin.

4.3.3. Substrate bioconversion

Bioconversion of each substrate was performed as described in Chapter 2. The Ec-NtGT2 was treated with 50 μM flavonols (quercetin and kaempferol), 20 μM flavones (apigenin and luteolin) or 50 μM flavanone (naringenin). The Ec-NtGT3 was treated with 50 μM flavonols (quercetin and kaempferol) or 100 μM phenolic compounds (1-naphthol, 2-naphthol, and *p*-nitrophenol). The bioconversion process was carried out at 30 °C for 1 h.

4.3.4. Scale-up production of the flavonoid 7-*O*-glucosides

A total of 400–760 mL cell cultures were sequentially administered 20 μM of apigenin or luteolin, 40 μM of kaempferol, and 50 μM of quercetin or naringenin every 30–60 min in five to eight cycles, as in Chapter 2. The culture was then incubated at 30 °C for 4–9 h.

4.3.5. Purification of the products

The cells were centrifuged, and the supernatant was applied to a Sep-Pak C18 column (35 cc, Silica C18-10g; Waters) prewashed with 100% methanol and equilibrated with 20% methanol, as in Chapter 2. Each compound was then eluted using a linear gradient of 150 mL of 20–60% methanol, and the fraction containing the final product was concentrated by rotary evaporation, followed by centrifugation.

4.3.6. UPLC-MS analysis

The condition and method of UPLC-MS was described in Chapter 2. For flavonoid glucosides, LC-Method A and B were used. For naphthol glucosides, the column was eluted with 20% solvent B (methanol supplemented with 0.1% formic acid) in solvent A (0.1% formic acid) for 30 s, followed by a gradient from 20–60% solvent B for 4 min, 60% solvent B in A for 1 min 30 s, and 20% solvent B in A for 2 min at a flow rate of 0.25 mL min^{-1} and temperature of 40 °C (**LC-Method D**). For *p*-nitrophenol glucoside, the column was eluted with 5% solvent B in solvent A for 30 s, followed by a gradient from 5–20% solvent B for 3 min, 20% solvent B in solvent A for 30 s, then a gradient from 20–60% solvent B for 2 min, 60% solvent B in solvent A for 2 min 30 s, and 5% solvent B in solvent A for 1 min 30 s at the same rate and temperature (**LC-Method E**). The eluent was monitored at 275 (2-naphthol), 285 (1-naphthol), and 315 nm (*p*-nitrophenol).

4.3.7. NMR analysis

NMR analysis was performed using the same equipment as Chapter 2. The recorded spectrometer was compared with the reported NMR spectra of related compounds (Markham et al. 1978; Shimoda et al. 2010). The NMR assignments are listed in **Table 4-1 A–C**.

Table 4-1 A NMR spectroscopy assignment of the kaempferol 7-*O*-glucoside and quercetin 7-*O*-glucoside produced by the Ec-NtGT2 system (400 MHz, dimethyl sulfoxide-d_6 (DMSO-d_6))

	Kaempferol 7-*O*-glucoside			Quercetin7-*O*-glucoside		
	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)
Flavonoid						
2	-	-	148.0	-	-	148.4
3	-	-	136.5	-	-	136.6
4	-	-	176.6	-	-	176.5
5	-	-	160.8	-	-	160.8
6	6.43	*d* (2.1)	99.2	6.43	*d* (2.1)	99.2
7	-	-	163.2	-	-	163.2
8	6.81	*d* (2.1)	94.8	6.78	*d* (2.1)	94.7
9	-	-	156.2	-	-	156.2
10	-	-	105.2	-	-	105.1
1′	-	-	122.0	-	-	122.3
2′	8.08	*dd* (9.0, 2.8)	130.1	7.73	*d* (2.2)	115.8
3′	6.95	*dd* (9.0, 2.8)	116.0	-	-	145.5
4′	-	-	159.9	-	-	148.0
5′	6.95	*dd* (9.0, 2.8)	116.0	6.91	*d* (8.5)	116.1
6′	8.08	*dd* (9.0, 2.8)	130.1	7.56	*dd* (8.5, 2.2)	120.5
Glucose moiety						
1″	5.08	*d* (7.4)	100.3	5.09	*d* (7.4)	100.3
2″	3.25-3.34	*m*	73.6	3.25-3.32	*m*	73.6
3″	3.25-3.34	*m*	76.9	3.25-3.32	*m*	76.9
4″	3.19	*dd* (8.9, 8.9)	70.0	3.19	*dd* (8.8, 8.8)	70.0
5″	3.44-3.51	*m*	77.6	3.44-3.50	*m*	77.6
6″	3.72	*d* (9.8)	61.1	3.72	*d* (10.0)	61.1
	3.44-3.51	*m*		3.44-3.50	*m*	
Aromatic-OH						
	12.5	1H, *s*		12.52	1H, *s*	
	10.18	1H, *s*				
	9.55	1H, *s*		9.52	3H, *br*	
Glucose-OH						
				5.41	1H, *br*	
				5.10	2H, *br*	
				4.63	1H, br	

Table 4-1 B NMR spectroscopy assignment of the apigenin 7-*O*-glucoside and luteolin 7-*O*-glucoside produced by the Ec-NtGT2 system (400 MHz, dimethyl sulfoxide-d_6 (DMSO-d_6))

	Apigenin 7-*O*-glucoside			Luteolin 7-*O*-glucoside		
	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	^{1}H (δ)	*J* (Hz)	^{13}C (δ)
Flavonoid						
2	-	-	164.8	-	-	164.8
3	6.87	*s*	103.5	6.68	*s*	103.5
4	-	-	182.5	-	-	182.5
5	-	-	161.6	-	-	161.6
6	6.45	*d* (2.2)	100.0	6.38	*d* (2.1)	100.0
7	-	-	163.4	-	-	163.4
8	6.84	*d* (2.1)	95.3	6.72	*d* (1.9)	95.3
9	-	-	157.4	-	-	157.4
10	-	-	105.8	-	-	105.8
1′	-	-	121.4	-	-	121.4
2′	7.96	*d* (8.8)	129.1	7.36	*s*	129.1
3′	6.95	*d* (8.8)	116.5	-	-	116.5
4′	-	-	162.0	-	-	162.0
5′	6.95	*d* (8.8)	116.5	6.84	*d* (8.0)	116.5
6′	7.96	*d* (8.8)	129.1	7.38	*d* (8.4)	129.1
Glucose moiety						
1″	5.11	*d* (13.2)	100.4	5.02	*d* (7.3)	100.4
2″	3.26-3.31	*m*	73.6	3.18-3.27	*m*	73.6
3″	3.30-3.35	*m*	76.9	3.18-3.27	*m*	76.9
4″	3.20	*dd* (8.8, 8.8)	70.0	3.12	*dd* (7.6, 7.6)	70.0
5″	3.44-3.49	*m*	77.6	3.37-3.44	*m*	77.6
6″	3.73	*d* (10.2)	61.1	3.65	*d* (10.3)	61.1
	3.46-3.52	*m*		3.37-3.44	*m*	
Aromatic-OH						
	13.00	1H, *s*		12.92	1H, *s*	
Glucose-OH						
	5.41	2H, *br*				
	4.64	1H, *br*				

Table 4-1 C NMR spectroscopy assignment of the naringenin 7-*O*-glucoside produced by the Ec-NtGT2 system (400 MHz, dimethyl sulfoxide-d_6 (DMSO-d_6))

	Naringenin 7-*O*-glucoside			
	^{1}H (δ)	*J* (Hz)	^{13}C (δ)	
Flavonoid				
2	5.50	*dd* (12.8, 2.9)	79.2	
3	3.31-3.40	*m*	42.5	
	2.74	*dd* (17.0, 2.6)		
4	-	-	197.7	(197.7)
5	-	-	163.4	(163.4)
6	6.14	*d* (2.2)	97.0	
7	-	-	165.8	(165.7)
8	6.16	*d* (2.1)	95.9	
9	-	-	163.3	(163.2)
10	-	-	103.7	
1′	-	-	129.1	(129.1)
2′	7.33	*d* (8.6)	128.9	
3′	6.8	*d* (8.5)	115.7	
4′	-	-	158.3	
5′	6.8	*d* (8.5)	115.7	
6′	7.33	*d* (8.6)	128.9	
Glucose moiety				
1″	4.98	*dd* (9.4, 7.6)	100.1	(99.9)
2″	3.20-3.28	*m*	73.5	
3″	3.20-3.28	*m*	77.6	
4″	3.15	*m*	69.9	
5″	3.31-3.40	*m*	76.8	
6″	3.66	*d* (11.5)	61.0	
	3.41	*dd* (12.2, 5.7)		
Aromatic-OH				
	12.06	1H, *s*		
Glucose-OH				
	4.55	6H, *br*		

4.4. RESULTS

4.4.1. Bioconversion of flavonoids to their 7-*O*-glucosides

Flavonoid 7-*O*-glucosides have received attention because of their various biological activities. The production of flavonoid 7-*O*-glucosides has been reported, but the production yield is lower than that of flavonoid 3-*O*-glucosides (Zhu et al. 2020). Thus, the NtGT2 that was isolated by our group and catalyzes regiospecific glucosylation of the 7-hydroxyl group of flavonols was analyzed for the production of flavonoid 7-*O*-glucosides (Taguchi et al. 2003b). The recombinant protein was expressed in *E. coli* (**Fig. 4-2**). However, it was unstable after protein extraction and unsuitable for the production of glucosides using a purified enzyme. Therefore, the Ec-NtGT2 system was examined as a whole-cell catalyst for bioconversion of some flavonoids to 7-*O*-glucosides. First, the system was administered 20 μM apigenin or luteolin. This low concentration of substrate was selected to prevent substrate precipitation, similar to the production of flavonoid *C*-glucosides, as mentioned in Chapter 2. Apigenin began to accumulate in the cells immediately after being added to the medium and was converted to its glucoside, which was detected in the medium. The glucoside peak (a1) presented an $[M-H]^-$ ion at *m/z* 431, which was confirmed to be apigenin 7-*O*-glucoside (A7G), when compared to the standard compound (**Fig. 4-3**). The concentration of apigenin in the medium started to decrease immediately after its addition; therefore, the amount of apigenin at 0 min was significantly less than the initial amount of administered substrate (20 μM) (**Fig. 4-3 B, D**). Approximately 19.6 μM of apigenin 7-*O*-glucoside was detected in the medium after 1 h, with a conversion rate of 98%. Luteolin was converted to its glucoside, presenting an $[M-H]^-$ ion at *m/z* 447 with a 67% conversion rate (peak l2, **Fig. 4-4 B–D**), and this glucoside was confirmed to be luteolin 7-*O*-glucoside (L7G) when compared to the standard compound. Minor peaks were also observed, one of which presented an $[M-H]^-$ ion at *m/z* 609 (peak l1, **Fig. 4-4 C–D**), corresponding to the di-glucoside of luteolin.

