Studies on the functional metabolites in citrus

（カンキツの機能性代謝産物に関する研究）

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agricultural Science

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Declaration

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ABBREVIATIONS

Abbreviation	Elaboration
%	Percentage
°C	Degree Celsius
ANOVA	Analysis of variance
BA	Bile acid
BAT	Brown adipose tissue
BLAST	Basic Local Alignment Search Tool
cAMP	Cyclic adenosine monophosphate
CcOMT	*Citrus clementine O*-methyltransferase
CDC	Chenodeoxycholic acid
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CRE	cAMP response element
CreOMT	*Citrus reticulata O*-methyltransferase
CTAB	Cetyltrimethylammonium bromide
dATP	Deoxyadenosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli.	*Escherichia coli*
EtOH	Ethanol
FAOSTAT	The Food and Agriculture Organization Corporate Statistical Database
FBS	Fetal bovine serum
FOMT	Flavonoid *O*-methyltransferase
g	Gram
gDNA	Genomic deoxyribonucleic acid
GLP-1	Glucagon-like peptide-1
GPBAR1	G protein-coupled bile acid receptor1
GPCRs	G protein-coupled receptors
HEK293 cells	Human embryonic kidney 293 cells
HPLC	High-performance liquid chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
L	Liter
LB	Luria-Bertani
MEMα	Alpha-modified minimum essential medium
mg	Milligrams
min	Minutes

mL	Milliliter
MT	Methyltransferase
N-J	Neighbor-joining
OMT	*O*-methyltransferase
PCR	Polymerase chain reaction
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PKA	Protein kinase A
PMFs	Polymethoxyflavones
PPARs	Peroxisome proliferator-activated receptors
qRT-PCR	Quantitative RT-PCR
r.t.	Room temperature
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse-transcription
RT-PCR	Reverse-transcription polymerase chain reaction
SAM	*S*-adenosylmethionine
SDS	Sodium dodecyl sulfate
SDW	Sterilized distilled water
SQ	Semi-quantitative
Taq polymerase	*Thermus aquaticus* polymerase
TGR5	Takeda G protein-coupled receptor 5
UCP	Uncoupling protein
USDA	United States Department of Agriculture
UV	Ultraviolet
μL	Microliter
μm	Micrometer
μM	Micromolar
ARS	The Agricultural Research Service

LIST OF TABLES

LIST OF FIGURES

CONTENTS

Chapter 1: General Introduction

1.0 General Introduction

1.1 Origin and distribution of citrus

The genus *Citrus* L. belonging to the family Rutaceae and subfamily Aurantiodeae is one of the most important and widely cultivated fruit crops worldwide. Citrus genera are covered in a wide range of edible and commercial citrus cultivars (Shimizu et al., 2016). Citrus species originated in southeast foothills of the Himalayas, in a region that includes the eastern area of Assam, northern Myanmar, and western Yunnan and spread during the Middle Ages, later to became established in all continents (Calabrese 1994; Spiegel-Roy and Goldschmidt, 1996).

1.2 Taxonomic position of citrus

According to the USDA-ARS (https://www.ars-grin.gov/) the taxonomy of citrus plants follow the order;

Taxonomic position of citrus		
Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Rosidae
Order	:	Sapindales
Family	:	Rutaceae
Genus	:	*Citrus*

1.3 Production of citrus fruit and its utilization

Citrus fruit is considered a major evergreen fruit crop in world trade. The production of major citrus cultivars in sub-tropical and temperate areas are over 100 million metric tons per year (https://www.fas.usda.gov/data/citrus). China, Brazil, Mexico and the United States (U.S.) are among the leading commercial producers of citrus (https://www.fas.usda.gov/data/citrus). The U.S. is the world's largest citrus producer after Brazil (Patil, 2006). Wakayama, Ehime, and Shizuoka prefectures are the main citrus producing areas in Japan (FAOSTAT, http://www.fas.usda.gov/data/citrus-world-markets-and-trade). The fruit's special structure and

long shelf life have facilitated its large-scale export as fresh fruit. Fresh citrus fruit is consumed directly. Citrus fruit is also used for processed citrus-products and by-products. Globally, more than 40% of total citrus production is utilized for processed citrus products. The beverage industry mainly uses orange for its juice flavors (Ferguson, 1990; Yano et al., 1999). The most important processed citrus fruit-product is orange juice (Spiegel-Roy and Goldschmidt, 1996). Citrus industry occupies a vital position in the fruit industry and in the global agricultural economy (Webber, 1967).

1.4 Nutritional value of citrus

Citrus fruit is rich sources of essential health-promoting nutrients. Citrus fruit provide adequate nutrition including carbohydrates (fructose, glucose, and sucrose), non-starch polysaccharides (pectin, cellulose, and hemicellulose), glutarate, furocoumarins, vitamin C (ascorbic acid plus dehydroascorbic acid), folate, calcium, thiamin, niacin, vitamin B6, riboflavin, pantothenic acid, phosphorus, magnesium, copper, and various bioactive secondary metabolites (Economos and Clay, 1999; Kaur and Kaur, 2015). The nutritional composition of fresh citrus fruit is given in Table 1-1.

Table 1-1. Nutritional composition of fresh citrus fruits.

	Orange	Grapefruit	Tangerine
Weight (g)	131	236	84
Energy (kcal)	62	78	37
Fiber content (g)	3.1	2.5	1.7
Ascorbic acid (mg)	70	79	26
Folate (mcg)	40	24	17
Potassium (mg)	237	350	132

[Source: Gutherie et al., 1995]

1.5 Citrus secondary metabolites

Numerous phytochemicals are produced by citrus plants primarily to serve the purpose of defense to insects and microbial attack. These phytochemicals are also known as plant secondary metabolites. Secondary metabolites play a vital role in both health benefits and disease prevention. The major secondary metabolites of citrus fruits are flavonoids, limonoids, coumarins, alkaloids, terpenoids, carotenoids, phenolic acids, and essential oils (Hasegawa and Herman, 1992; Manners, 2007; Patil et al., 2009).

1.6 Human health benefits of citrus secondary metabolites

Citrus secondary metabolites are a large and diverse group of specialized metabolites specific to certain groups of citrus. Citrus specialized metabolites, flavonoids and limonoids, have been reported to exhibits diverse bioactivity on human health, including effects against memory impairment of Alzheimer's disease (Braidy et al., 2017; Kimura et al., 2018; Onozuka et al., 2008), as well as the activities of anti-cancer, anti-hyperglycemic, anti-obesity, insulin resistance, and anti-microbial (Chowdury et al., 2003; Govindachari et al., 2000; Jacob et al., 2000; Kawaii et al., 1999; Lam et al., 1994; Lee et al, 2010, 2013; Miller et al., 2004; Minagawa et al., 2001; Miyata et al., 2008; Surichan et al., 2018).

1.7 Utilization of citrus by-products

Citrus fruits are commercially grown across the globe and consumed either fresh or in processed form. Approximately 50% of citrus fruit remains unconsumed and discarded as waste. The waste contains almost 50% of the original fruit mass, of which ~40–55% are the peels, ~30–35% internal tissues and ~10% seeds (Sharma et al., 2018) (Fig. 1-1). Direct disposal of these bioactive compounds to the open landfills causes bad odor and spread of diseases, and disposal to water bodies or seepage to the underground water table deteriorates water quality and harms aquatic life. In this regard, many researchers are focused their research on the development of better reuse methods to obtain value-added phytochemicals as well as

for safe disposal. The phytochemicals such as flavonoids, essential oils, citric acid, and pectin obtained from citrus and its by-products are attracting great attention in industrial, food and synthetic chemistry-related research (Rafiq et al., 2018; Sharma et al., 2018).

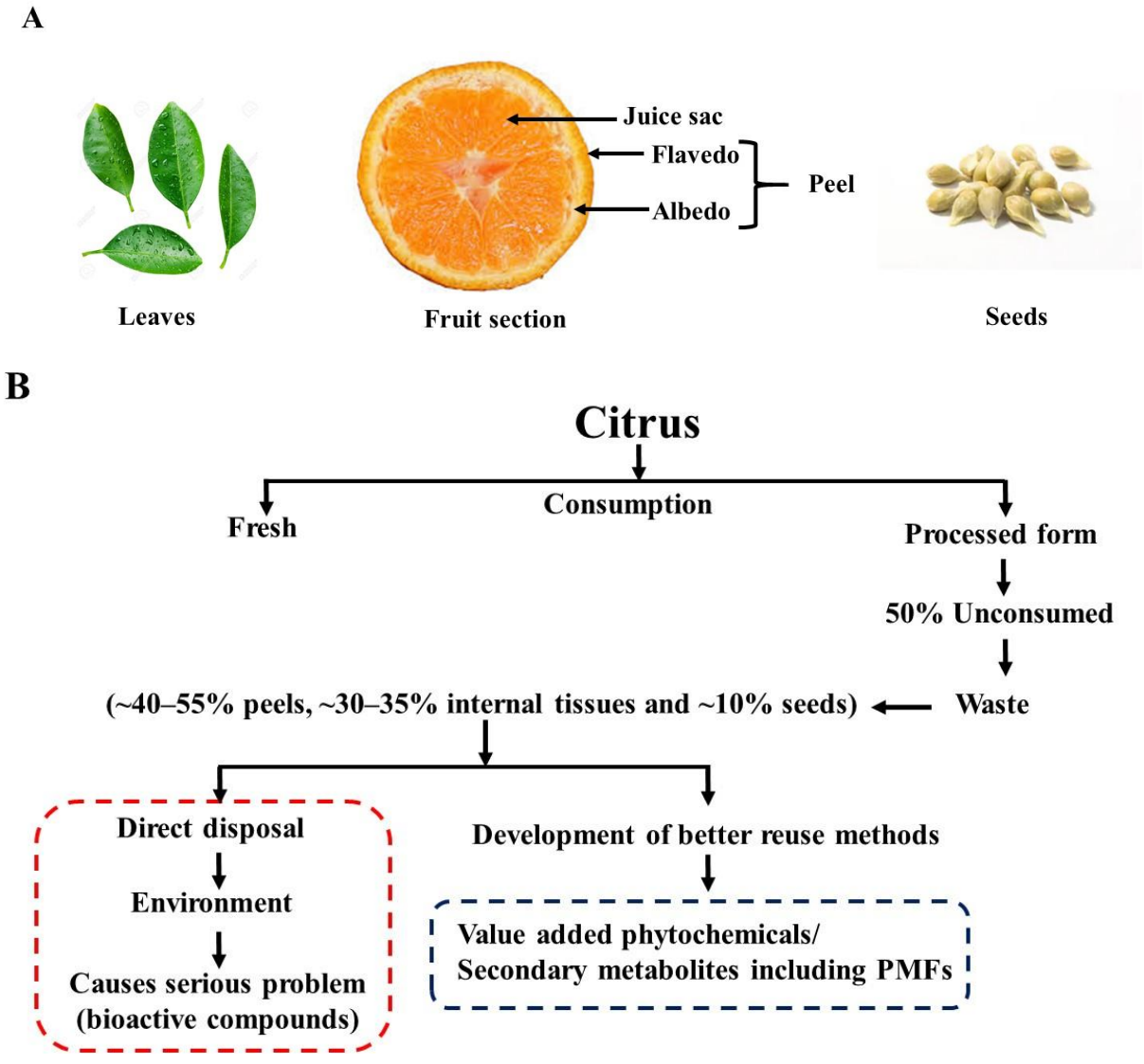


Fig. 1-1. Citrus leaves, internal fruit parts and it's by-products use flow diagram.

(A) Citrus young green leaves, fruit section, and seeds. (B) Uses of citrus fruit parts and it's by-products.

1.8 Citrus polymethoxyflavone (PMF)

Polymethoxyflavone (PMF) is a highly *O*-methylated (methoxylated) flavones and abundant in citrus (Nogata, 2006). Major citrus PMFs are sinensetin (3',4',5,6,7-pentamethoxyflavone), nobiletin (3',4',5,6,7,8-hexamethoxyflavone), heptamethoxyflavone (3,5,6,7,8,3',4',-heptamethoxyflavone) and tangeretin (4',5,6,7,8-pentamethoxyflavone) (Fig. 1-2). It has been reported that there are many physiological activities in PMF, among them most prevalent PMF nobiletin attracts the greatest attention for its potential role of human health beneficial functions in recent years.