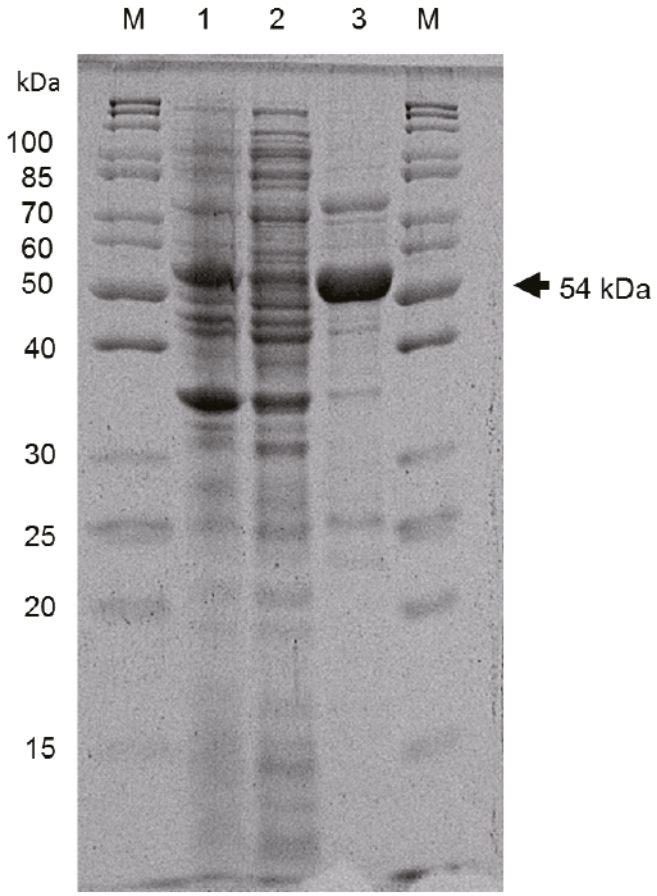


Figure 4-2 SDS-PAGE analysis of the recombinant NtGT2 protein expressed in *E. coli*. Proteins were separated on a 12.5% SDS-PAGE. Lane M, Size marker (PageRuler unstained protein ladder, ThermoFisher Scientific); lane 1 and 2, insoluble (**1**) and soluble (**2**) fraction of the cell-free extract; 3, recombinant NtGT2 protein purified with the 6×Histidine tag. Arrowhead indicates the size of the recombinant NtGT2 protein.

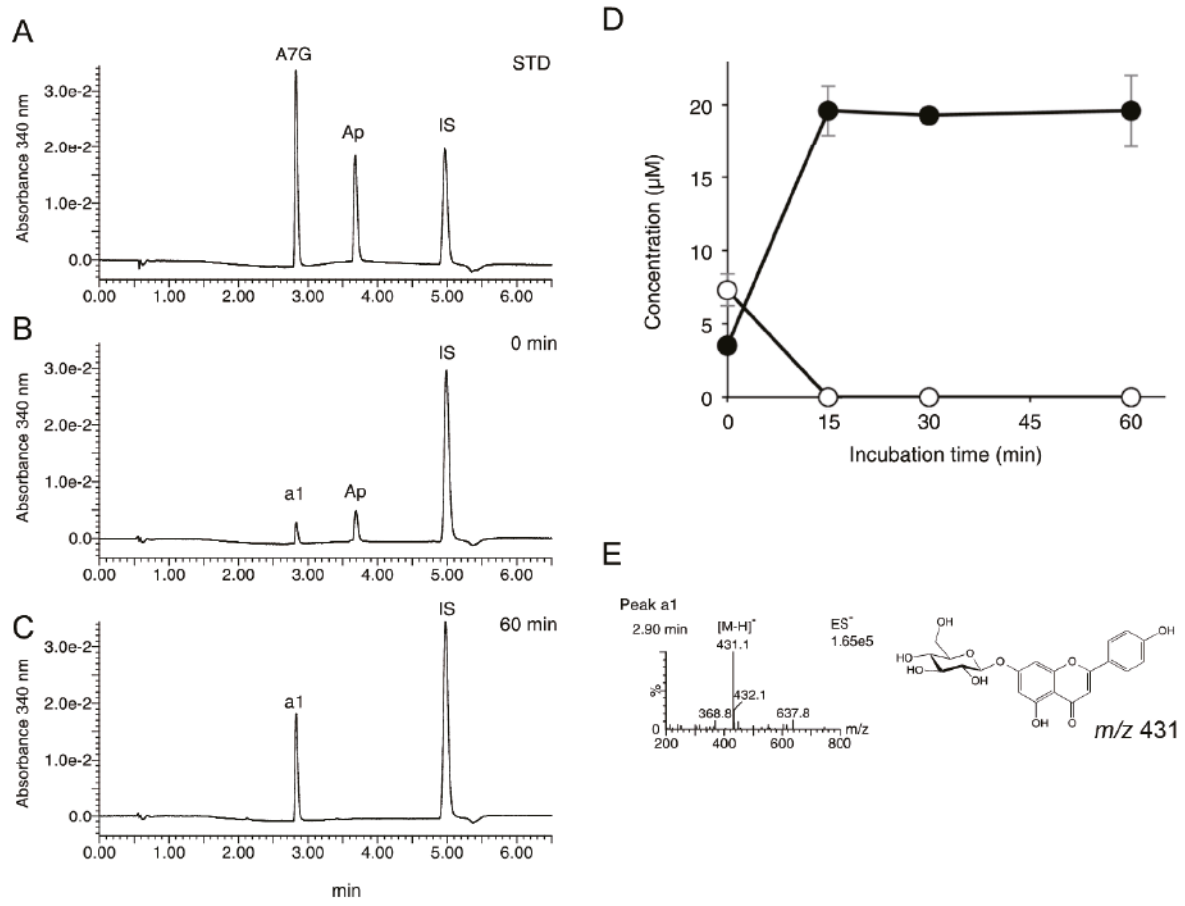


Figure 4-3 Bioconversion of apigenin using the Ec-NtGT2 system. A–C UPLC profiles of the standard of apigenin and apigenin 7-*O*-glucoside (A), and culture media at 0 min (B) and 60 min (C) after addition. Peak identification: Ap, apigenin; A7G, apigenin 7-*O*-glucoside; a1, product peak of apigenin conversion; and IS, internal standard (chrysin). Time course of the production of apigenin 7-*O*-glucoside after addition of 20 μM apigenin (D). Open and closed circles indicate the concentrations of apigenin and the product, respectively. Mass spectrum and chemical structure of the product (peak a1) (E).

In addition, the Ec-NtGT2 system was tested to examine the conversion of other flavonoids, including flavonols (kaempferol and quercetin) and a flavanone (naringenin). The system converted these flavonoids (50 μM) into *O*-glucosides within 1 h. The conversion of kaempferol yielded a single product (peak k1) presenting an $[M-H]^-$ ion at *m/z* 447. This product was confirmed to be kaempferol 7-*O*-glucoside (K7G) when compared to the standard compound (**Fig. 4-5 A–D**). Naringenin was also converted to a single product (peak n1) with an $[M-H]^-$ ion at *m/z* 433, which was confirmed to be naringenin 7-*O*-glucoside (N7G) when compared to the standard compound (**Fig. 4-5 E–H**). Quercetin was converted to the main product (peak q2) and some minor products (peak q1 and asterisks), similar to luteolin (**Fig. 4-4 G**). The main product (peak q2) presented with an $[M-H]^-$ ion at *m/z* 463, and it was confirmed as quercetin 7-*O*-glucoside (Q7G) when compared to the standard compound (**Fig. 4-4 E–G**). A minor product at a retention time of 2.35 min (peak q1, 2.41 min for MS analysis) presented with an $[M-H]^-$ ion at *m/z* 625, corresponding to the di-glucoside of quercetin (peak q1, **Fig. 4-4 H**). Both luteolin and quercetin possess two hydroxyl groups on the B-ring, whereas the other tested flavonoids had one hydroxyl group. Luteolin and quercetin were likely further glucosylated at another hydroxyl group (possibly 3′-OH) during bioconversion. Unfortunately, these byproducts could not be identified owing to their low yields. The conversion rates of kaempferol, quercetin, and naringenin to kaempferol 7-*O*-glucoside, quercetin 7-*O*-glucoside, and naringenin 7-*O*-glucoside, respectively, were 78%, 77%, and 93%.