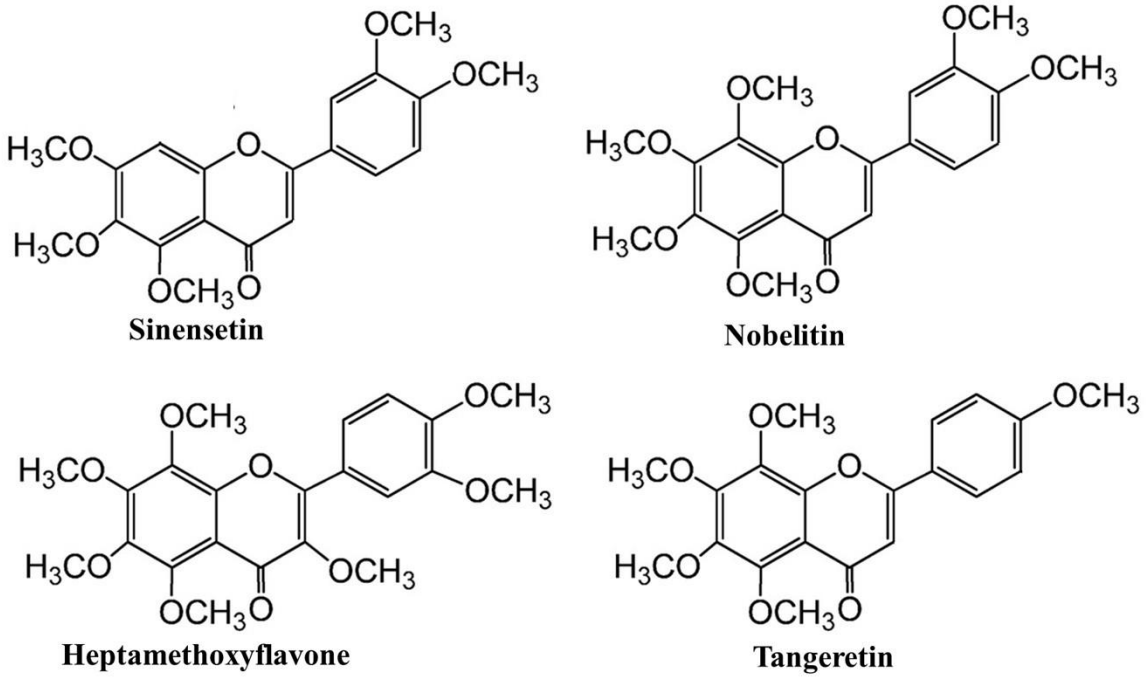


Fig. 1-2. Chemical structure of citrus four major polymethoxyflavone (PMF).

1.9 PMF biosynthesis in citrus and other plant species

Methylation of the hydroxy group of flavonoids in plants is carried out by *O*-methyltransferase (OMT). OMTs transfer a methyl group from *S*-adenosyl-L-methionine (SAM) onto a hydroxyl group (Fig. 1-3) (Ibrahim et al., 1998). Therefore, OMTs are considered to be involved in the synthesis of PMF in citrus. Plant OMTs can be distinguished between Class I OMTs, Class II OMTs and Class III OMTs, based on their primary amino acid sequence (Noel et al., 2003). These families have been generally implicated in the modification of flavonoids (Class I), the methylation of caffeoyl CoA, which is a step in lignin formation (Class II), and the modification of small phenolic compounds such as salicylic acid (Class III) (Lam et al., 2007; Noel et al., 2003). Many OMT genes have been reported so far, and function analysis of them has been advanced. As an example, in wheat *TaOMT2*, which is involved in continuous *O*-methylation of the flavone, tricetin to its 3'-methyl-(selgin), tricine, 3',5'-dimethyl-(tricin) and 3',4',5'-trimethyl ether derivatives (Zhou et al., 2010). The *Brachypodium distachyon*, caffeic acid *O*-methyltransferase (*BdCOMT4*) has the function of *O*-methylating caffeic acid, which is a precursor of lignin biosynthesis (Trabucco et al., 2013). In Alphonso mango, *MiOMTS* showed substrate specificity towards furaneol and protocatechuic aldehyde synthesizing mesifuran and vanillin, respectively, specifically expressed in the fruit of Alphonso mango, involved in the synthesis of mesifuran and vanillin (Chidley et al., 2016). The methylation of anthocyanins is catalyzed by *S*-adenosyl-L-methionine: anthocyanin *O*-methyltransferase (AOMT) (Harbourne, 1958). A cDNA encoding *S*-adenosylmethionine: anthocyanin 3',5'-*O*-methyltransferase (*A3',5'OMT*) are derived from *Torenia hybrida* petals, involved in the modification of flower color (Nakamura et al., 2015). *AnthOMT*, an *O*-methyltransferase (OMT) mediating the methylation of anthocyanins in the seedlings of tomato (*Solanum lycopersicon* var. MoneyMaker) (Roldan et al., 2014). Five flavonoid-*O*-methyltransferase (*FOMT*) genes were isolated from *Citrus depressa* (*CdFOMT1*, *3*, *4*, *5*, and

6). Of these five *FOMT* genes, *CdFOMT5* was successfully expressed as a soluble homodimer enzyme in *Escherichia coli* that exhibited *O*-methyltransferase activity for quercetin, naringenin, (-)-epicatechin, and equol using *S*-adenosyl-L-methionine as a methyl donor, thus, *CdFOMT5* is an *O*-methyltransferase possessing a broad range of substrate specificity and regioselectivity for flavonoids (Itoh et al., 2016).

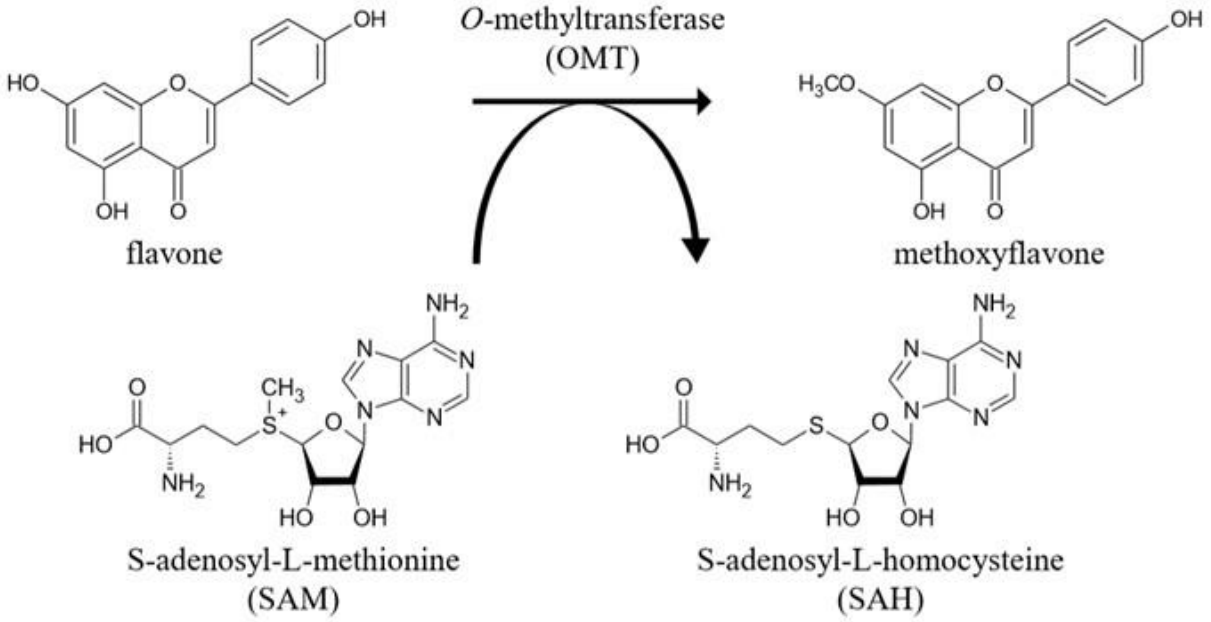


Fig. 1-3. Methylation of the hydroxyl group of flavonoids in the plant is carried out by *O*-methyltransferase (OMT) using *S*-adenosyl-L-methionine (SAM) as a methyl group donor (Ibrahim et al., 1998).

1.10 G protein-coupled bile acid receptor, GPBAR1 (TGR5), a bile acid receptor and its functional roles in human health

G protein-coupled receptors (GPCRs) are a large family of receptors, playing important roles in multiple pathways (Cvijic et al., 2015). Recently, GPCRs have attracted great attention for the treatment of many diseases due to their important functional roles in different cell signaling pathways (Guo et al., 2016). G protein-coupled bile acid receptor 1 (GPBAR1), also known as Takeda G protein-coupled receptor 5 (TGR5), is a member of 7 transmembrane-bound G protein-coupled receptors (GPCRs) family. TGR5 was discovered in 2002 (Foord et al., 2005; Kawamata et al., 2003; Maruyama et al., 2002; Pols et al., 2011). In humans, the TGR5 gene locates on chromosome position 2q35 and its open reading frame has 993 base pairs, encoding 330 amino acids. High levels of TGR5 mRNA were detected in several organs such as small intestine, adipose tissue, skeletal muscle, stomach, liver, lung, especially placenta and spleen in humans (Kawamata et al., 2003; Keitel et al., 2007; Tiwari and Maiti, 2009).

In humans, the liver is the major cholesterol synthesis organ and synthesize around 1 g of cholesterol per day (Grundy and Metzger, 1972; Jones, 1997). Through several enzymatic processes, the liver can dispose of excess cholesterol by converting it to bile acids and excreting them in the bile (Chiang, 2009; Russell and Setchell, 1992). Bile acid plays an important role in lipid absorption in the small intestine and regulation of cholesterol metabolism (Morimoto et al., 2013; Tiangang and Chiang, 2009). In the 2000s, TGR5 was found as a receptor binding to bile acid in the blood (Kawamata et al., 2003; Maruyama et al., 2002).

It has been reported that TGR5 activation could have beneficial effects on several metabolic syndromes (Sasaki et al. 2018; Thomas et al., 2008; Watanabe et al., 2006). Therefore, TGR5 agonists could be potential drugs for the treatment of metabolic, inflammation and digestive disorders (Broeders et al., 2015; Kumar et al., 2012). Recently, several potential TGR5 agonists

have been identified among a variety of plant extracts such as oleanolic acid (Sato et al., 2007), betulinic acid (Genet et al., 2010), and nomilin (Ono et al., 2011).

1.11 Pathways by which TGR5 agonists exert their actions on the small intestine, BAT, and skeletal muscle

Ligand binding to TGR5 induced an intracellular cAMP by transduction through a G protein and receptor trafficking. It has been reported that lithocholic acid, deoxycholic acid, chenodeoxycholic acid, and cholic acid could increase intracellular cAMP (Kawamata et al., 2003; Li et al., 2011). The previous study demonstrated that TGR5 is highly expressed in the gallbladder causing smooth muscle relaxation (Li et al., 2011). Intake of a high-fat diet for 2 months by TGR5-deficient mice showed higher fat accumulation with body weight gain, suggesting an important role of TGR5 in energy homeostasis (Maruyama et al., 2006). Mainly, energy homeostasis is regulated through TGR5 activation by bile acids (Watanabe et al., 2006). Treatment of cholic acid to diet-induced obese mice normalized their body weight and increases energy expenditure in BAT(brown adipose tissue) (Fig. 1-4). The bile acids effect is dependent on the induction of the cAMP-dependent thyroid hormone-activating enzyme type 2 iodothyronine deionase (D2). D2 converts inactive thyroxine (T4) into the active thyroid hormone 3,2,3'-tri-iodothyronine (T3) (Bianco and Kim, 2006). The T3 generation is providing a higher concentration of intracellular active T3 that necessary to occupy the number of thyroid hormone receptors (TRs) in selected tissues (de Jesus et al., 2001).

Increased intracellular cAMP levels mediated by TGR5 activation is also induced gene expression of peroxisome proliferator-activated receptor γ-coactivator 1α (PGC-1α). PGC-1α regulates energy expenditure by interacting with a selected subset of nuclear receptors, including the peroxisome proliferator-activated receptors (PPARs) and TRs. PPARγ or PPARα activation regulates the expression of uncoupling protein isoforms (UCP) isoforms (Kelly et al., 1998). Transcriptional regulation of genes related to β-oxidation of fatty acids is also under

the control of a combination of PPARα and PGC-1α (Vega et al., 2000). Consequently, PGC-1α activation is stimulating energy expenditure through modulation of mitochondrial activity.

1.12 Roles of TGR5 in intestinal cells

The previous study has been demonstrated the potential functional roles of TGR5 in intestinal cells (Fig. 1-4). Through a TGR5 activation and induction of intracellular cAMP levels mechanism, bile acids induce GLP-1 secretion from intestinal enteroendocrine secretin tumor cells (Katsuma et al., 2005).