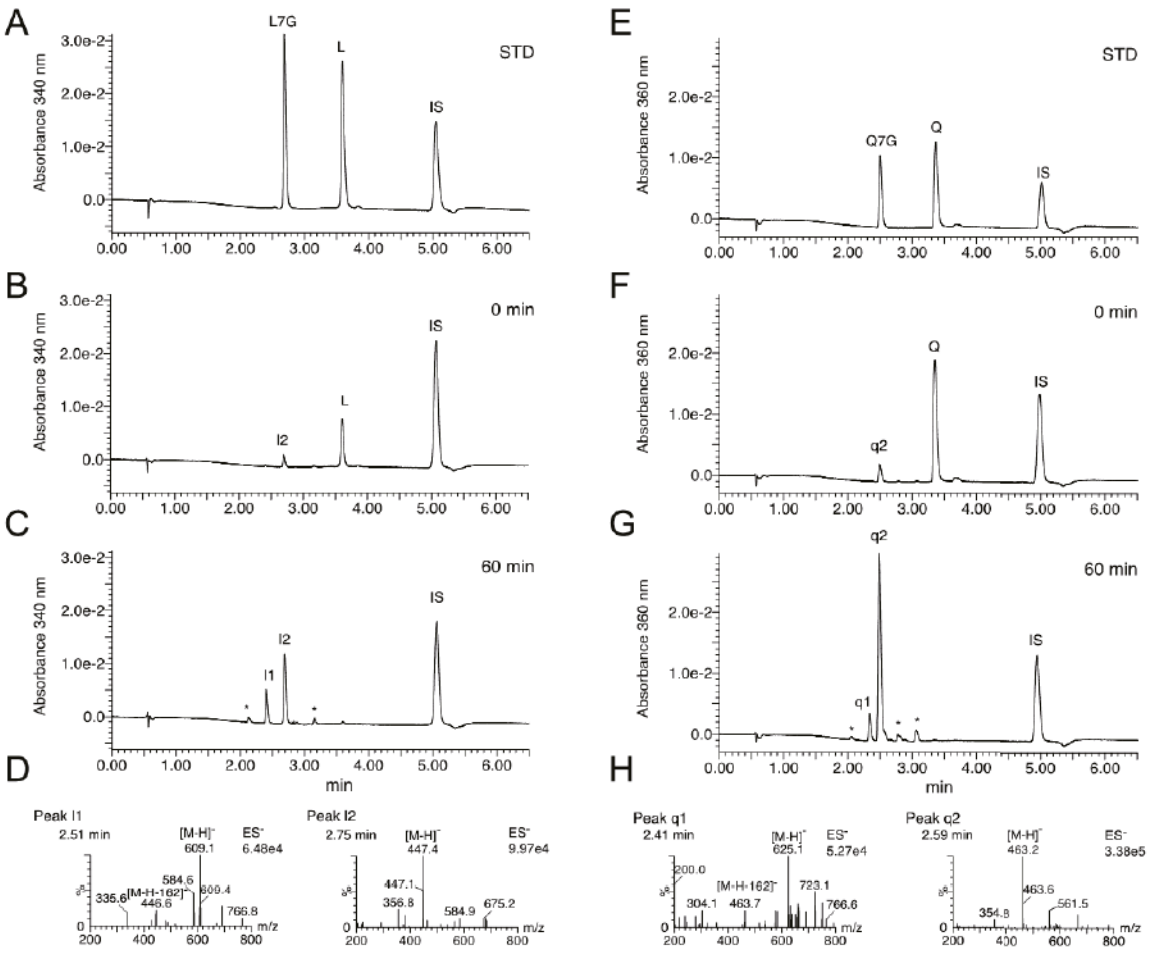


Figure 4-4 Bioconversion of luteolin and quercetin using the Ec-NtGT2 system. Each panel shows the UPLC-MS results of luteolin (A–D) and quercetin (E–H), which represent the standard compounds (A, E), culture media at 0 min (B, F) and 60 min (C, G), and mass spectrum of the product (D, H). Peak identification: L, luteolin; L7G, luteolin 7-*O*-glucoside; Q, quercetin; Q7G, quercetin 7-*O*-glucoside; l1, l2, q1, and q2, product peaks of luteolin and quercetin conversions; and IS, internal standard (chrysin).

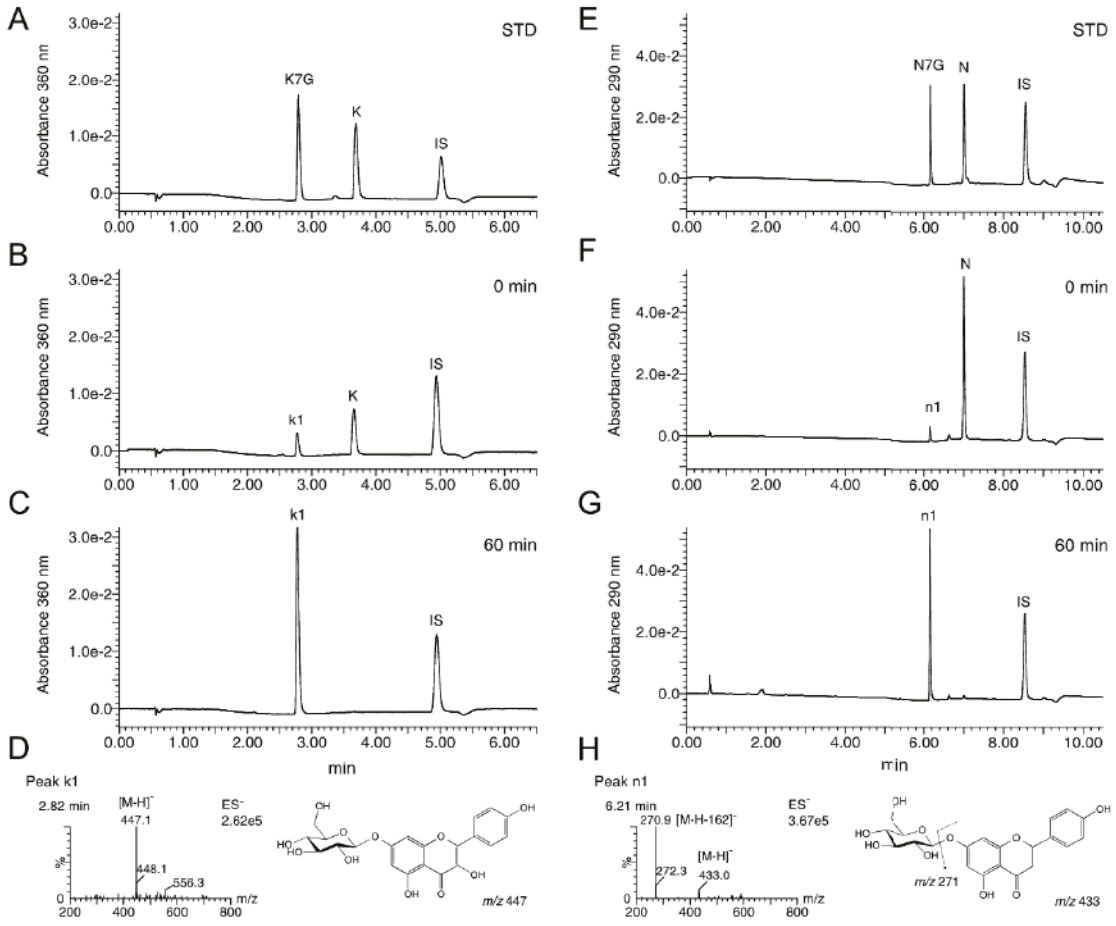


Figure 4-5 Bioconversion of kaempferol and naringenin using the Ec-NtGT2 system. Each panel shows the UPLC-MS results of kaempferol (A–D) and naringenin (E–H), which represent the standard compounds (A, E), culture media at 0 min (B, F) and 60 min (C, G), and MS spectra of the product (D, H). The MS analyses of the products showed $[M-H]^-$ ion at *m/z* 447 and 433, corresponding to kaempferol glucoside and naringenin glucoside, respectively. Peak identification: K, kaempferol; K7G, kaempferol 7-*O*-glucoside; N, naringenin; N7G, naringenin 7-*O*-glucoside; k1 and n1, products of kaempferol and naringenin; and IS, internal standard (chrysin).

Scale-up production of flavonoid 7-*O*-glucosides has been attempted using these flavonoids as substrates. In Chapter 2, a high concentration of flavonoids was immediately precipitated in the medium after administration, and their conversion rates were low. In contrast, flavonoids that were administered repeatedly at low concentrations did not precipitate immediately and were efficiently converted into their products. Therefore, the sequential administration method was selected for this study. Firstly, quercetin 7-*O*-glucoside was produced using the Ec-NtGT2 system. Seven cycles of 50 μM quercetin (total of 60 mg) was added to 600 mL of the Ec-NtGT2 system every 45–60 min (**Fig. 4-6 A**). After a 6 h bioconversion reaction, approximately 77% of quercetin was converted to 7-*O*-glucoside, and the rest was converted to di-glucoside and other metabolites, with only 1.6% of the total added quercetin remaining unreacted (**Fig. 4-6 B–C**). The products in the medium began to precipitate during storage at 4 °C. Therefore, the *E. coli* cells centrifuged from the culture medium were extracted with methanol and analyzed using UPLC to confirm whether the substrate (quercetin) and its product (quercetin 7-*O*-glucoside) precipitated during the conversion process. Only small amounts of quercetin (approximately 0.7%) and its product (approximately 1.1%) were detected in the extract, suggesting that most of the administered quercetin was converted to its glucosides and remained in the culture medium. Next, apigenin 7-*O*-glucoside was produced by the addition of 20 μM apigenin every 30 min to 400 mL of the Ec-NtGT2 system (a total of 15 mg was added seven times). Approximately 97% of the apigenin was converted to 7-*O*-glucoside after 4 h (**Fig. 4-7**) and some of the substrate and product precipitated in the medium. The 7-*O*-glucosides from quercetin and apigenin were purified and analyzed using NMR. The ^{1}H and ^{13}C NMR spectra (**Table 4-1 A, B**) corresponded to a Chapter 2, and correlations between H-1″ (glucose moiety, δ 5.09 and 5.11) and C-7 (163.2 and 163.4) were observed in the HMBC analysis of the products, showing that they were quercetin 7-*O*-glucoside and apigenin 7-*O*-glucoside, respectively (**Fig. 4-8**). Then, luteolin (a total of 35 mg in eight cycles

in 8 h), kaempferol (a total of 25 mg in five cycles in 6 h), and naringenin (a total of 49 mg in eight cycles in 9 h) were added to Ec-NtGT2, for the scale-up production of their 7-*O*-glucosides. Approximately, 72% of luteolin, 98% of kaempferol, and 96% of naringenin were converted to their 7-*O*-glucosides (**Fig. 4-9**). These compounds were also confirmed as their 7-*O*-glucoides by NMR analysis (**Table 4-1 A–C**). The yields of apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside, quercetin 7-*O*-glucoside, kaempferol 7-*O*-glucoside, and naringenin 7-*O*-glucoside were approximately 59, 52, 118, 90, and 166 mg/L, respectively. These results indicated that the Ec-NtGT2 system with sequential administration is a simple and efficient system for producing large amounts of flavonoid 7-*O*-glucosides.

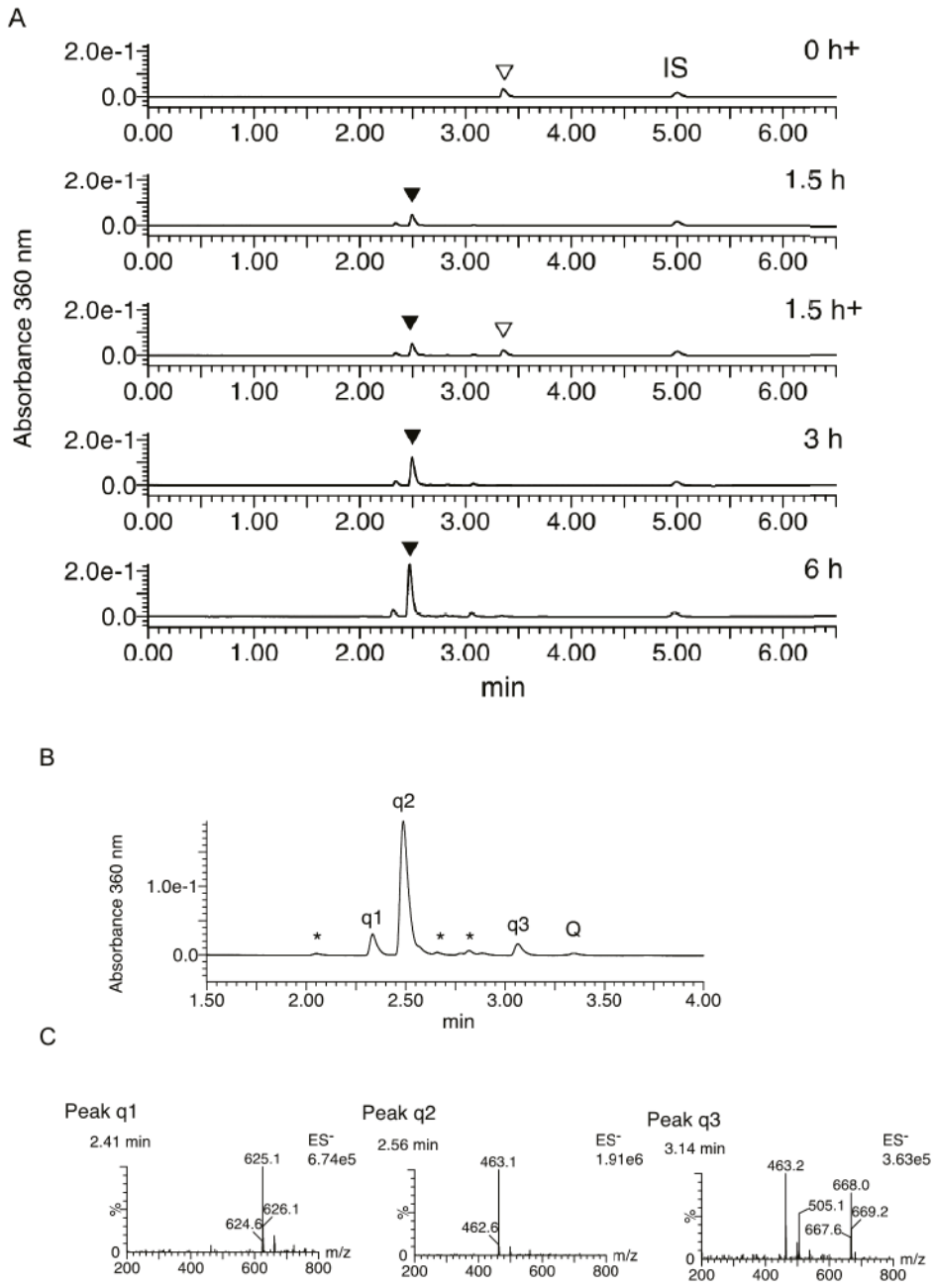


Figure 4-6 Production of quercetin 7-*O*-glucoside by the Ec-NtGT2 system. Quercetin (50 μM) was administered sequentially to the Ec-NtGT2 system every 45–60 min (total of 7 times). (A) UPLC profiles after adding quercetin at 0 and 1.5 h (indicated as "+" in the figure), and products at 1.5, 3, and 6 h are shown. Closed and open triangles indicate the product (quercetin 7-*O*-glucoside) and substrate (quercetin) addition, respectively. (B) Magnified UPLC profiles of culture media after conversion. Peak identification: Q, quercetin; q1, q2, and q3, product peaks of quercetin; asterisks, other minor products. (C) Mass spectrum of the main product at 2.49 min (2.56 min for mass spectrometry analysis) and minor products.

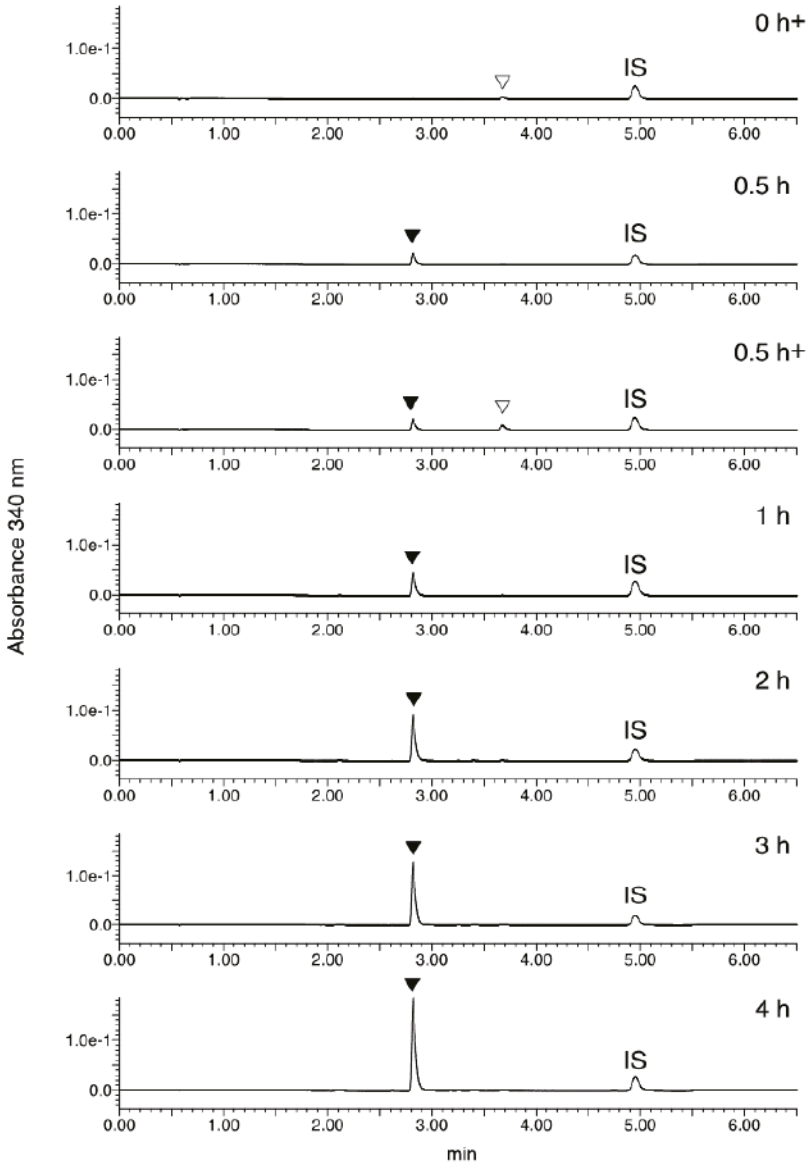
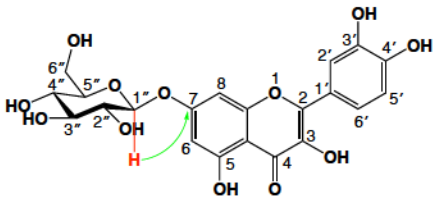
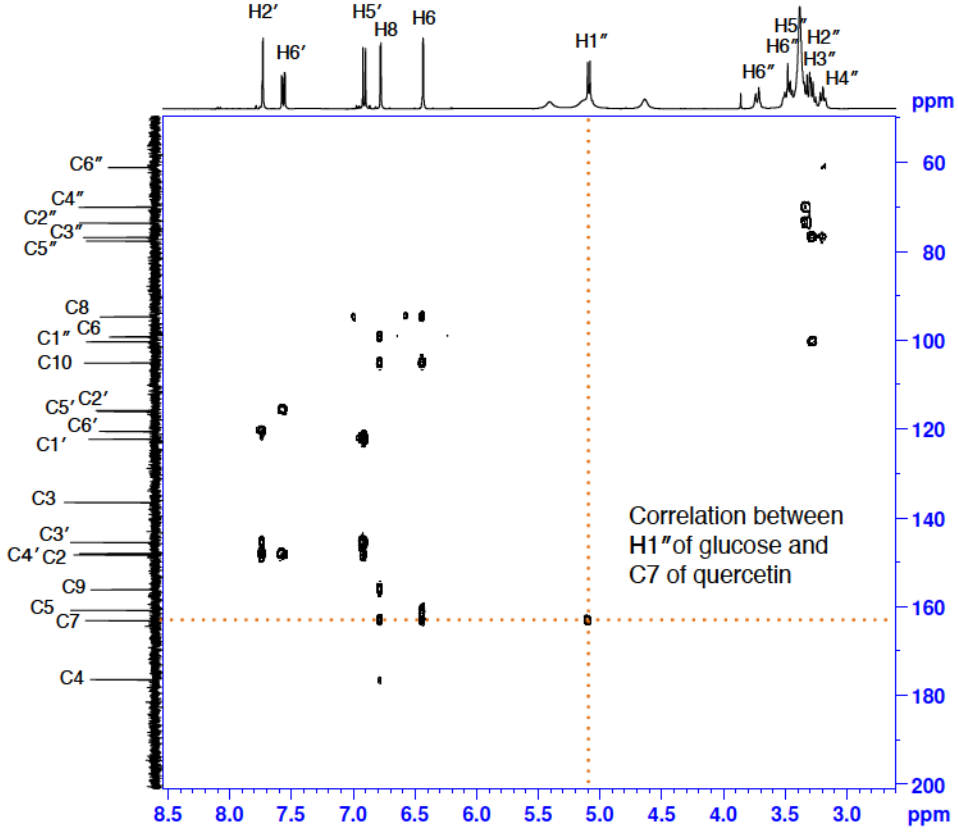


Figure 4-7 Production of apigenin 7-*O*-glucoside using the Ec-NtGT2 system. Apigenin (20 μM) was added sequentially to the Ec-NtGT2 system every 30 min (total of 7 times). The results of the first two additions up to 1 h and the products at every hour up to 4 h are shown. The "+" indicates the addition of apigenin. Closed and open arrowheads indicate the product (apigenin 7-*O*-glucoside) and the addition of the substrate (apigenin), respectively. IS: internal standard (chrysin).