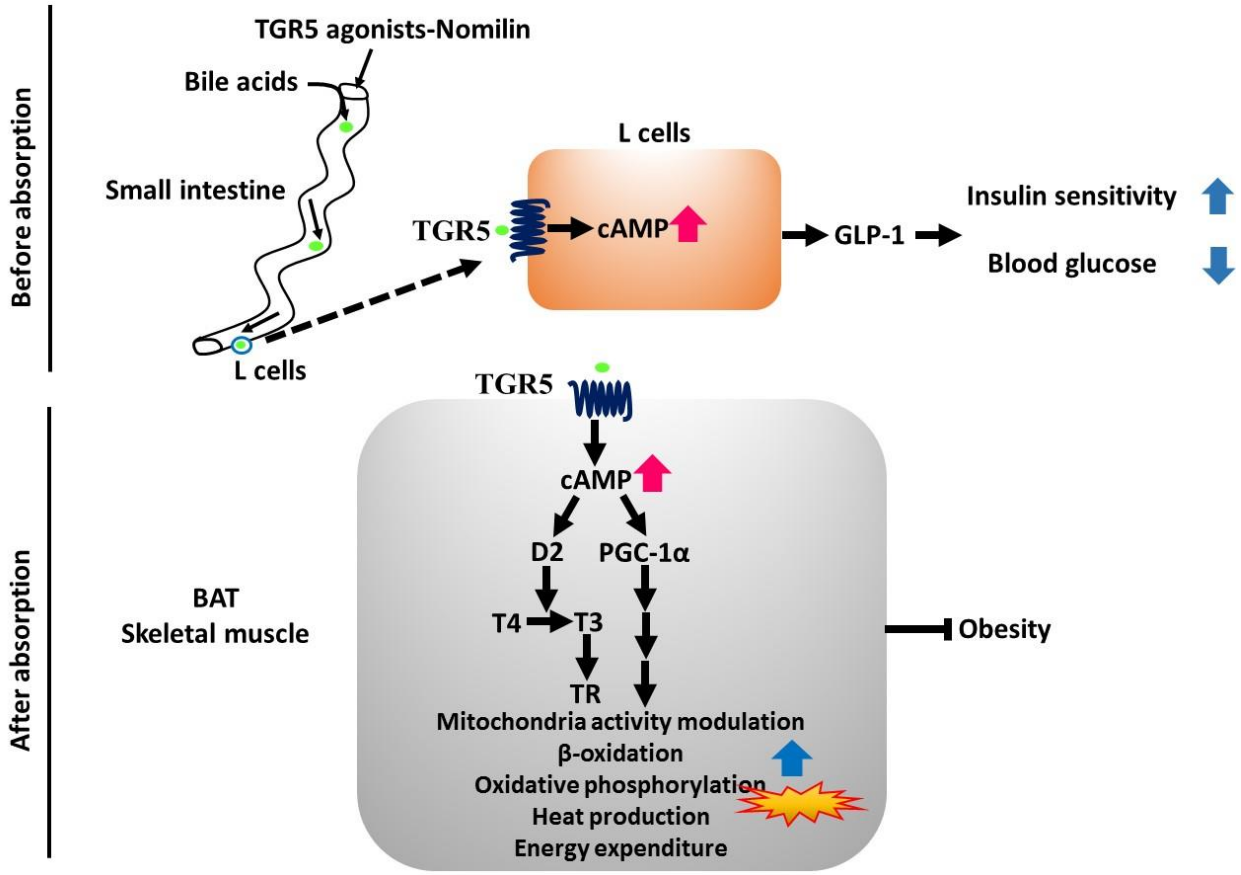


Fig. 1-4. Proposed pathways by which TGR5 agonists exert their action on the small intestine, BAT, and skeletal muscle. (Sato, 2013).

1.13 Luciferase assay system and screening of TGR5 agonists

Bile acids regulate glucose and energy metabolism through TGR5. So, researchers are paying their attention to the development of potent small molecule agonists of TGR5 and also trying to screen naturally occurring compounds that stimulate the bile acid functions (Thomas et al., 2008; Tiwari and Maiti, 2009). TGR5 is activated by its agonists and induces the intracellular cAMP levels. In a luciferase assay system, the cAMP response element (CRE)-driven reporter gene is enhanced by the endogenously activated CRE-binding protein. Several TGR5 agonists such as betulic, oleanolic, ursolic acid, and nomilin were found using a luciferase assay system (Genet et al., 2010; Ono et al., 2011; Sato et al., 2007). Ono et al. (2011) established the luciferase assay system using human HEK293 cells that overexpress human TGR5 together with a CRE-containing (four repeats) reporter gene (Fig. 1-5). Approximately 160 commercially available purified food constituents and their derivatives, which are categorized as flavonoids (45%), terpenoids (20%), and steroids (15%), have been evaluated as potential TGR5 agonists. Ono et al. (2011) have found a citrus limonoid-nomilin, to be the most potent agonist using the luciferase assay system.

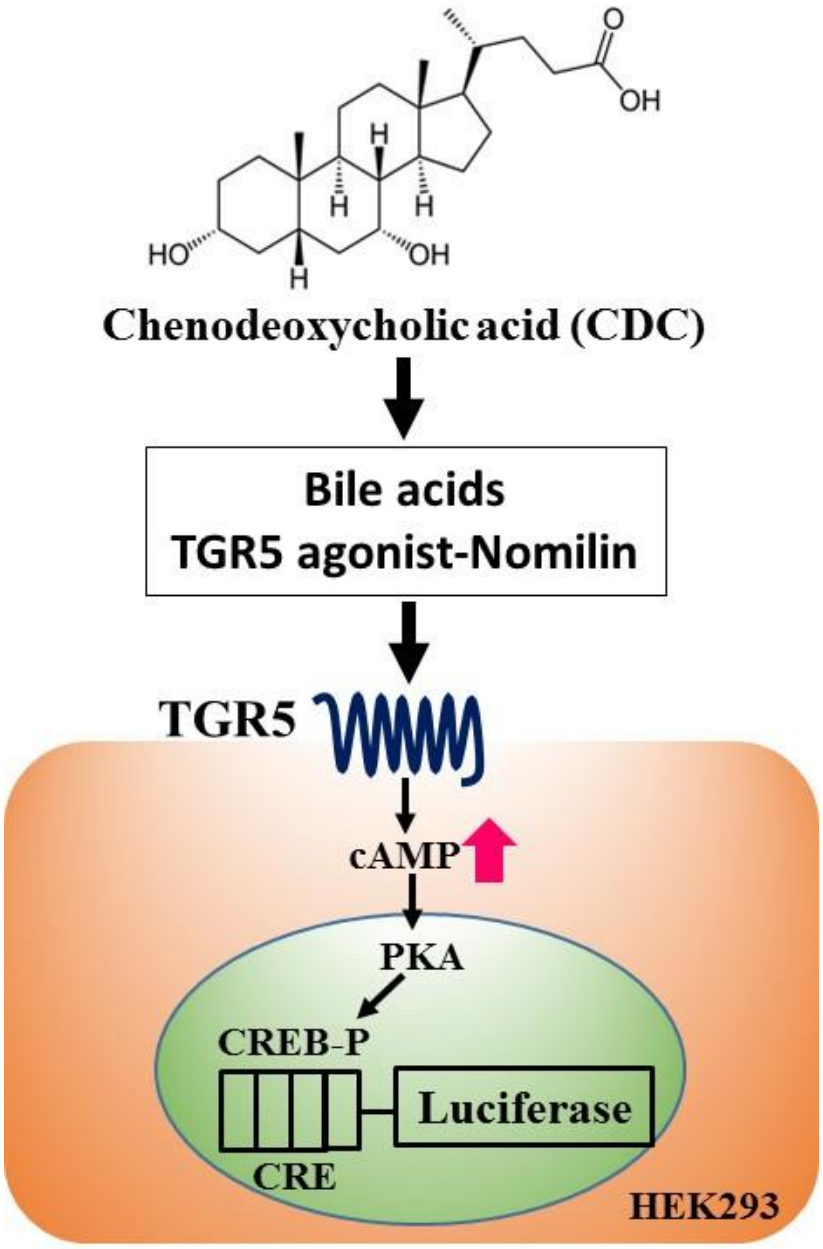


Fig. 1-5. A citrus limonoid, nomilin, as a TGR5 agonist. A schematic diagram of isolation of a novel TGR5 agonists, HEK293 cells expressing in human TGR5 and the CRE-driven reporter were cultured in the presence of citrus nomilin (Ono et al., 2011; Sato, 2013).

1.14 Citrus limonoids, a potential agonist of TGR5

Limonoids are highly oxygenated, modified triterpenoid compounds. These specialized compounds are naturally found in the Rutaceae and Meliaceae plant families (Manners, 2007). Limonoids are synthesized through the acetate-mevalonate pathway in citrus (Roy and Saraf, 2006). Citrus seed and fruit contain a significant amount of limonoids as aglycones and glycosides. Citrus and their closely related species contain 36 limonoid aglycones and 17 limonoid glucosides (Patil et al., 2009). The major limonoids are limonin, nomilin, obacunone, deacetylnomilin and limonin glucoside (limonin 17-β-D-glucopyranoside) (Fig. 1-6). The limonin and nomilin are the two major bitter components in citrus juice (Dea et al., 2013; Endo et al., 2002; Hasegawa et al., 1973; Higby, 1938).

It has been reported that citrus limonoids showed several significant physiological and biological activities such as anti-cancer (Govindachari et al., 2000; Guthrie et al., 2000; Tian et al., 2001), anti-malarial (Manners, 2007; Roy et al., 2006), anti-microbial (Abdelgaleil et al., 2004, 2005), insect-killing or eating disorders, growth regulation (Jacob et al., 2000; Lam et al., 1994; Miller et al., 2004), and anti-HIV activity (Battinelli et al., 2003). Recently, the citrus limonoid nomilin is considered as a novel TGR5 agonist, which can bind to the bile acid receptor and showed the significant effects on anti-obesity and antihyperglycemic in mice fed a high-fat diet (Ono et al., 2011).

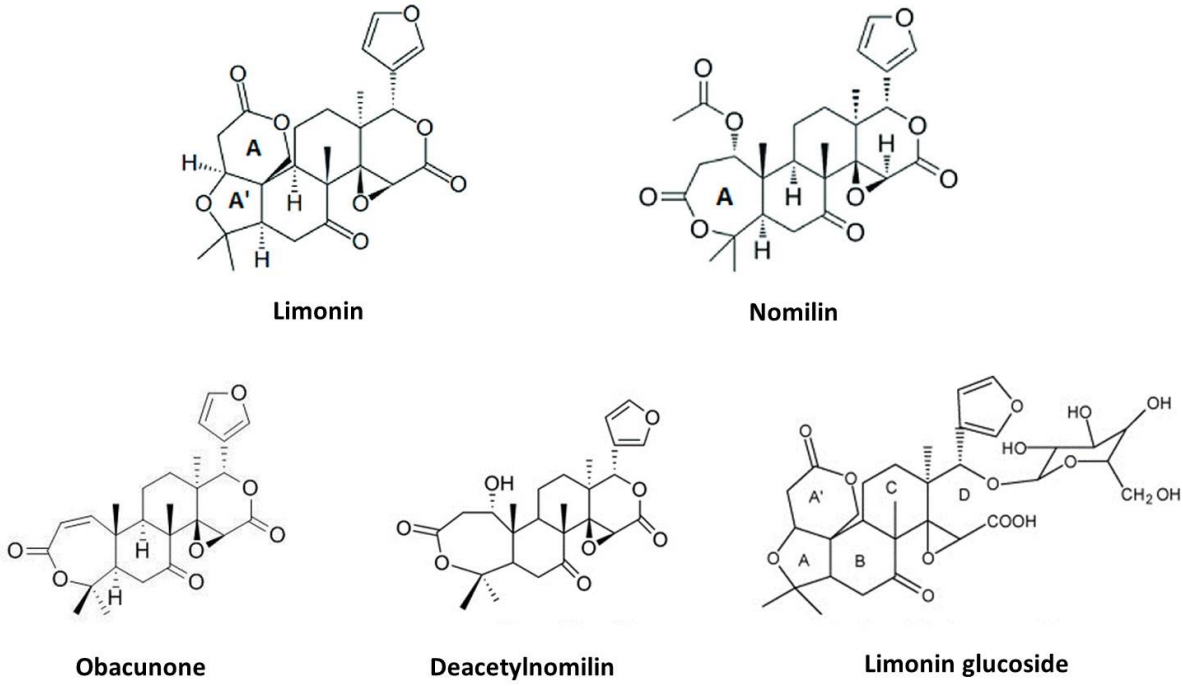


Fig. 1-6. Chemical structure of major citrus limonoids.