A



quercetin 7-*O*-glucoside



B

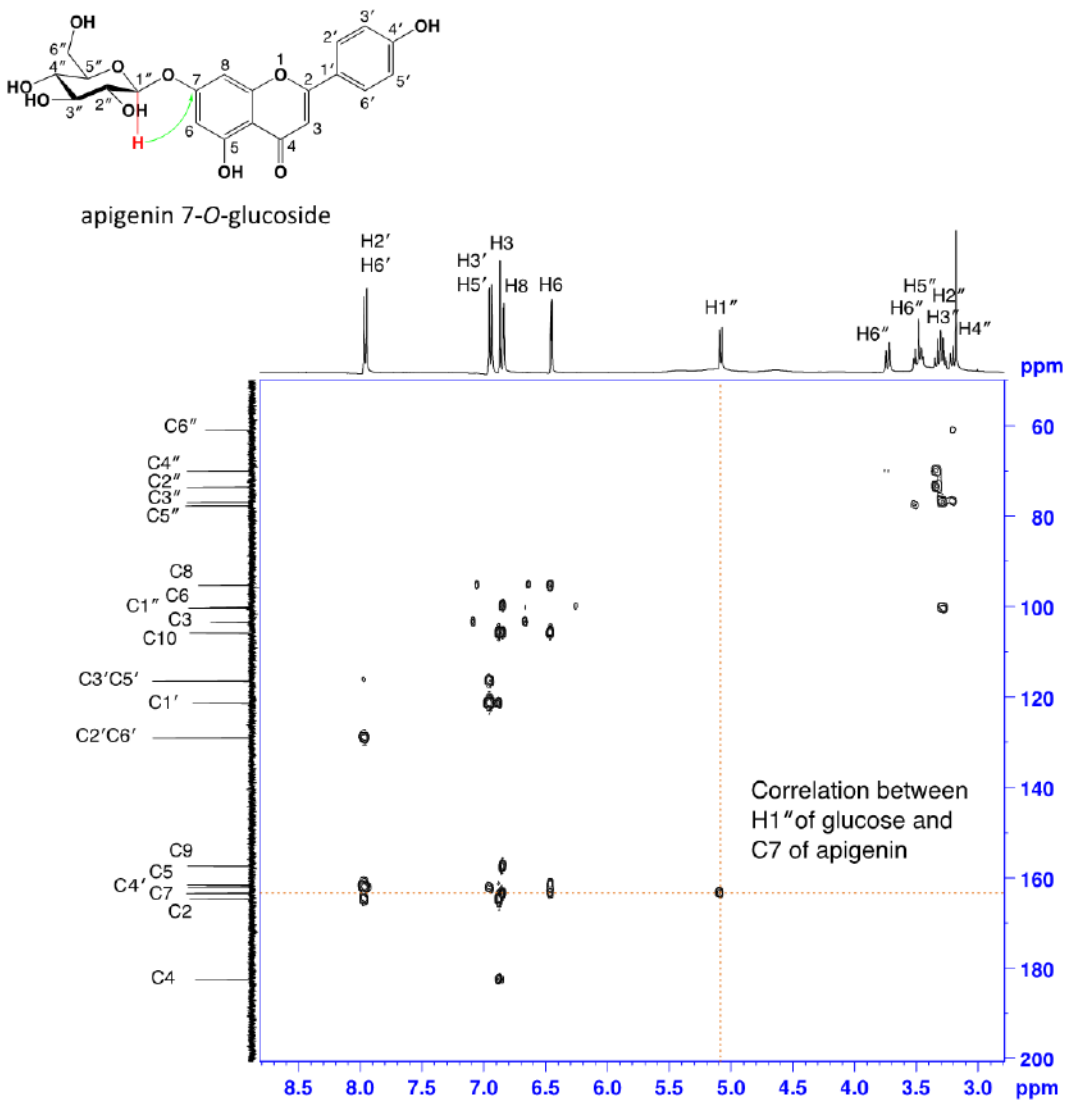


Figure 4-8 Heteronuclear multiple bond correlation (HMBC) analysis of the quercetin 7-*O*-glucoside and apigenin 7-*O*-glucoside produced using the Ec-NtGT2 system. The purified products were dissolved in dimethyl sulfoxide (DMSO-d_6) and analyzed by nuclear magnetic resonance (NMR) spectroscopy. Each panel shows the HMBC analyses results of quercetin 7-*O*-glucoside (A) and apigenin 7-*O*-glucoside (B), respectively. Green arrows in the chemical structures indicate the HMBC correlation between H-1″ of glucose and the carbon of the flavonoid.

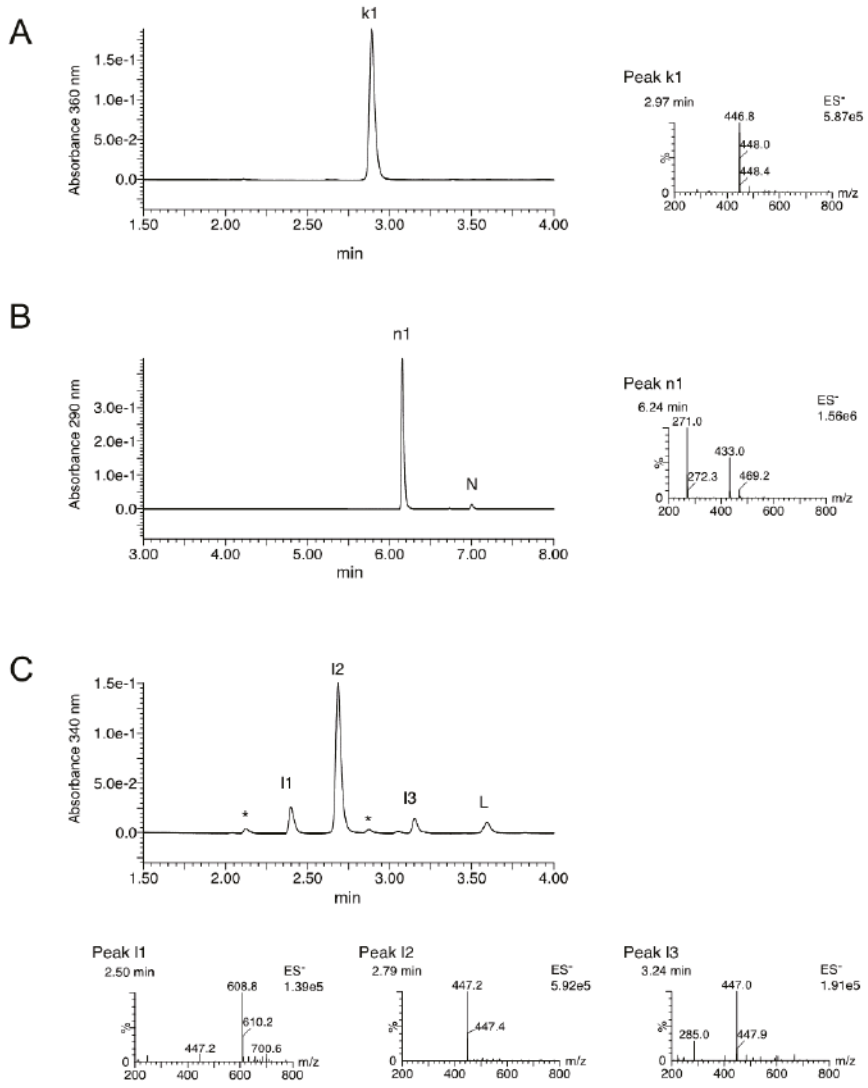


Figure 4-9 UPLC-MS analysis of the final product obtained from the scaled-up bioconversion of kaempferol (A), naringenin (B), and luteolin (C). Each volume of 760, 440, and 450 mL of the Ec-NtGT2 was added 20 μM luteolin (a total of 35 mg in eight cycles), 40 μM kaempferol (a total of 25 mg in five cycles), or 50 μM naringenin (a total of 49 mg in eight cycles), respectively, every 1 h. MS spectra of the products are shown. Peak identification: k1, product peak of kaempferol (kaempferol 7-*O*-glucoside); N, naringenin; n1, product peak of naringenin (naringenin 7-*O*-glucoside); L, luteolin; l1, l2, and l3; product peaks of luteolin (l2 corresponded to luteolin 7-*O*-glucoside); and asterisks, other minor products.

4.4.2. Bioconversion of flavonoids and phenolics to their glucosides

A recombinant NtGT3 (SDS-PAGE analysis of the purified enzyme showed in **Fig. 4-10**) catalyzed a glucosylation toward a broader range of substrates including 3-hydroxyl group of flavonoid, 7-hydroxyl group of coumarin, and naphthol (Taguchi et al. 2003a). Thus, *E. coli* harboring pET-NtGT3 (Ec-NtGT3) was examined for bioconversion of flavonoids and phenolic compounds to their glucosides. First, the Ec-NtGT3 system was administered 50 μM of quercetin or kaempferol. According to the MS spectrum signals obtained, all of the products from flavonols were assigned to be *O*-glucosides. The main products from quercetin and kaempferol corresponded to the standard compounds of quercetin 3-*O*-glucoside and kaempferol 3-*O*-glucoside, respectively, suggesting that the Ec-NtGT3 can be used for the production of flavonol 3-*O*-glucosides. The minor peaks of unexpecting byproducts of quercetin and kaempferol appeared, which presented with a $[M-H]^-$ ion at *m/z* 625.2 (from quercetin) and 609.2 (from kaempferol), suggesting that their di-glucosides were also produced (**Fig. 4-11**). In addition, the Ec-NtGT3 system was tested to examine the conversion of 1-naphthol, 2-naphthol, and *p*-nitrophenol. This system converted these compounds (100 μM) into their glucosides within 1 h. The conversion of each substrate yielded a single product, because of one hydroxyl group. The final products from the medium were confirmed as a 1-naphthol glucoside (1NAG), 2-naphthol glucoside (2NAG), and *p*-nitrophenol glucoside (pNPG) by comparing with their standard compounds (**Fig. 4-12**). Approximately 42.8 μM of quercetin 3-*O*-glucoside, 47.8 μM of kaempferol 3-*O*-glucoside, 75.3 μM of 1-naphthol glucoside, 75.1 μM of 2-naphthol glucoside, and 96.1 μM of *p*-nitrophenol glucoside were produced within 1 h. The time course of each produced glucoside was shown in **Fig. 4-13**. The conversion of quercetin and kaempferol finished at 0.5–1 h with high conversion rates of 84% and 95%, respectively (**Fig. 4-13 A**). The highest conversion rate of 1-naphthol, 2-naphthol, and *p*-nitrophenol were 79%, 76%, and 96%, respectively, at 0.5 h and 1 h (**Fig. 4-13 B**).

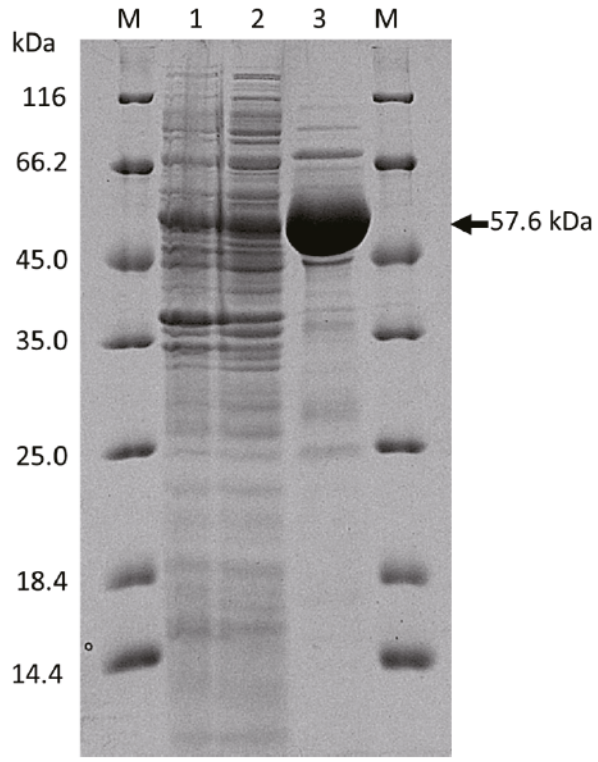


Figure 4-10 SDS-PAGE analysis of the recombinant NtGT3 protein expressed in *E. coli*. Proteins were separated on a 12.5% SDS-PAGE. Lane M, Size marker (Unstained protein molecular weight marker, ThermoFisher Scientific); lane 1 and 2, insoluble (**1**) and soluble (**2**) fraction of the cell-free extract; 3, recombinant NtGT3 protein purified with the 6×Histidine tag. Arrowhead indicates the size of the recombinant NtGT3 protein.