1.15 Objectives of this research

(1) To determine the accumulation of major PMFs, nobiletin and tangeretin, in the leaves and flavedos of selected citrus cultivars;

(2) To determine the correlation between nobiletin and tangeretin in the leaves and flavedos of selected citrus cultivars;

(3) To identify the *OMT* genes involved in the PMF biosynthesis;

(4) To determine the expression of candidate *OMT* genes in the leaves and flavedos of selected citrus cultivars;

(5) To determine the limonoid content in different tissues of sour orange (*Citrus aurantium*) using different solvents;

(6) To measure TGR5 ligand activity for sour orange derived extracts;

Chapter 2: Accumulation of polymethoxyflavone and *O*-methyltransferase gene expression in various citrus cultivars

2.1 Introduction

Citrus is one of the most popular and widely cultivated fruits in the world. Many citrus species are considered to be rich in secondary metabolites (present in the peel, pulp, seed, pressed oil, juice or whole fruit) that play important roles in plant cells and human health due to their functional properties (Patil et al., 2006; Tripoli et al., 2007). Polymethoxyflavone (PMF) are naturally occurring *O*-methylated flavonoid compounds found exclusively in citrus belonging to the Rutaceae family as well as in the plant species in the Lamiaceae and Asteraceae families (Fig. 2-1; Berim and Gang, 2016; Kefford and Chandler, 1970; Li et al., 2009; Patnayak et al., 1942). The peels of citrus fruits contain higher amounts of PMF than other edible parts of the fruit (Ke et al., 2017; Nogata et al., 2006). Citrus fruits are used in the juice industry for juice production as a raw material as well as in the food, beverage, cosmetic, and pharmaceutical industries as food additives, spices, cosmetic ingredients, and chemoprophylactic drugs, respectively (Braddock, 1999; Lv et al., 2015). The peels are often discarded as waste materials from the juice and other industries and citrus peel byproducts are utilized as a source of functional ingredient (Braddock, 1999; Manthey and Grohmann, 2001; Rafiq et al., 2018). Nobiletin and tangeretin are two major PMF distinctively abundant in citrus peels (Choi et al., 2007; Inafuku-Teramoto et al., 2011; Nogata et al., 2006). Recently, citrus PMF have attracted great interest due to their effects against memory impairment in Alzheimer's disease (Braidy et al., 2017; Kimura et al., 2018; Onozuka et al., 2008), as well as their activities of anti-tumor, anti-hyperglycemia, anti-obesity, and anti-neuroinflammation and insulin resistance (Ho and Kuo, 2014; Kawaii et al., 1999; Lee et al., 2010, 2013; Minagawa et al., 2001; Miyata et al., 2008; Onda et al., 2013; Surichan et al., 2018; Wang et al., 2014).

In plants, *O*-methyltransferase (OMT) is considered a major group of methyltransferase (MT). Methylation of the hydroxy group of flavonoids in plants is carried out by OMT using *S*-adenosyl-L-methionine (SAM) as a methyl group donor (Ibrahim et al., 1998; Roje, 2006;

Struck et al., 2012). In the PMF, the hydroxy group(s) is replaced with a methoxy group(s). Citrus PMFs are a special group of *O*-methylated flavonoids and have many methoxy groups in their parent molecules. OMT proteins that are encoded by the *OMT* gene family transfer a methyl group from SAM onto the hydroxy group, and they are considered to be involved in the biosynthesis of PMF (Berim and Gang, 2016; Itoh et al., 2016). The functions of plant *OMT* genes in flavonoid biosynthesis have been investigated in many plant species, such as barley, Madagascar periwinkle, mango, and rice (Byeon et al., 2015; Chidley et al., 2016; Christensen et al., 1998; Schröder et al., 2004). Recently, five flavonoid-*O*-methyltransferase (FOMT) genes were identified from Shiikuwasha (*Citrus depressa* Hayata) that are known to accumulate nobiletin in the fruits peels (Itoh et al., 2016). Moreover, 58 *OMT* genes were identified from the entire *C. sinensis* genome (Liu et al., 2016). However, a little is known about the PMF accumulation, the genes responsible for PMF biosynthesis, or their expression in the leaves and flavedos of various citrus cultivars. Quantification of nobiletin and tangeretin in various citrus cultivars and analysis of related *OMT* genes are needed because of their broad spectrum of biological activities and the value of citrus as a functional food.

In this study, therefore, the accumulation of nobiletin and tangeretin in the leaves and flavedos and the expression of the related *OMT* genes that are putatively involved in PMF biosynthesis was investigated in various citrus cultivars. Notably, the expression of two *OMT* genes, *CreOMT1* and *CreOMT4* were possibly related to the accumulation of PMF. Identifying the *OMT* genes related to PMF biosynthesis and elucidating their functions in relation to PMF accumulation in leaves and flavedos would play an important role in studying the detailed mechanism of PMF accumulation in citrus.

2.2 Materials and Methods

2.2.1 Screening of citrus cultivars and collection of plant materials

In total, 125 citrus accessions, including wild citrus plants that are growing in the citrus germplasm collections at Saga University, Japan were used for preliminary screening based on the PMFs accumulation in leaves. According to the PMF accumulation, classified them into three categories, e.g. high, medium and low PMF accumulating plants (Yuri Itami, Graduation thesis, 2018, Department of Applied Biological Sciences, Laboratory of Fruit Sciences, Faculty of Agriculture, Saga University, Japan). Among them, in this study 11 citrus cultivars were selected from the high, medium and low group. Tissue samples (leaves and flavedos) of 11 citrus cultivars, 'Amanatsu' (*Citrus natsudaidai* Hayata), 'Aoshima' (*C. unshiu* Marcow), 'Benibae' ('HF No. 9' × 'Encore'), 'Hirakishu' (*C. kinokuni* hort. ex Tanaka), 'Kiyomi' (*C. unshiu* Marcow × *C. sinensis* Osbeck), 'Mihaya' ('Tsunonozomi' × 'No. 1408'), 'Ogimi kuganii' (*C. depressa* Hayata), 'Ota' Ponkan (*C. reticulata* Blanco), 'Seinannohikari' ('EnOw No. 21' × 'Youkou'), 'Shiranuhi' ['Kiyomi' × 'Nakano No.3' (*C. reticulata*)], and 'Yoshida' Ponkan (*C. reticulata*) grafted onto trifoliate orange [*Poncirus trifoliata* (L.) Raf.], were collected in January 2018 from the citrus germplasm collections at Saga University, Japan. Out of the 11 cultivars, 9, 10, and 11 cultivars were used for semi-quantitative reverse-transcription (RT)-polymerase chain reaction (PCR), quantitative RT-PCR (qRT-PCR), and quantitative analysis for PMF, respectively. The mature leaves were collected randomly, whereas flavedos were collected from the ripened citrus fruits (flavedos were sliced from the peels), and both tissues were quickly frozen in liquid nitrogen and stored at -70°C until further analysis.

2.2.2 Sample preparation for high-performance liquid chromatography (HPLC) analysis

Tissue samples were freeze-dried for overnight and crushed to a fine powder by a multi-beads shocker (Yasui Kikai Corp., Osaka, Japan) using a standard program. Fifty milligrams of the powdered sample was added to 500 μL of 80% methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and mixed by vortex, then extracted using an ultrasonic cleaner US-3R (AS ONE Corp., Osaka, Japan) for 10 min. The mixture was stored in the dark at room temperature

(r.t.). Subsequently, the sample was centrifuged at 10000 rpm with a TOMY MX-200 (TOMY Seiko Co., Ltd., Tokyo, Japan) for 1 min at r.t., and the supernatant was collected. Then 500 μL of 80% methanol was added, mixed by vortex, and centrifuged at 10000 rpm for 1 min at r.t. (this step was repeated twice). Finally, extracted supernatant was adjusted up to 2.5 mL by adding 80% methanol and filtered through a 0.45 μm RephiQuik PTFE syringe filter (RephiLe Bioscience, Ltd., Shanghai, China) into a 2 mL vial (Agilent Technology, Santa Clara, CA, USA) and stored at -20°C until analysis. The experiment was performed in three biological replicates.

2.2.3 HPLC analysis

Samples were analyzed using an HPLC system (Pump: PU-2089 plus, Autosampler: AS-2057 Plus, Detector: UV-2075, Jasco Corp., Tokyo, Japan; Column oven: CTO-10AC, Shimadzu Corporation, Kyoto, Japan) equipped with an Inertsil® ODS-3 column (3 μm, 4.6 × 33 mm; GL Sciences Inc., Tokyo, Japan) under the following conditions: solvent A was 10% methanol with 0.1% phosphoric acid, and solvent B was 90% methanol with 0.1% phosphoric acid; the HPLC separation condition was as follows: 87.5% solvent A from 0 to 4 min, 87.5-40.0% A from 4 to 10 min, 40.0% A from 10 to 20 min, 40-87.5% A from 20 to 21 min, 87.5% A from 21 to 30 min. The flow rate was 1.2 $mL \cdot min^{-1}$, and the UV was measured at 254 nm. The temperature of the column oven was set at 40°C and the sample injection volume was 5.0 μL. The analysis time was 30 min per sample.

2.2.4 Identification of OMT genes using C. clementina genome database

To identify flavonoid *OMT*-related genes across the latest *C. clementina* genome database (ver. 1.0), a Basic Local Alignment Search Tool (BLAST) search was conducted in Phytozome (https://phytozome.jgi.doe.gov/), the Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute, based on the amino acid sequence of a flavonoid *7-OMT* gene CAA54616) in barley. A flavonoid *7-OMT* gene in barley has been found to have a function of

O-methylating apigenin and the hydroxy group at the 7th-position of naringenin (Christensen et al., 1998). From this search, 16 candidate genes for citrus *OMT* (designated as *CcOMT*) were obtained in descending order of homology (Table 2-1).

2.2.5 Isolation of OMT genes from 'Yoshida' Ponkan

2.2.5.1 Primer Design

The genomic sequences of three putative candidate *CcOMT1, CcOMT2*, and *CcOMT4* genes were used to design primers for gene isolation. The designed primers are shown in Table 2-2.

2.2.5.2 Total RNA isolation

The leaves of 'Yoshida' Ponkan were used to isolate Total RNA by cetyltrimethylammonium bromide (CTAB) method. In the CTAB method, four 1.5 mL tubes containing 700 µL of 2 × CTAB (0.5% 2-mercaptoethanol) were prepared. The sample (0.15 g/tube) was ground in liquid nitrogen and 100 mg was placed in the prepared tube. Incubate at 65°C for 15 minutes, then add an equal volume of chloroform isoamyl alcohol and vortex. After centrifugation (14,000 rpm, 10 min, 10°C; hybrid high-speed refrigerated centrifuge 6200; KUBOTA CORPORATION, Tokyo, Japan), the supernatant was taken and transferred to a new 1.5 mL tube. Add chloroform isoamyl alcohol again to this tube and vortex. After centrifugation, the supernatant was removed and transferred to a new 1.5 mL tube. Add the 3/4-fold amount of isopropanol and leave at room temperature for 10 minutes. After centrifugation, the precipitate was dissolved in 100 µL SDW (sterilized distilled water). Add 1/4-fold amount of 10 M lithium chloride and leave at -30°C for 2 hours. After centrifugation, the precipitate was dissolved in 100 µL SDW. After dissolution, 4 tubes were merged into 1 tube and proceeded to 400 µL/tube. Add an equal volume of phenol/chloroform and vortex, centrifuge and transfer the supernatant to a new 1.5 mL tube. Add an equal volume of chloroform isoamyl alcohol and vortex, centrifuge and transfer the supernatant to a new 1.5 mL tube. 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% EtOH were added and mixed, the mixture was allowed to

stand at -80°C for 20 minutes, centrifuged, and the supernatant was discarded. 500 µL of 70% EtOH was added, lightly tapped and centrifuged (14,000 rpm, 10 minutes), and the supernatant was discarded.

Air-dried at 37°C for 8 minutes and dissolved in 100 µL of SDW 9 µL of SDW was added to a new 1.5 mL tube, 1 µL of RNA solution was added, and the absorbance was measured with BioSpec-mini (SHIMADZU CORPORATION, Kyoto, Japan). In addition, 1 µL of GelRed (Biotium, Inc., Fremont, Calif., USA) diluted 50 times with 6 µL of RNA Buffer and 6 × Loading Buffer was mixed with 3 µL of the obtained RNA solution and heat denaturation at 65°C for 15 minutes. After that, it was placed on ice for 5 minutes and electrophoresed using a gel for RNA migration to confirm the band.

2.2.5.3 Synthesis of complementary DNA (cDNA)

RT reaction was performed using QuantiTect Reverse Transcription Kit (QIAGEN). 'Yoshida' Ponkan total RNA isolated from the leaves were as a template. The method is shown below;

RT reaction

gDNA wipeout Buffer (7 ×)	:	4 µL
Template RNA	:	2 µg
RNase-free water	:	up to 28 µL

React the above mixture at 42°C for 2 minutes and immediately transfer onto ice.

RTase	:	2 µL
RT Buffer	:	8 µL
Primer mix	:	2 µL
gDNA elimination reaction	:	28 µL

The above mixture was allowed to react at 42°C for 30 minutes and at 95°C for 3 minutes, then stored frozen at -80°C.

2.2.5.4 RT-PCR (Reverse transcriptase-polymerase chain reaction)

RT products of 'Yoshida' Ponkan leaves were used to amplify the cDNA of three *OMT* genes corresponding to *CcOMT1*, *CcOMT2*, and *CcOMT4* by PCR using KOD FX (TOYOBO, Osaka, Japan. The generation of mixtures during RT-PCR are given below;

RT-PCR mixture (KOD FX)		
Template	:	1.0 µL
KOD FX	:	0.5 µL
KOD FX buffer	:	12.5 µL
dNTP	:	5.0 µL
Sense primer (10 pmol/µL)	:	0.5 µL
Anti-sense primer (10 pmol/µL)	:	0.5 µL
SDW	:	up to 25 µL

2.2.5.5 Gel electrophoresis

One µl of the 25 µl reaction mixture was electrophoresed on a 1% agarose gel.