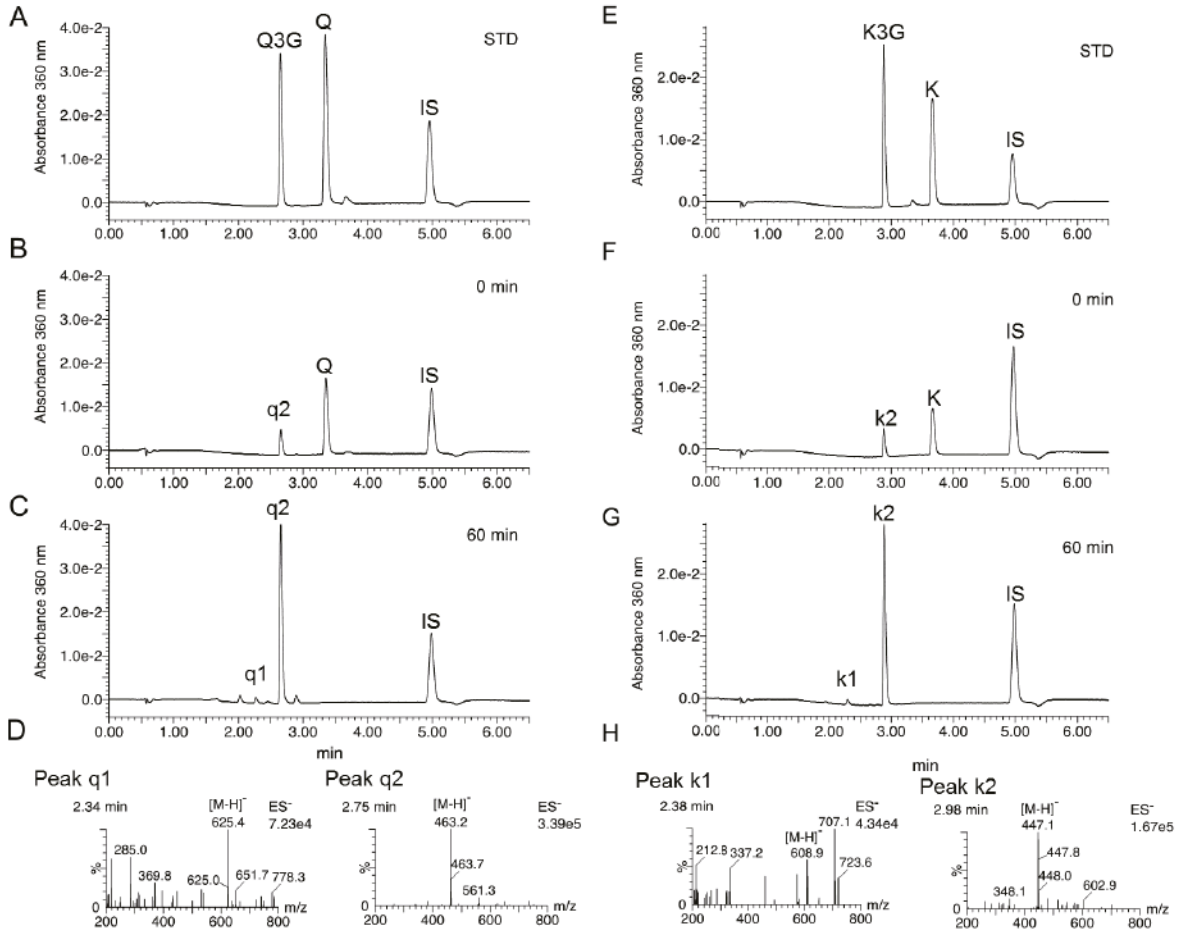


Figure 4-11 Bioconversion of quercetin and kaempferol using the Ec-NtGT3 system. Each panel shows the UPLC-MS results of quercetin (A–D) and kaempferol (E–H), which represent the standard compounds (A, E), culture media at 0 min (B, F) and 60 min (C, G), and mass spectrum of the product (D, H). Peak identification: Q, quercetin; Q3G, quercetin 3-*O*-glucoside; K, kaempferol; K3G, kaempferol 3-*O*-glucoside; q1, q2, k1, and k2, product peaks of quercetin and kaempferol conversions; and IS, internal standard (chrysin).

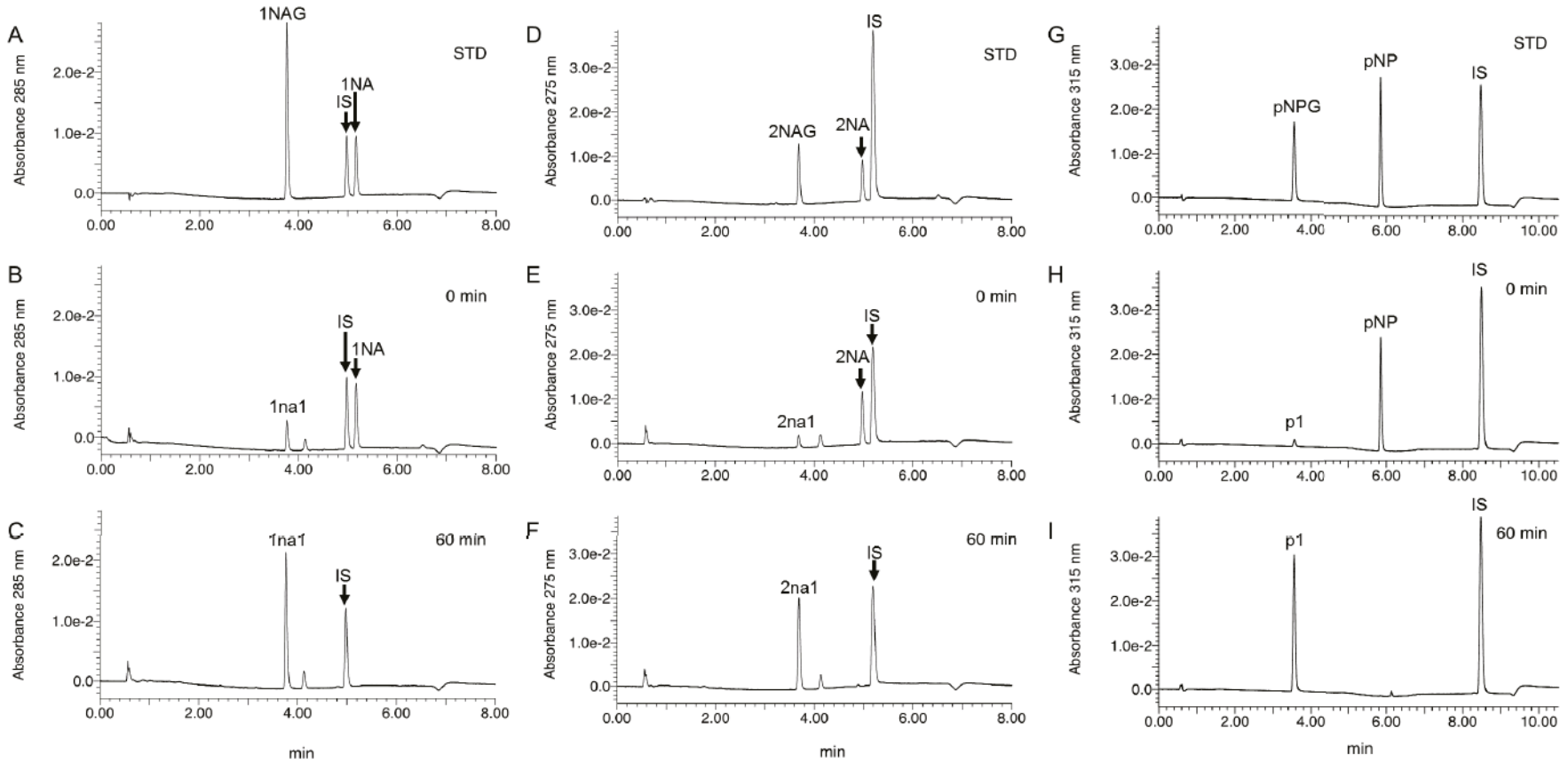
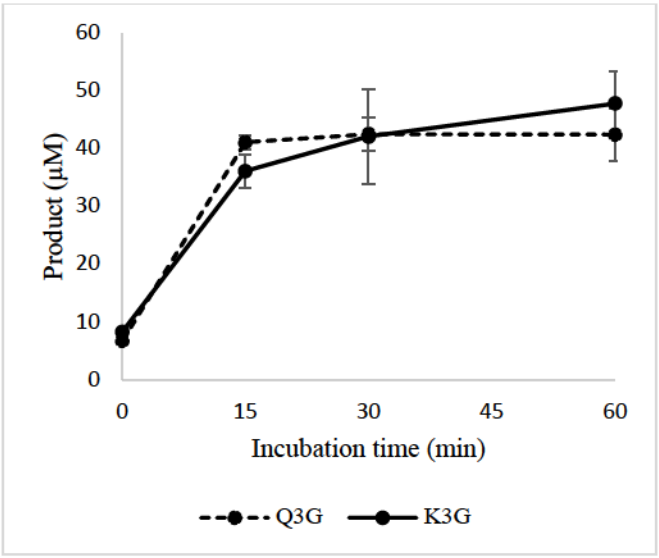


Figure 4-12 Bioconversion of 1-naphthol, 2-naphthol, and *p*-nitrophenol using the Ec-NtGT3 system. Each panel shows the UPLC-MS results of 1-naphthol (A–C), 2-naphthol (D–F), and *p*-nitrophenol (G–I), which represent the standard compounds (A, D, G), culture media at 0 min (B, E, H) and 60 min (C, F, I). Peak identification: 1NA, 1-naphthol; 1NAG, 1-naphthol glucoside; 2NA, 2-naphthol; 2NAG, 2-naphthol glucoside; pNP, *p*-nitrophenol; pNPG, *p*-nitrophenol glucoside; 1na1, 2na1, and p1, products of 1-naphthol, 2-naphthol, and *p*-nitrophenol; and IS, internal standard (2-naphthol for 1-naphthol, kaempferol for 2-naphthol, and chrysin for *p*-nitrophenol).

A



B

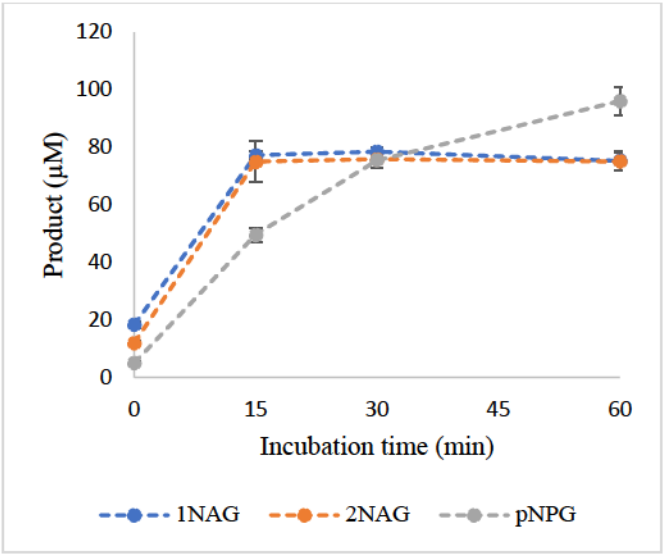


Figure 4-13 Time course of production of flavonol 3-*O*-glucosides and phenolic glucosides by the Ec-NtGT3 system. The amounts of products from quercetin and kaempferol (A) and 1-naphthol, 2-naphthol, and *p*-nitrophenol (B) are shown over time. Abbreviation: Q3G, quercetin 3-*O*-glucoside; K3G, kaempferol 3-*O*-glucoside; 1NAG, 1-naphthol glucoside; 2NAG, 2-naphthol glucoside; pNPG, *p*-nitrophenol glucoside.

4.5. DISCUSSION

Flavonoid 7-*O*-glucosides have attracted considerable attention because of their numerous biological activities. For instance, apigenin 7-*O*-glucoside shows significant activities against gastric cancer and anxiety, which are common diseases. Moreover, this compound is not abundant in plants and is expensive. There are only a few studies conducted on flavonoid 7-*O*-glucosides production using a whole-cell system have been reported (He et al. 2008; Koirala et al. 2019; Penso et al. 2014; Thuan et al. 2018; Zhu et al. 2020). For example, quercetin 7-*O*-glucoside and kaempferol 7-*O*-glucoside were produced with a conversion rate of more than 70% over 48–72 h using *E. coli* cells expressing genetically modified glucosyltransferase (PaUGT1-Q19A) originating from liverwort. Furthermore, additional expression of flavone synthase and sucrose synthase in the same *E. coli* strain enabled the production of 35.0 mg/L of apigenin 7-*O*-glucoside and 39.6 mg/L of luteolin 7-*O*-glucoside with 81% and 88.6% conversion rates at 48 h, respectively (Zhu et al. 2020). Similarly, *E. coli* expressing UGT73C8 derived from barrel medic produced 9 mg/L of luteolin 7-*O*-glucoside in 24 h at a conversion rate of 75% (He et al. 2008). A co-culture of two *E. coli* strains that convert *p*-coumaric acid into apigenin and apigenin into apigenin 7-*O*-glucoside produced 16.6 mg/L of apigenin 7-*O*-glucoside from *p*-coumaric acid within 60 h, with a conversion rate of 38.5% (Thuan et al. 2018). Other systems, such as fungi and bacteria, have also been examined for the production of flavonoid 7-*O*-glucosides. For instance, the fungi *Beauveria bassiana* converted quercetin to its 7-*O*-glucoside in 96 h with a 38% yield (Penso et al. 2014), and the bacteria *Bacillus amyloliquefaciens* converted naringenin to a mixture of naringenin derivatives, including naringenin 7-*O*-glucoside, with a 57% conversion rate in 48 h (Koirala et al. 2019). Compared with these systems, the Ec-NtGT2 system efficiently converted several flavonoids into their 7-*O*-glucosides in a short time. The production yields of apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside, quercetin 7-*O*-glucoside, kaempferol 7-*O*-glucoside, and naringenin 7-*O*-glucoside

reached 59, 52, 118, 90, and 166 mg/L, with 97%, 72%, 77%, 98%, and 96% conversion rates, respectively, after sequential administration of a low concentration (20–50 μM) of aglycones at 4–9 h, similar as WjGT1 (Chapter 2) and IbGT1 (Chapter 3). These results suggested that Ec-NtGT2 is a simple and efficient system for the production of flavonoid 7-*O*-glucosides.

Flavonoid 3-*O*-glucosides have been successfully produced using a whole-cell system; moreover, production yield has been achieved by deleting or inserting genes in microorganisms for relevant enzymes. For example, *E. coli* cells lacking *pgi* gene (coding phosphoglucose isomerase) and expressing UGT73B3 produced 4 g/L of quercetin 3-*O*-glucoside in 56 h (Xia and Eiteman 2017). The combination of AtUGT78D2, flavanone synthase, flavonol synthase, sucrose synthase, and an additional UDP-glucose synthesis pathway was introduced into *E. coli*, and a recombinant strain successfully produced 1.7 g/L of kaempferol 3-*O*-glucoside from naringenin (Pei et al. 2019). A broader promiscuity 3-*O*-glycosyltransferase SbGT1 with an ambiguous substrate specificity was able to produce kaempferol 3-*O*-glucoside *in vivo* at a conversion rate of more than 98% (Wang et al. 2019). Here, *E. coli* expressing only tobacco-derived glucosyltransferase NtGT3 (Ec-NtGT3) showed the potential for converting various substrates such as flavonols (quercetin and kaempferol) and phenolic compounds (1-naphthol, 2-naphthol, and *p*-nitrophenol) to flavonol 3-*O*-glucosides and phenolic glucosides. Approximately 85% of quercetin, 96% of kaempferol, 75 % of 1-naphthol, 75% of 2-naphthol, and 96% of *p*-nitrophenol were converted to their glucosides in 1 h. This result indicates that the system can be expanded for bioconversion of useful flavonoid 3-*O*-glucosides and phenolic compounds in the future.

4.6. REFERENCES

De Bruyn F, Van Brempt M, Maertens J, Van Bellegem W, Duchi D, De Mey M (2015) Metabolic engineering of *Escherichia coli* into a versatile glycosylation platform: production of bio-active quercetin glycosides. Microb Cell Fact 14:138

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55:481-504

He X-Z, Li W-S, Blount JW, Dixon RA (2008) Regioselective synthesis of plant (iso) flavone glycosides in *Escherichia coli*. Appl Microbiol Biotechnol 80:253-260

Ji Y, Li B, Qiao M, Li J, Xu H, Zhang L, Zhang X (2020) Advances on the *in vivo* and *in vitro* glycosylations of flavonoids. Appl Microbiol Biotechnol 104:6587-6600

Kim BG, Yang SM, Kim SY, Cha MN, Ahn J-H (2015) Biosynthesis and production of glycosylated flavonoids in *Escherichia coli*: current state and perspectives. Appl Microbiol Biotechnol 99:2979-2988

Koirala M, Lee YK, Kim MS, Chung YC, Park J-S, Kim S-Y (2019) Biotransformation of naringenin by *Bacillus amyloliquefaciens* into three naringenin derivatives. Nat Prod Commun 14(5)

Markham K, Ternai B, Stanley R, Geiger H, Mabry T (1978) Carbon-13 NMR studies of flavonoids—III: Naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron 34:1389-1397

Pandey RP, Malla S, Simkhada D, Kim B-G, Sohng JK (2013) Production of 3-*O*-xylosyl quercetin in *Escherichia coli*. Appl Microbiol Biotechnol 97:1889-1901

Pei J, Chen A, Dong P, Shi X, Zhao L, Cao F, Tang F (2019) Modulating heterologous pathways and optimizing fermentation conditions for biosynthesis of kaempferol and astragalin from naringenin in *Escherichia coli*. J Ind Microbiol Biotechnol 46:171-186

Penso J, Cordeiro KC, da Cunha CR, da Silva Castro PF, Martins DR, Lião LM, Rocha ML, de Oliveira V (2014) Vasorelaxant activity of 7-*β*-*O*-glycosides biosynthesized from flavonoids. Eur J Pharmacol 733:75-80

Shimoda K, Kubota N, Taniuchi K, Sato D, Nakajima N, Hamada H, Hamada H (2010) Biotransformation of naringin and naringenin by cultured *Eucalyptus perriniana* cells. Phytochemistry 71:201-205

Taguchi G, Nakamura M, Hayashida N, Okazaki M (2003a) Exogenously added naphthols induce three glucosyltransferases, and are accumulated as glucosides in tobacco cells. Plant Sci 164:231-240

Taguchi G, Ubukata T, Hayashida N, Yamamoto H, Okazaki M (2003b) Cloning and characterization of a glucosyltransferase that reacts on 7-hydroxyl group of flavonol and 3-hydroxyl group of coumarin from tobacco cells. Arch Biochem Biophys 420:95-102

Thuan NH, Chaudhary AK, Van Cuong D, Cuong NX (2018) Engineering co-culture system for production of apigetrin in *Escherichia coli*. J Ind Microbiol Biotechnol 45:175-185

Wang Z, Wang S, Xu Z, Li M, Chen K, Zhang Y, Hu Z, Zhang M, Zhang Z, Qiao X (2019) Highly promiscuous flavonoid 3-*O*-glycosyltransferase from *Scutellaria baicalensis*. Org Lett 21:2241-2245

Williams CA (2006) Flavone and flavonol *O*-glycosides. In: Andersen ØM, Markham KR (eds) Flavonoids: chemistry, biochemistry and application. CRC Press, Boca Raton, FL, pp 749-824

Xia T, Eiteman MA (2017) Quercetin glucoside production by engineered *Escherichia coli*. Appl Biochem Biotechnol 182:1358-1370

Yang B, Liu H, Yang J, Gupta VK, Jiang Y (2018) New insights on bioactivities and biosynthesis of flavonoid glycosides. Trends Food Sci Technol 79:116-124

Zhu T-T, Liu H, Wang P-Y, Ni R, Sun C-J, Yuan J-C, Niu M, Lou H-X, Cheng A-X (2020) Functional characterization of UDP-glycosyltransferases from the liverwort *Plagiochasma appendiculatum* and their potential for biosynthesizing flavonoid 7-*O*-glucosides. Plant Sci 299:110577

Chapter 5

Production of gentiobiosides of phenolics and flavonoids by bioconversion using *Escherichia coli* expressing a novel glucosyltransferase from tobacco (NtGGT2)

5.1. ABSTRACT

A phenolic glucoside 1,6-glucosyltransferase from *Nicotiana tabacum* (NtGGT2) exhibited significant activity against phenolic glucosides and flavonol 7-*O*-glucosides and produced their gentiobiosides. *Escherichia coli* cells expressing NtGT2, NtGT3, and NtGGT2 were examined for potential to produce gentiobiosides of *p*-nitrophenol, naphthol, and flavonoid. *E. coli* expressing NtGT3 and NtGGT2 converted 100 μM each of *p*-nitrophenol, 1-naphthol, and 2-naphthol into their gentiobiosides with a conversion rate of 35–56%. *E. coli* expressing both NtGT2 and NtGGT2 converted 40–50 μM of apigenin, kaempferol, and naringenin into their di-glucosides (a high possibility is 7-*O*-gentiobioside) with a conversion rate of 14–31%.