2.2.5.6 Addition of nucleotide 'A'

For TA cloning, dATP addition to the 3' end of the RT-PCR product was performed. In this case, 10 × A-attachment Mix (TOYOBO) was used and the steps are mention below;

1. Add 0.5 µL of 10 x A-attachment Mix to 4.5 µL RT-PCR product and mix well.

2. Incubate at 60°C for 10 minutes and transferred on ice.

2.2.5.7 TA cloning

As a vector, pGEM®-T Easy vector (Promega, Madison, WI, USA) was used. 2.5 µL of the insert was added to 0.5 µL of vector and an equivalent amount of Ligation Mix (TAKARA BIO) was added on ice. Thereafter, the reaction was carried out at 16°C for 30 minutes. Competent cell DH5α was used for the introduction of the plasmid into *E. coli.* About 100 µL of competent cells thawed on ice was added to the reaction solution, and the mixture was allowed to stand on ice for 30 minutes, then kept at 42°C for 30 seconds, and again placed on ice for 2 minutes. 500 µL of Super Optimal broth with Catabolite repression (SOC) medium was added and shake cultured at 37°C for 1 hour. Thereafter, it was coated on ampicillin-containing (100 mg/L) Luria-Bertani (LB) broth agar medium previously coated with 50 µL of X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 100 µL of Isopropyl-*β*-D-thiogalactopyranoside (IPTG) for 17 hours. Thereafter, clones of the formed colonies were checked. In the clone check, a small number of colonies on the plate were scraped off at the tip of a toothpick and suspended in 30 µL of SDW and then boiled for 10 minutes. 0.5 µL of the

boiled product was added to the PCR reaction solution and PCR was performed. Ex-taq (TAKARA BIO) was used as a PCR enzyme. The PCR program was repeated for 35 cycles after one cycle of heating at 95°C for 2 minutes, one cycle consisting of 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 30 seconds.

2.2.5.8 Plasmid extraction by alkaline-SDS method

Plasmid extraction was performed by the alkaline SDS method. In order to perform plasmid extraction, a toothpick with a colony attached was placed in 2 mL of LB liquid medium containing 100 mg/L ampicillin and cultured with shaking at 37°C for 17 hours. The culture was transferred to a 2 mL tube, and after centrifugation (14,000 rpm, 1 minute, room temperature), the supernatant was discarded. 100 µL of Solution I was added and the precipitate was suspended. 200 µL 0.2 N NaOH + 1% SDS solution was added and placed on ice for 2 minutes. 150 µL of 3 M sodium acetate was added and mixed. Add 450 µL of phenol/chloroform and vortex. After centrifugation (14,000 rpm, 5 minutes, room temperature), the supernatant was collected. Add an equal volume of chloroform isoamyl alcohol and vortex. After centrifugation, the supernatant was removed, 1 mL of 100% EtOH was added, and mixed by inversion. After centrifugation, the supernatant was discarded. 500 µL 70% EtOH was added and lightly tapped. After centrifugation, the supernatant was discarded and dried at 37°C for 8 minutes. 100 µL SDW was added to dissolve. Add 1 µL of 10 $mg \cdot mL^{-1}$ RNase and incubate at 37 °C for 1 hour. Add an equal amount of phenol/chloroform and stir, then centrifuge. The supernatant was taken in a new tube. Add an equal amount of chloroform isoamyl alcohol, stir, and then centrifuge. The supernatant was taken in a new tube. 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100 % EtOH were added and mixed, the mixture was allowed to stand at -80°C for 20 minutes, centrifuge (14,000 rpm, 10 minutes), and the supernatant was discarded. Add 100 µL of 70% EtOH, lightly tap and centrifuge

(14,000 rpm, 10 min). The supernatant was discarded and air-dried at 37°C for 8 minutes. 100 µL of SDW was added and dissolved.

2.2.5.9 Plasmid digestion by restriction enzyme

Restriction enzyme treatment was performed on the plasmid in order to confirm whether or not the extracted plasmid was normally inserted into the vector. Not I was used for *CcOMT1*, and *CcOMT2* and EcoR I was used for *CcOMT4*. The enzymatic reaction times were 3 h at 37° C.

2.2.5.10 Gel electrophoresis

The mixture of each restriction enzyme was electrophoresed on a 1% agarose gel.

2.2.5.11 Big Dye sequencing of isolated plasmid

Big Dye sequence was performed using a plasmid confirmed that the target gene had been introduced by plasmid restriction enzyme treatment. In order to perform the sequence, the extracted plasmid DNA was adjusted to a concentration of 500 $ng \cdot \mu L^{-1}$. To prepare the sequencing product, first add 1.0 µL of plasmid DNA, 1.5 µL of 5 × Sequencing Buffer, 0.5 µL of Big Dye (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1.0 µL of sequence primer and 10.25 I made a reaction solution up to µL. After heating the reaction solution at 96°C for 30 seconds for 1 cycle, 35 cycles were repeated with 1 cycle of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes as one cycle. After the reaction, add 2.5 µL Stop Solution and 30 µL 100 % EtOH and centrifuge (3,500 rpm, 30 min). Carefully remove the supernatant, add 30 µL 70 % EtOH and centrifuge (3,500 rpm, 15 min). The supernatant was carefully removed and dried at 37°C thereafter, it was dissolved in 17 µL of HiDi Formamide, placed in ice for 5 minutes after heat shock at 96°C and heat-denatured. The prepared sequencing products were sequenced with an Applied Biosystems 3130 Genetic Analyzer (Life Technologies Corporation, Carlsbad, Calif., USA). The primer sets used in gene cloning are listed in Table 2-2.

2.2.5.12 Sequence analysis of isolated plasmid

The data obtained by the Big Dye sequence is converted to Gene Studio™ Professional Edition ver 2.2.0.0 (http://www.genestudio.com) and BioEdit program ver. 7.7.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit), the obtained sequences of *CcOMT1*, *CcOMT2*, and *CcOMT4* are identical with the sequences deposited in the *C. clementine* genome database.

2.2.5.13 **Renamed of isolated *OMT genes***

The isolated genes were designated as *CreOMT1* (accession no. LC507211), *CreOMT2* (acc. no. LC507212), and *CreOMT4* (acc. no. LC507213), respectively.

2.2.6 Multiple sequence alignment and construction of a phylogenetic tree

In order to predict the function of identified *OMT* genes in citrus, putative amino acid sequences were analyzed using the ClustalX2 mμLtiple sequence alignment program ver. 2.0.5 (Jeanmougin et al., 1998) and BioEdit program ver. 7.7.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit) with the known genes from other plant species that are related to the *O*-methylation of flavonoids. The tree was constructed using the neighbor-joining (N-J) method for the deduced amino acid sequences of the *OMT* genes from alfalfa [*Medicago sativa* (L10211)], American golden saxifrage [*Chrysosplenium americanum*(U16794)], Arabidopsis [*Arabidopsis thaliana* (U70424)], 242 barley [*Hordeum vμLgare* (CAA54616)], corn [*Zea mays* (DR811764)], Madagascar periwinkle [*Catharanthus roseus* (AY127568)], mango [*Mangifera indica* (KP993176)], peppermint [*Mentha* × *piperita* (AY337457, AY337458, AY337459, AY337460, AY337461)], Shiikuwasha [*C. depressa* (LC126059)], rice [*Oryza sativa* (DQ288259, DQ530257)], vanilla [*Vanilla planifolia* (DQ400399, DQ400400)], wheat [*Triticum aestivum* (DQ223971)] with Clementine [*C. clementina* hort. ex Tanaka (*CcOMT1 to CcOMT16*)] and 'Yoshida' Ponkan (*CreOMT1*, *CreOMT2*, and *CreOMT4*). The phylogenetic tree was displayed using the N-J plot with bootstrap values for 100 trials in each branch. In addition, a multiple sequence alignment was

conducted for *CreOMT1*, *CreOMT2*, and *CreOMT4* with three other OMT proteins from barley (CAA54616), Madagascar periwinkle (AY127568) and peppermint (AY337459).

2.2.7 Expression analysis of citrus OMT genes

2.2.7.1 Total RNA isolation

Total RNA was isolated from the leaves of 9 citrus cultivars (‘Amanatsu’, ‘Aoshima’, ‘Benibae’, ‘Hirakishu’, ‘Kiyomi’, ‘Ogimi kuganii’, ‘Ota’ Ponkan, ‘Seinannohikari’, and ‘Shiranuhi’) using a CTAB-based method (as described previously) using an SV Total RNA Isolation System (Promega) according to the manufacturer's instruction. Isolated total RNA was purified with an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany).

2.2.7.2 Determination of quality and concentration of isolated total RNA

The quality and concentration of RNAs were determined using a BioSpec-mini (Shimadzu). Prior to the cDNA synthesis, the concentration of extracted RNA was adjusted to 200 $ng \cdot \mu L^{-1}$.

2.2.7.3 cDNA synthesis

The first-strand cDNA was synthesized from 1 μg of total RNA in 20 μL of a reaction mixture using a QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instruction.

2.2.7.4 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR for *CreOMT1*, *CreOMT2*, and *CreOMT4* was performed with 1 μL of the first-strand cDNA as a template in a total volume of 20 μL using PrimeTaq DNA Polymerase (GENETBIO Inc., Daejeon, Korea) with gene-specific primer sets (Table 2-2). *CuActin* (*C. unshiu* actin) was used as an internal control.

2.2.7.5 Gel electrophoresis

The RT-PCR products were separated by 2% agarose gel.

2.2.7.6 Expression analysis of citrus CreOMT1 and CreOMT4 by qRT-PCR

Further expression analysis of *CreOMT1* and *CreOMT4* was carried out with qRT-PCR. One microliter of the first-strand cDNA was used as a template in a total volume of 12.5 μL using SYBR® Green Real-Time PCR Master Mix (TOYOBO). The qRT-PCR was performed using a Thermo Scientific™ PikoReal™ Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA) as follows: 95°C for 1 min, 40 cycles of 95°C for 15s, and 60°C for 1 min for *CreOMT1*, *CreOMT4*, and *CuActin*. *CuActin* was used as a reference gene for normalization of the transcript levels of *CreOMT1* and *CreOMT4*, as described in Kotoda et al. (2016). Three technical replicates were performed for each reaction. The primer sets used in the qRT-PCR are listed in Table 2-2.

2.2.7 Statistical analysis

The relationship between PMF content in leaves and flavedos or between PMF content and gene expression was investigated using Pearson's correlation analysis and non-correlation test. All statistical analyses were performed at a significant level of $P < 0.05$ using R-3.2.0 (R Core Team, 2015).

2.3 Results

2.3.1 Accumulation of nobiletin and tangeretin in the leaves and flavedos of citrus

Investigated the PMF, nobiletin, and tangeretin accumulation, in the leaves and flavedos of 11 citrus cultivars were shown in Figure 2-2. Nobiletin and tangeretin were widely distributed among the citrus cultivars and varied in the degree of accumulation between the tissues. Nobiletin and tangeretin were accumulated in flavedos (Fig. 2-2C, D) more than in leaves (Fig. 2-2A, B).

In the leaves, a higher level of nobiletin was found in 'Benibae' (6.19 $mg \cdot g^{-1}$ DW), followed by 'Hirakishu', 'Ogimi kuganii', 'Yoshida' Ponkan, and 'Ota' Ponkan, whereas nobiletin was present at a lower level in 'Seinannohikari' (1.64 $mg \cdot g^{-1}$ DW), followed by 'Shiranuhi',

'Kiyomi', 'Aoshima', 'Mihaya', and 'Amanatsu' (Fig. 2-2A). Similarly, tangeretin was highly accumulated in the leaves of 'Benibae' (5.90 mg·g^{-1} DW), followed by 'Hirakishu', 'Yoshida' Ponkan, 'Ogimi kuganii', and 'Ota' Ponkan, but a low content of tangeretin was observed in 'Seinannohikari' (0.43 mg·g^{-1} DW), followed by 'Kiyomi', 'Shiranuhi', 'Aoshima', 'Amanatsu', and 'Mihaya' (Fig. 2-2B).

In the flavedos, nobiletin was accumulated highly in 'Hirakishu' (14.81 mg·g^{-1} DW), followed by 'Ogimi kuganii', 'Ota' Ponkan, 'Benibae', and 'Yoshida' Ponkan, but a lower amount of nobiletin was accumulated in 'Kiyomi' (4.38 mg·g^{-1} DW), followed by 'Shiranuhi', 'Seinannohikari', 'Aoshima', 'Amanatsu', and 'Mihaya' (Fig. 2-2C). Similarly, tangeretin was accumulated at a higher level in the flavedos of 'Ota' Ponkan (12.01 mg·g^{-1} DW), followed by 'Hirakishu,' 'Yoshida' Ponkan, 'Ogimi kuganii', and 'Benibae', whereas a lower accumulation of tangeretin was observed in 'Kiyomi' (0.74 mg·g^{-1} DW), followed by 'Amanatsu', 'Shiranuhi', 'Seinannohikari', 'Aoshima', and 'Mihaya' (Fig. 2-2D).

The nobiletin and tangeretin contents were highest in the leaves of 'Benibae' (6.19 mg·g^{-1} DW and 5.90 mg·g^{-1} DW, respectively), and the lowest content was found in 'Amanatsu' (0.17 mg·g^{-1} DW) and 'Mihaya' (0.09 mg·g^{-1} DW), respectively. The highest amounts of nobiletin and tangeretin were accumulated in the flavedos of 'Hirakishu' (14.81 mg·g^{-1} DW) and 'Ota' Ponkan (12.01 mg·g^{-1} DW), respectively. The lowest nobiletin and tangeretin content were found in 'Mihaya' (0.17 mg·g^{-1} DW and 0.09 mg·g^{-1} DW, respectively).

2.3.2 Correlation of nobiletin content in the leaves and flavedos of citrus

To evaluate the distribution of nobiletin content in both the leaves and flavedos of 11 citrus cultivars was performed a correlation analysis using the R program (R-3.2.0) (R Core Team, 2015). The correlation analysis revealed that nobiletin content was positively and significantly correlated (r = 0.824, P = 0.00182) between the leaves and flavedos of 11 citrus cultivars (Fig.

2-3). In addition, the nobiletin content was significantly correlated with the tangeretin content in both leaves ($r = 0.978$, $P < 0.000001$) and flavedos ($r = 0.913$, $P < 0.0001$) (Fig. 2-4).

2.3.3 Isolation of OMT genes from 'Yoshida' Ponkan

In total 16 *OMT* related genes in the *C. clementina* genome database (ver. 1.0) by conducting a BLAST search (Table 2-1). To infer the function of *CcOMT1*, *CcOMT2*, and *CcOMT4* genes in citrus, the cDNA was isolated from these corresponding genes from the leaves of 'Yoshida' Ponkan. To evaluate the evolutionary relationships of 16 putative *OMT* genes from the *C. clementina* genome (assigned as *CcOMT*), three isolated genes from 'Yoshida' Ponkan [assigned as *CreOMT*: *CreOMT1* (LC507211), *CreOMT2* (LC507212), and *CreOMT4* (LC507213)] and 18 previously reported *OMT* genes, including *Hv7-OMT* of barley, from other plant species, a phylogenetic tree was constructed based on the deduced amino acid sequences (Fig. 2-5). The phylogenetic tree revealed that CreOMT1, CreOMT2, and CreOMT4 were closely clustered with Mp8-OMT (AY337459) of peppermint, Cat3'5'-OMT (CrOMT2, AY127568) of Madagascar periwinkle, and Hv7-OMT (CAA54616) of barley. Hv7-OMT is involved in the *O*-methylation of the hydroxy group at the 7th-position of the flavonoid (Christensen et al., 1998). Moreover, Mp8-OMT of peppermint and Cat3'5'-OMT of Madagascar periwinkle, which was more closely related to CreOMT1, CreOMT2, and CreOMT4 than Hv7-OMT, are involved in the oxidation and *O*-methylation of the hydroxy group at the 8- and 3', 5'-position of flavonoid, respectively (Cacace et al., 2003; Willits et al., 2004). The identity (similarity) between CreOMT1 and CreOMT2, CreOMT1 and CreOMT4, and CreOMT2 and CreOMT4 was 94.5% (96.4%), 54.1% (70.8%), and 54.4% (71.4%), respectively. CreOMT1 and CreOMT2 were closely related as shown in the phylogenetic tree. In addition, multiple sequence alignment of CreOMT1, CreOMT2, and CreOMT4 with Hv7-OMT, Mp8-OMT, and Cat3'5'-OMT showed five consensus sequences (Fig. 2-6). These motifs (motif I to V) are the residues found in SAM-dependent *O*-methyltransferase.

2.3.4 Expression analysis of CreOMT1, CreOMT2, and CreOMT4

To clarify whether or not *CreOMT1*, *CreOMT2*, and *CreOMT4* are expressed in the leaves of nine citrus cultivars where PMF accumulate, was analyzed the expression of these genes by semi-qRT-PCR (Fig. 2-7). *CreOMT1* was highly expressed in 'Hirakishu', 'Ota' Ponkan, and 'Ogimi kuganii' with a lower expression in 'Benibae' and 'Amanatsu', whereas no expression was found in 'Seinannohikari', 'Shiranuhi', 'Kiyomi', and 'Aoshima'. On the other hand, no expression was detected for *CreOMT2* in any of the investigated cultivars. *CreOMT4* was expressed in 'Benibae', 'Hirakishu', 'Ota' Ponkan, 'Ogimi kuganii', and 'Amanatsu', whereas it was not expressed in 'Seinannohikari', 'Shiranuhi', 'Kiyomi', or 'Aoshima'.

The qRT-PCR was performed to further confirm the transcripts of *CreOMT1* and *CreOMT4* in the leaves and flavedos of 10 citrus cultivars. The transcripts of *CreOMT1* and *CreOMT4* were detectable in both tissues of all citrus cultivars (Fig. 2-8). The transcript of *CreOMT1* was highly accumulated in the leaves of 'Hirakishu', 'Ota' Ponkan, 'Amanatsu', and 'Ogimi kuganii', but lower accumulation was observed in 'Benibae', 'Mihaya', 'Kiyomi', 'Shiranuhi', 'Aoshima', and 'Seinannohikari' (Fig. 2-8A). In contrast, the transcripts of *CreOMT1* were relatively abundant in the flavedos of 'Ogimi kuganii', 'Ota' Ponkan, 'Shiranuhi', and 'Hirakishu', with a little transcripts in 'Benibae', 'Mihaya', 'Aoshima', 'Seinannohikari', 'Amanatsu', and 'Kiyomi' (Fig. 2-8B). The transcript of *CreOMT4* accumulated primarily in the leaves of 'Ota'Ponkan, 'Amanatsu', 'Benibae', and 'Hirakishu' and remained constant at lower levels in 'Mihaya', 'Kiyomi', 'Ogimi kuganii', 'Shiranuhi', 'Seinannohikari', and 'Aoshima' (Fig. 2-8C). In flavedos, *CreOMT4* expressed at higher levels in 'Ota' Ponkan, 'Ogimi kuganii', and 'Shiranuhi', whereas the fewer amount of transcripts were detected in 'Kiyomi', 'Mihaya', and 'Seinannohikari' (Fig. 2-8D).

To investigate the correlation between nobiletin content and *CreOMT* gene expression, correlation analysis was conductated as shown in Figure 2-9. The result showed a significant

correlation of the nobiletin content and *CreOMT1* expression in the flavedos (r = 0.700, P = 0.0243; Fig. 2-9B) with an apparent correlation between the nobiletin content and *CreOMT4* in the flavedos (r = 0.588, P = 0.0740; Fig. 2-9D), the nobiletin content and *CreOMT1* in the leaves (r = 0.420, P = 0.2278; Fig. 2-9A), and the nobiletin content and *CreOMT4* in the leaves (r = 0.306, P = 0.3906; Fig. 2-9C).

2.4 Discussion

In 11 citrus cultivars tested, the nobiletin and tangeretin content were generally higher in flavedos than in leaves, except for nobiletin in 'Seinannohikari' and tangeretin in 'Mihaya' (Fig. 2-2). Similarly, a higher accumulation of PMF in flavedos/peels are observed in various citrus cultivars as compared to the other tissues such as the fruit, juice vesicle, albedo, and segment epidermis (Nogata et al., 2006). Of the 11 cultivars, five cultivars ('Benibae', 'Hirakishu', 'Ogimi kuganii', 'Ota' Ponkan, and 'Yodhida' Ponkan) accumulated a higher level of PMF. Because the parents of 'Benibae' is HF No.9 ('Hayashi' Satsuma mandarin × 'Fukuhara' orange) and 'Encore', the high content of PMF in 'Benibae' may be derived from 'Encore', which accumulated 3.73 $mg \cdot g^{-1}$ DW of nobiletin in leaves (unpublished result). Regarding three mandarins, Kishu ('Hirakishu'), Shiikuwasha ('Ogimi kuganii'), and Ponkan ('Ota' and 'Yoshida'), their genetic background and genealogy are unclear at present. Fundamentally, our results were consistent with those of Nogata et al. (2006) and Yamamoto et al. (2019). The nobiletin content was higher than the tangeretin content in the leaves of the same cultivar (Fig. 2-2A, B). The flavedos also had more nobiletin than tangeretin, except for 'Amanatsu' (Fig. 2-2C, D). These results revealed that nobiletin tended to accumulate more than tangeretin and that there was a significant correlation between the accumulation of nobiletin and tangeretin in leaves (r = 0.978, P < 0.000001; Figure 10A) and flavedos (r = 0.913, P < 0.0001; Fig. 2-4B) in the citrus tested. Nogata et al. (2006) comprehensively investigated the flavonoids, including PMF, in the flavedos of 45 citrus cultivars, and similar

results were obtained in about 76% of 21 cultivars with some nobiletin content (> 0.2 $mg \cdot g^{-1}$ FW). These results may be due to the fact that nobiletin has six methoxy groups and tangeretin has five, and the structural difference is only one methoxy group (Fig. 2-1), suggesting that their biosynthetic pathway is similar. In addition, the nobiletin content in the leaves was found to have a positive correlation ($r = 0.824$, $P = 0.00182$) with the nobiletin content in the flavedos (Fig. 2-3). Similarly, tangeretin content was shown to be positively correlated in the leaves and flavedos (data not shown). The result suggests that a set of genes that synthesize PMF is present in both leaves and flavedos. In the citrus breeding program, this could be utilized for early selection of seedlings in the juvenile phase, which are expected to accumulate a higher amount of PMF in fruit, resulting in shortening the breeding period.

The OMT multigene family have been identified in many plant species, including alfalfa (Maxwell et al., 1993), American golden saxifrage (Gauthier et al., 1995), *Arabidopsis* (Zhang et al., 1997), barley (Christensen et al., 1998; Zhou et al., 2008), corn (Zhou et al., 2008), Madagascar periwinkle (Cacace et al., 2003), mango (Chidley et al., 2016), peppermint (Willits et al., 2004), rice (Kim et al., 2006), vanilla (Li et al., 2006), and wheat (Zhou et al., 2006; Wang et al., 2018). Recently, five *OMT* genes for methylation of flavones were isolated from Shiikuwasha (Itoh et al., 2016). In this study, 16 putative *OMT* genes were identified in the *C. clementina* genome database (ver. 1.0) and isolated three putative *OMT* genes, *CreOMT1*, *CreOMT2*, and *CreOMT4* from 'Yoshida' Ponkan. Phylogenetic analysis showed that they were closely related to *OMT* genes such as *Mp8-OMT* (AY337458), *Cat3'5'-OMT* (AY127568), and *Hv7-OMT* (CAA54616). Barley *Hv7-OMT* is involved in the *O*-methylation of the hydroxy group at the ^{7th}-position of the flavones (Christensen et al., 1998). Similarly, Madagascar periwinkle *Cat3'5'-OMT* (*CrOMT2*) is involved in the two sequential *O*-methylations of the hydroxy group at the 3'- and 5'-position of flavonols and in their biosynthesis (Cacace et al., 2003). In peppermint, *Mp8-OMT* (*MpOMT2*) is found to be involved in the *O*-methylation of

the hydroxy group at the C8-position of flavones (Willits et al., 2004). Because *Hv7-OMT* and *Mp8-OMT* methylate hydroxyflavones at C7 and C8, respectively, these citrus *OMT*-like genes might be involved in the biosynthesis of nobiletin and tangeretin, which are methylated at C7 and C8, respectively (Fig. 2-1). Especially, CreOMT1 and CreOMT4 might catalyze the hydroxy group at the ^{8th}-position of flavones, considering that they were more related to Mp8-OMT than to Cat3'5'-OMT or Hv7-OMT in the phylogenetic tree.

Multiple sequence alignment of CreOMT1, CreOMT2, and CreOMT4 with Hv7-OMT, Cat3'5'-OMT, and Mp8-OMT showed several consensus sequence motifs (motif I to V; Fig. 2-6). Basically, consensus sequence motifs I to IV are characteristic of SAM-dependent *O*-methyltransferase, which transfers a methyl group from SAM onto a hydroxy group and SAM-dependent methylations are important in the biosynthesis of flavonoids (Christensen et al., 1998; Gana et al., 2013; Ibrahim et al., 1998; Vidgren et al., 1994; Willits et al., 2004). Thus, *CreOMT1*, *CreOMT2*, and *CreOMT4* might be expected to be involved in the biosynthesis of PMF in citrus.