Chapter 6

General Discussion and Conclusion

6.1. GENERAL DISCUSSION AND CONCLUSION

In this thesis, the production of mono- and di-glucoside of various compounds using the recombinant *E. coli* expressing plant-derived glycosyltransferases was studied.

In chapter 1, I described the background and objective of this study. Last decades, *de novo* production of plant secondary metabolites having pharmaceutical use through the bioconversion process using a combination of microbial cells and glycosyltransferases has been much attention because it has become possible of advances in molecular and synthetic biology. Microbial cells expressing foreign genes that originated from plants are promising for the large-scale production of valuable metabolites that are difficult to obtain from plants because of their limited content. Moreover, this approach is simple, efficient, and eco-friendly as compared to chemical synthesis methods. A typical example is the bioconversion of flavonoids to their glycosides. Several attempts to produce flavonoid glucosides by yeast or *E. coli* cells expressing plant genes have been performed successfully (Brazier-Hicks and Edwards 2013; De Bruyn et al. 2015; Kim et al. 2012; Kim et al. 2013). Similarly, aromatic compounds such as geraniol glucoside were produced successfully on a large scale (Effenberger et al. 2019; Priebe et al. 2021).

In chapter 2, flavone (apigenin and luteolin) 6-*C*-glucosides, flavonol (quercetin and kaempferol) 6-*C*-glucosides, and flavanone (naringenin) *C*-glucoside were produced by *E. coli* expressing wasabi-derived WjGT1 (UGT84A57). The conversion rates successfully reached 89–99% after sequential administrations of apigenin, quercetin, and kaempferol at low concentrations. The yields of isovitexin, kaempferol *C*-glucoside, and quercetin *C*-glucoside reached 172, 105, and 145 mg/L-culture, respectively.

In chapter 3, the cinnamate glucosyltransferase (IbGT1, UGT84A20) from sweet potato was confirmed to react toward ferulic acid, sinapic acid, *p*-coumaric acid, and caffeic acid in acidic and alkaline conditions. *E. coli* cells expressing IbGT1 were successfully used for the

production of sinapoyl glucoside and *p*-hydroxybenzoyl glucoside and yields of these compounds reached 159 and 177 mg/L-culture, respectively. The conversion rates of sinapic acid and *p*-hydroxybenzoic acid were 69% and 98%, respectively, through sequential administrations.

In chapter 4, *E. coli* cells expressing tobacco-derived NtGT2 (UGT75L3) or NtGT3 (UGT71A11) were used for the conversion of flavonoids and phenolic compounds into their *O*-glucosides. *E. coli* cells expressing NtGT2 (UGT75L3) converted flavonoids into 7-*O*-glucosides with 67–98% conversion rates in 1 h, and the yields of flavone (apigenin and luteolin) 7-*O*-glucosides, flavonol (quercetin and kaempferol) 7-*O*-glucosides, and flavanone (naringenin) 7-*O*-glucoside reached 52–166 mg/L-culture, through sequential administrations in 4–9 h. Similarly, *E. coli* cells expressing NtGT3 (UGT71A11) converted flavonols (quercetin and kaempferol) and phenolic compounds (naphthols and *p*-nitrophenol) into glucosides with 75–96% conversion rates.

In chapter 5, the novel 1,6-GT was isolated from tobacco, and it was found to exhibit glycosylation activity toward phenolic glucosides, especially *p*-nitrophenol glucoside. *E. coli* cells expressing NtGT3 and NtGGT2 converted naphthols and *p*-nitrophenol into their gentiobiosides with conversion rates of 35–56%. Similarly, *E. coli* cells expressing NtGT2 and NtGGT2 converted apigenin, kaempferol, and naringenin into their di-glucosides with conversion rates of 14–31%.

In this thesis, I reported the biosystems to produce various glucosylated compounds using *E. coli* cells expressing diverse plant GTs. The systems were virtually effective for the production of flavonoid glucosides, cinnamate glucosides, and phenolic glucosides. These systems are a promising way for the large-scale production of valuable pharmaceuticals such as flavonol *C*-glucosides, cinnamate glucosides, and flavone 7-*O*-glucosides. For instance, only a mixture of flavonol 6- and 8-*C*-glucosides were produced using similar systems. But, in

this thesis, only flavonol 6-*C*-glucosides were successfully produced on a large scale using the constructed *E. coli* system (Dorjjugder et al. 2021). In addition, expensive and not abundant flavone 7-*O*-glucosides were efficiently produced using constructed *E. coli* system in a short time compared to other systems that required more than 24 h (Dorjjugder and Taguchi 2022).

6.2. REFERENCES

Brazier-Hicks M, Edwards R (2013) Metabolic engineering of the flavone-*C*-glycoside pathway using polyprotein technology. Metab Eng 16:11-20

De Bruyn F, Van Brempt M, Maertens J, Van Bellegem W, Duchi D, De Mey M (2015) Metabolic engineering of *Escherichia coli* into a versatile glycosylation platform: production of bio-active quercetin glycosides. Microb Cell Fact 14:138

Dorjjugder N, Hatano M, Taguchi G (2021) Production of flavonol and flavone 6-*C*-glucosides by bioconversion in *Escherichia coli* expressing a *C*-glucosyltransferase from wasabi (*Eutrema japonicum*). Biotechnol Lett 43:1913-1919

Dorjjugder N, Taguchi G (2022) Production of flavonoid 7-*O*-glucosides by bioconversion using *Escherichia coli* expressing a 7-*O*-glucosyltransferase from tobacco (*Nicotiana tabacum*). Appl Biochem Biotechnol 194:3320-3329

Effenberger I, Hoffmann T, Jonczyk R, Schwab W (2019) Novel biotechnological glucosylation of high-impact aroma chemicals, 3 (2H)-and 2 (5H)-furanones Sci Rep 9:1-9

Kim BG, Kim HJ, Ahn JH (2012) Production of bioactive flavonol rhamnosides by expression of plant genes in *Escherichia coli*. J Agric Food Chem 60:11143-11148

Kim HJ, Kim BG, Ahn JH (2013) Regioselective synthesis of flavonoid bisglycosides using *Escherichia coli* harboring two glycosyltransferases. Appl Microbiol Biotechnol 97:5275-5282

Priebe X, Hoang MD, Rüdiger J, Turgel M, Tröndle J, Schwab W, Weuster-Botz D (2021) Byproduct-free geraniol glycosylation by whole-cell biotransformation with recombinant *Escherichia coli*. Biotechnol Lett 43:247-259

APPENDIX

1. M9 salt (5×)

$Na_2HPO_4 \cdot 7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5.0 g
Deionized H_2O	Add to 1 L

Autoclave at 121˚C for 20 minutes.

2. M9 minimal media containing 2% glucose

M9 salts (5×)	20 ml
1 M $MgSO_4$ (filtered)	0.2 ml
20% glucose (filtered)	10 ml
1 M $CaCl_2$ (sterilized)	10 µl
Sterile Deionized H_2O	Add to 100 ml

LIST OF PUBLICATION

Original article

1. **Dorjjugder N.**, Hatano M., and Taguchi G.

   Production of flavonol and flavone 6-*C*-glucosides by bioconversion in *Escherichia coli* expressing *C*-glucosyltransferase from wasabi (*Eutrema japonicum*). *Biotechnology Letters* **43**: 1913–1919, 2021

2. **Dorjjugder N.**, and Taguchi G**.**

   Production of 7-*O*-glucosides by bioconversion using *Escherichia coli* expressing a 7-*O*-glucosyltransferase from tobacco (*Nicotiana tabacum*). *Applied Biochemistry and Biotechnology* **194**: 3320–3329, 2022

Reference articles

1. Phan D-N., **Dorjjugder N.**, Khan M-Q., Saito Y., Taguchi G., Lee H., Mukai Y., and Kim I-S.

   Synthesis and attachment of silver and copper nanoparticles on cellulose nanofibers and comparative antibacterial study. *Cellulose* **26** (11): 6629–6649, 2019

2. Phan D-N., **Dorjjugder N.**, Saito Y., Taguchi G., Lee H., Lee J-S., and Kim I-S.

   The mechanistic actions of different silver species at the surfaces of polyacrylonitrile nanofibers regarding antibacterial activities. *Materials Today Communications* **21**: 100622, 2019

3. Phan D-N., **Dorjjugder N.**, Saito Y., Taguchi G., Ullah A., Kharaghani D., and Kim I-S.

   The synthesis of silver-nanoparticle-anchored electrospun polyacrylonitrile nanofibers and a comparison with as-spun silver/polyacrylonitrile nanocomposite membranes

upon antibacterial activity. *Polymer Bulletin* **77**: 4197–4212, 2019

4. Kißling L., Schneider C., Seibel K., **Dorjjugder N.**, Busche T., Kalinowski J., and Mack M.

   The roseoflavin producer *Streptomyces davaonensis* has a high catalytic capacity and specific genetic adaptations with regard to the biosynthesis of riboflavin. *Environmental Microbiology* **22**: 3248–3265, 2020

5. Phan D-N., **Dorjjugder N.**, Saito Y., Khan M-Q., Ullah A., Bie X., Taguchi G., and Kim I-S.

   Antibacterial mechanisms of various copper species incorporated in polymeric nanofibers against bacteria. *Materials Today Communications* **25**: 101377, 2020

ACKNOWLEDGEMENTS

First, I would like to show my deepest appreciation to my supervisor, Prof. Goro Taguchi, for his great encouragement, kind support, enthusiastic attention, critical discussions, valuable advice, and guidance.

Also, I would like to express my sincere gratitude to all members of the Faculty of Textile, Science, and Technology, Global Leader Program for Fiber Renaissance, and Next-generation Advanced Human Resources/Local Leader Development Program for giving me this great opportunity. Their encouragement and support made my life very easy in Japan.

I wish to give special thanks to Prof. Makoto Shimosaka for his advice on my research and critical reviewing of my research papers.

I wish to thank Ms. Mayu Hatano, Ms. Rika Sato, and Mr. Keiichiro Taki for their help in the research for this thesis.

I wish to give special thanks to all members of the Taguchi Laboratory and Shimosaka Laboratory and all staff in Division of Gene Research Center, Research center for Advanced Science and Technology, Shinshu University, and the Research Center for Advanced Plant Factory, Shinshu University.

Special thanks to Ms. Sachiko Yoshioka for the NMR analysis.

My special thanks to all my friends for their constant support.

Finally, my special thanks to my mother Otgonbayar Bazar and my sister Burentegsh Dorjjugder for their deep love, real care, and encouragement at all times. I could not achieve this level without their great encouragement.