The expression of *CreOMT1*, *CreOMT2*, and *CreOMT4* in the leaves of nine citrus cultivars was investigated by semi-qRT-PCR at first. The results demonstrated that *CreOMT1* and *CreOMT4* were expressed in 'Benibae', 'Hirakishu', 'Ota' Ponkan, 'Ogimi kuganii', and'Amanatsu' (Fig. 2-7). This result reflected the accumulation s of nobiletin and tangeretin in the leaves of those cultivars, except for 'Amanatsu'. In contrast, no expression was detected for *CreOMT2* in any of the investigated cultivars, suggesting that *CreOMT2* may not be involved in the biosynthesis of PMF in the leaves of citrus cultivars. Therefore, two *OMT* genes, *CreOMT1* and *CreOMT4* were selected for further expression analysis in both the leaves and flavedos of 10 citrus cultivars. Transcripts of *CreOMT1* and *CreOMT4* were detected by qRT-PCR in both leaves and flavedos. However, there was a tendency that the transcripts of *CreOMT1* and *CreOMT4* in leaves were much higher than those in flavedos in the same

cultivars (Fig. 2-8). These results may suggest differences in the state of the RNA of the evergreen leaves and senescent flavedos (exocarps) in the maturation season. Flavedos almost cease growing in January, and RNA synthesis is not thought to be very active. Because previously it was confirmed a seasonal changes in the accumulation of PMF in the leaves and flavedos of Ponkan and Shiikuwasha (Kotoda et al., 2017; Yamaguchi et al., 2015), the expression of the genes and enzymes responsible for the biosynthesis of PMF might be varied according to developmental stages although enough PMF had accumulated in the tissues.

The transcripts of *CreOMT1* and *CreOMT4* were apparently associated with the accumulation of PMF in leaves and flavedos of the cultivars studied (Fig. 2-2 and 2-8). The expression of *CreOMT1* and *CreOMT4* tended to be more related to the accumulation of nobiletin in flavedos than in leaves (Fig. 2-9). Notably, there was a significant correlation (r = 0.700, P = 0.0243) between the *CreOMT1* expression and the nobiletin content in flavedos (Fig. 2-9). In leaves, however, *CreOMT1* was expressed relatively highly in ‘Amanatsu’, which accumulated a lower level of PMF, to the same degree as in ‘Ogimi kuganii’, which had a higher level of PMF. In contrast, there was not as much *CreOMT1* expression in ‘Benibae’ as expected. In flavedos, on the other hand, *CreOMT1* was expressed relatively highly in ‘Shiranuhi’, which was a low PMF-accumulating cultivar. In ‘Amanatsu’, which accumulated fewer PMF in both leaves and flavedos, *CreOMT1* and *CreOMT4* were expressed relatively highly, except for the lower expression of *CreOMT1* in flavedos. Based on these results, ‘Amanatsu’ might have inherited a part of the gene sets related to the biosynthesis of PMF from a PMF-accumulating cultivar, Kishu-mikan (*C. kinokuni* hort. ex Tanaka), which would be a pollen parent of ‘Amanatsu’ (Shimizu et al., 2016). In ‘Shiranuhi’, on the other hand, the higher expression of those *OMT* genes might be derived from the Ponkan genome because the pollen parent of ‘Shiranuhi’ is ‘Nakano No.3’ Ponkan (Matsumoto, 2001). Further study will be needed to clarify the biochemical function of *CreOMT1* and *CreOMT4*.

2.5 Conclusion

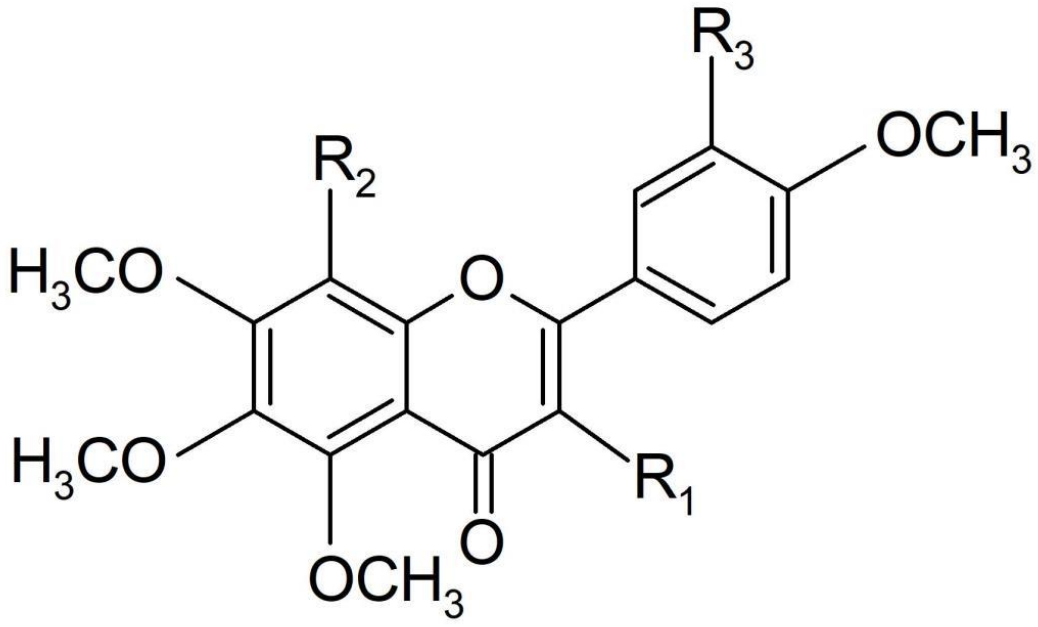
In this study, two PMF, nobiletin and tangeretin accumulation in the leaves and flavedos of the various citrus cultivars grown in Japan were clarified and found a positive correlation in the accumulation of PMF between leaves and flavedos. In addition, two candidate genes responsible for *O*-methylation in the biosynthesis of PMF were identified. Notably, the expression of *CreOMT1* showed a significant correlation with the accumulation of nobiletin in citrus cultivars used in this study. These results will be useful in breeding new cultivars accumulating higher PMF and elucidating the PMF biosynthetic pathway in citrus in the future.

Table 2-1. Identified flavonoid *OMT* related gene from the latest *C. clementina* (v1) in phytozome.

Gene ID	Locus name
CcOMT1	*Ciclev10015705m*
CcOMT2	*Ciclev10015708m*
CcOMT3	*Ciclev10015723m*
CcOMT4	*Ciclev10015630m*
CcOMT5	*Ciclev10015762m*
CcOMT6	*Ciclev10017559m*
CcOMT7	*Ciclev10018099m*
CcOMT8	*Ciclev10015724m*
CcOMT9	*Ciclev10015993m*
CcOMT10	*Ciclev10017872m*
CcOMT11	*Ciclev10017930m*
CcOMT12	*Ciclev10018226m*
CcOMT13	*Ciclev10015685m*
CcOMT14	*Ciclev10017683m*
CcOMT15	*Ciclev10017585m*
CcOMT16	*Ciclev10017649m*

Table 2-2. Primer sequence used in semi-quantitative and quantitative real-time RT-PCR.

Primer	Oligonucleotide sequence (5'→3')
Real-time RT-PCR	
qPCR_7OMT-1_F	TGTTGTACTCAAGTGGATATTG
qPCR_7OMT-1_R	TTCAGTAGACTCATCATTCCC
qPCR_7OMT-2_F	GGTTCTATTCAAGTGGATATTA
qPCR_7OMT-2_R	TTTCAATAGACTCAGAATCCCT
qPCR_7OMT-4_F	TACTCAAGTGGGTTCTGCATA
qPCR_7OMT-4_R	GGTTGAGTCCTTGTCTATGC
Reference gene	
CuActin_F	GAGCGATAGAGAGAATCGACA
CuActin_R	TATCCTCAGCATCGGCCATT



Compound	**R1**	**R2**	**R3**	**Total number of –OCH_3**
Sinensetin	H	H	OCH3	5
Tangeretin	H	OCH3	H	5
Nobiletin	H	OCH3	OCH3	6
Heptamethoxyflavone	OCH3	OCH3	OCH3	7

Fig. 2-1. Chemical structures of polymethoxyflavone found in citrus.

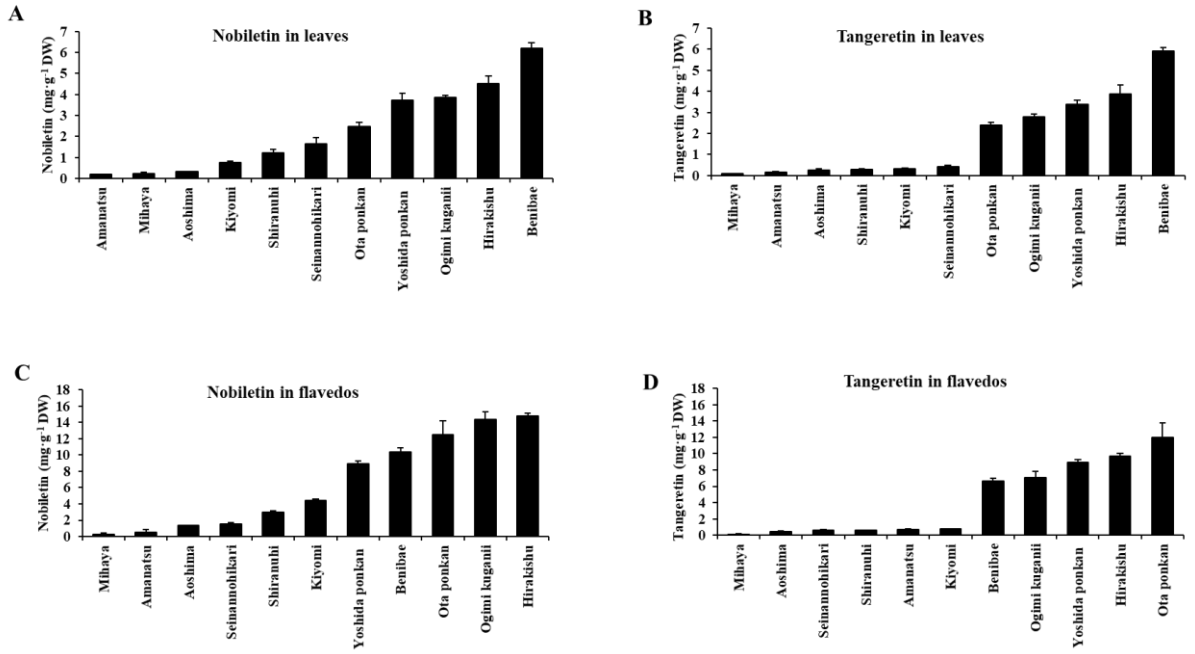


Fig. 2-2. Accumulation of nobiletin and tangeretin in the leaves and flavedos of 11 citrus cultivars, ‘Amanatsu’, ‘Aoshima’, ‘Benibae’, ‘Hirakishu’, ‘Kiyomi’, ‘Mihaya’, ‘Ogimi kuganii’, ‘Ota’ Ponkan, ‘Seinannohikari’, ‘Shiranuhi’, and ‘Yoshida’ Ponkan.

(A) The content of nobiletin in leaves. (B) The content of tangeretin in leaves. (C) The content of nobiletin in flavedos. (D) The content of tangeretin in flavedos. The values are means ± SD of the results from three biological replicates per cultivar.

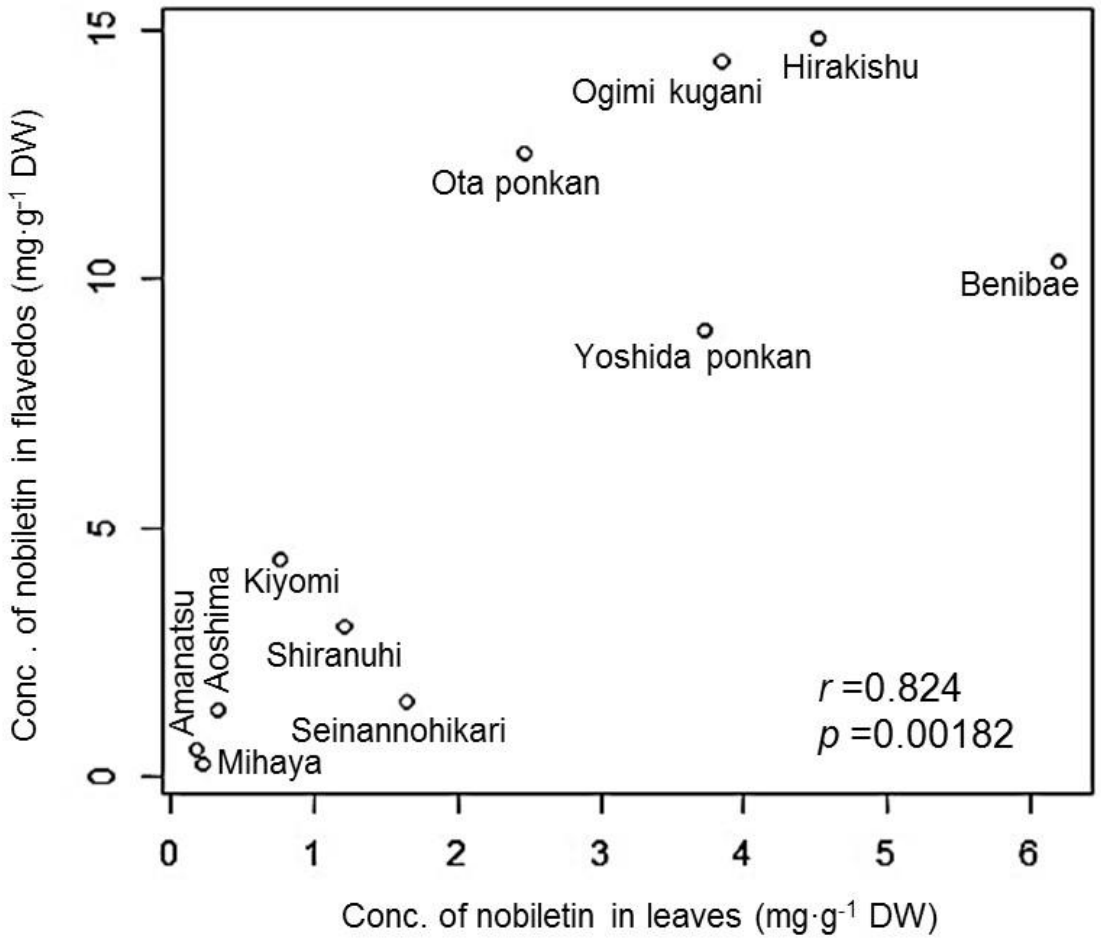


Fig. 2-3. Correlation of nobiletin content between the leaves and flavedos of 11 citrus cultivars, ‘Amanatsu’, ‘Aoshima’, ‘Benibae’, ‘Hirakishu’, ‘Kiyomi’, ‘Mihaya’, ‘Ogimi kuganii’, ‘Ota’ Ponkan, ‘Seinannohikari’, ‘Shiranuhi’, and ‘Yoshida’ Ponkan. Statistical analysis was performed using R-3.2.0 (R Core Team, 2015).

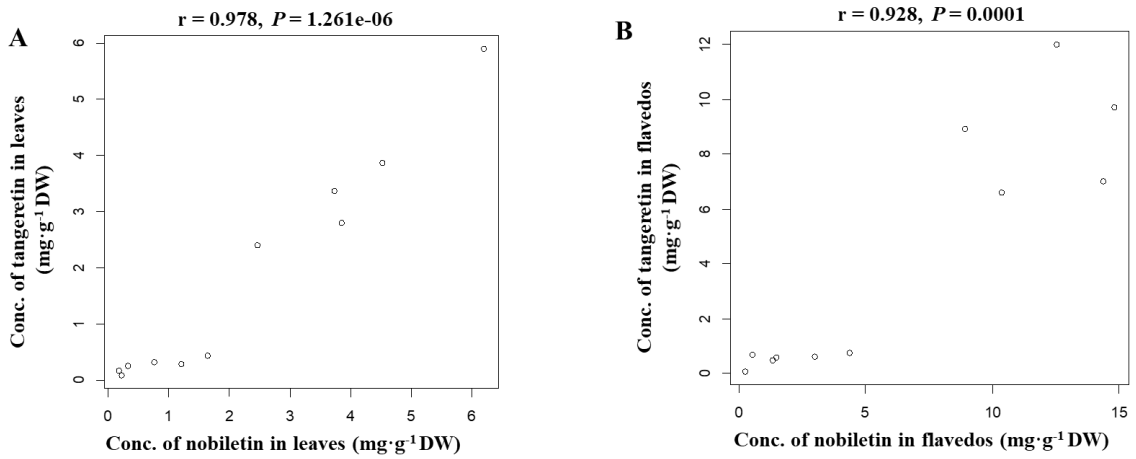


Fig. 2-4. Correlation of the nobiletin and tangeretin content in the leaves.

(A) and flavedos (B) of 11 citrus cultivars, ‘Amanatsu’, ‘Aoshima’, ‘Benibae’, ‘Hirakishu’, ‘Kiyomi’, ‘Mihaya’, ‘Ogimi kuganii’, ‘Ota’ Ponkan, ‘Seinannohikari’, ‘Shiranuhi’, and ‘Yoshida’ Ponkan. Displaying the scatter plot and the statistical analysis was performed using R-3.2.0 (R Core Team, 2015).

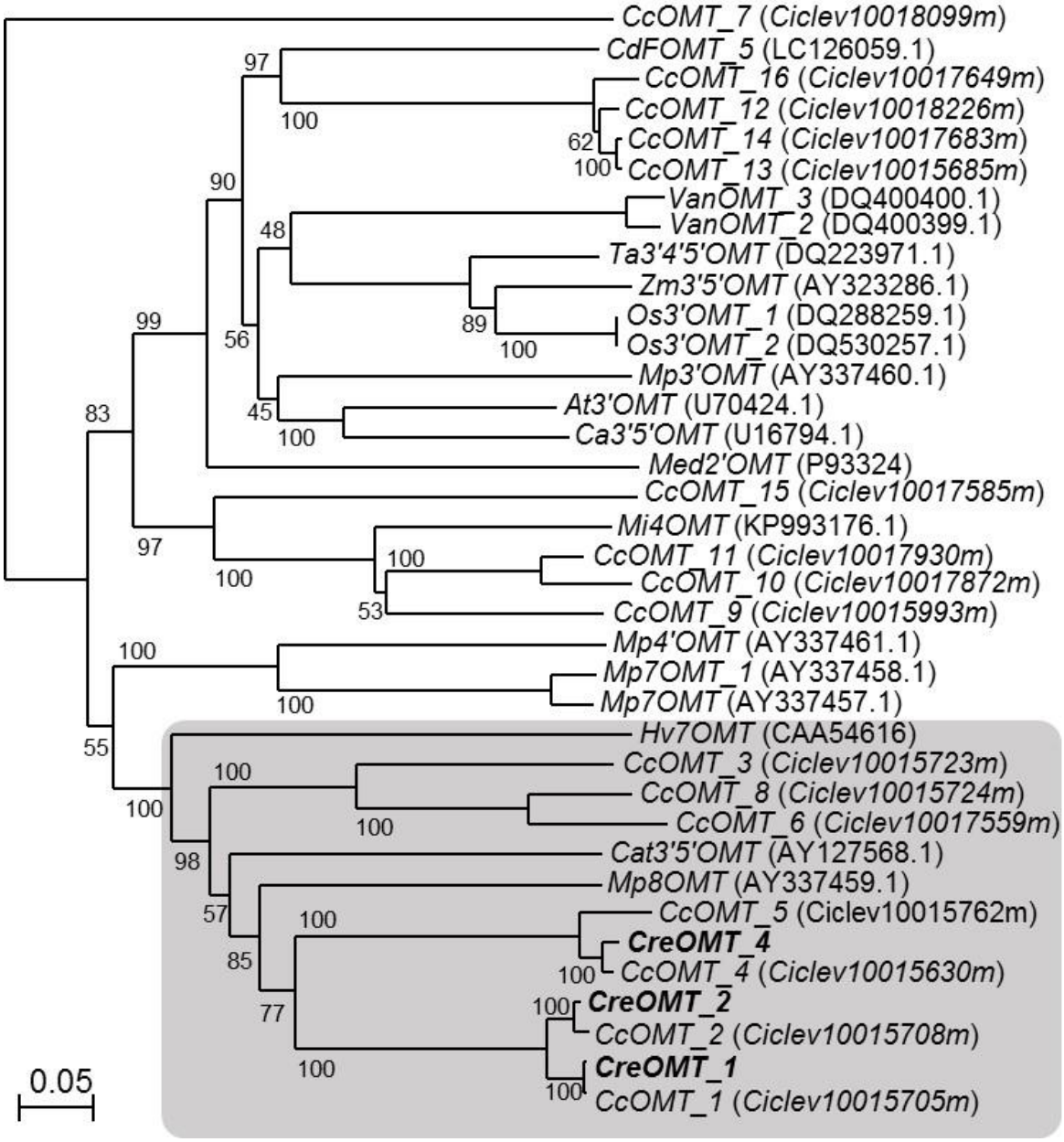


Fig. 2-5. Phylogenetic analysis of *OMT* genes of citrus together with other plant species.

The tree was constructed by the neighbor-joining (N-J) methods and 100 bootstrap trials for the deduced amino acid sequences of the *OMT* genes from alfalfa [*Med2'-OMT* (L10211)], arabidopsis Arabidopsis [*At3'-OMT* (U70424)], barley [*Hv7-OMT* (CAA54616)], corn [*Zm3'5'-OMT* (DR811764)], Madagascar periwinkle [*Cat3'4'-OMT* (AY12756), *Ca3'5'-OMT* (U16794)], mango [*Mi4-OMT* (KP993176)], peppermint [*Mp3'-OMT* (AY337457), *Mp4'-OMT* (AY337461), *Mp7-OMT* (AY337458), *Mp7-OMT1* (AY337460), *Mp8-OMT* (AY337459)], *Os3'-OMT2* (DQ530257)], rice [*Os3'-OMT1* (DQ288259), Shiikuwasha [*CdFOMT* (LC126059)], vanilla [*VanOMT2* (DQ400399), *VanOMT3* (DQ400400)], and wheat [*Ta3'4'5'-OMT* (DQ223971)] together with 16 *CcOMT* candidate genes from Clementin and three *CreOMT* genes [*CreOMT1* (LC507211), *CreOMT2* (LC507212), *CreOMT4* (LC507213)] isolated from 'Yoshida' Ponkan.

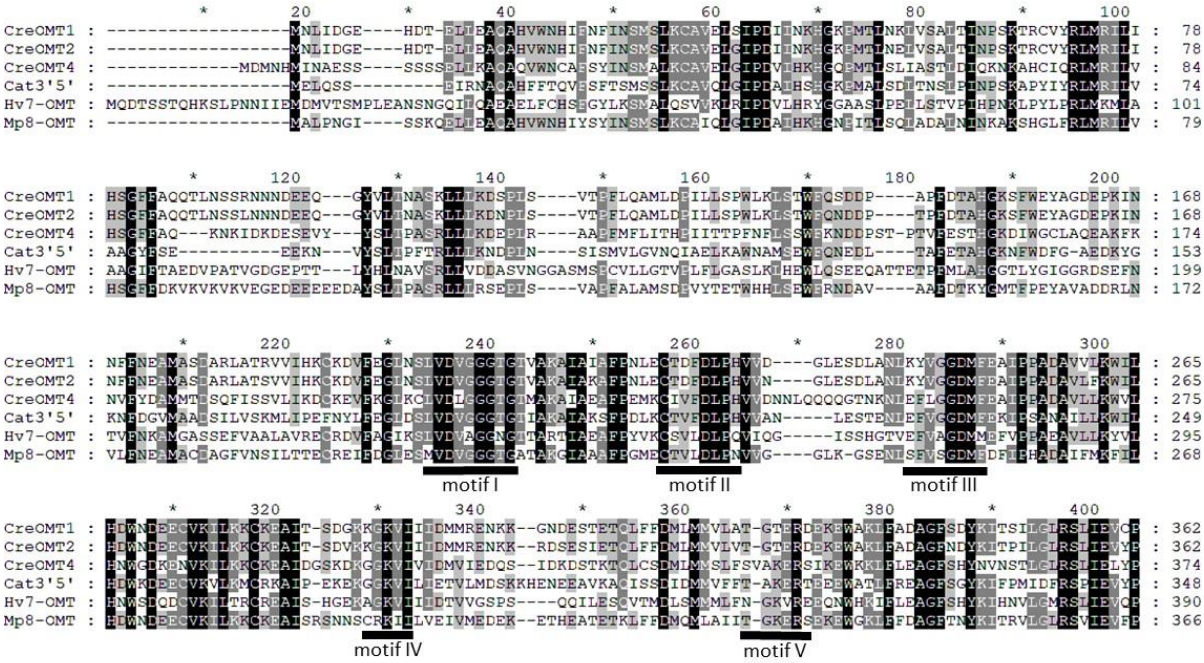


Fig. 2-6. Alignment of OMT proteins derived from 'Yoshida' Ponkan (*CreOMT1*, *CreOMT2*, and *CreOMT4*) and other plant species, Madagascar periwinkle [*Cat3'5'-OMT* (AY127568)], barley [*Hv7-OMT* (CAA54616)], and peppermint [*Mp8-OMT* (AY337458)]. Putative amino acid sequences were aligned using the ClustalX2 multiple sequence alignment program ver.2.0.5 (Jeanmougin et al., 1998).

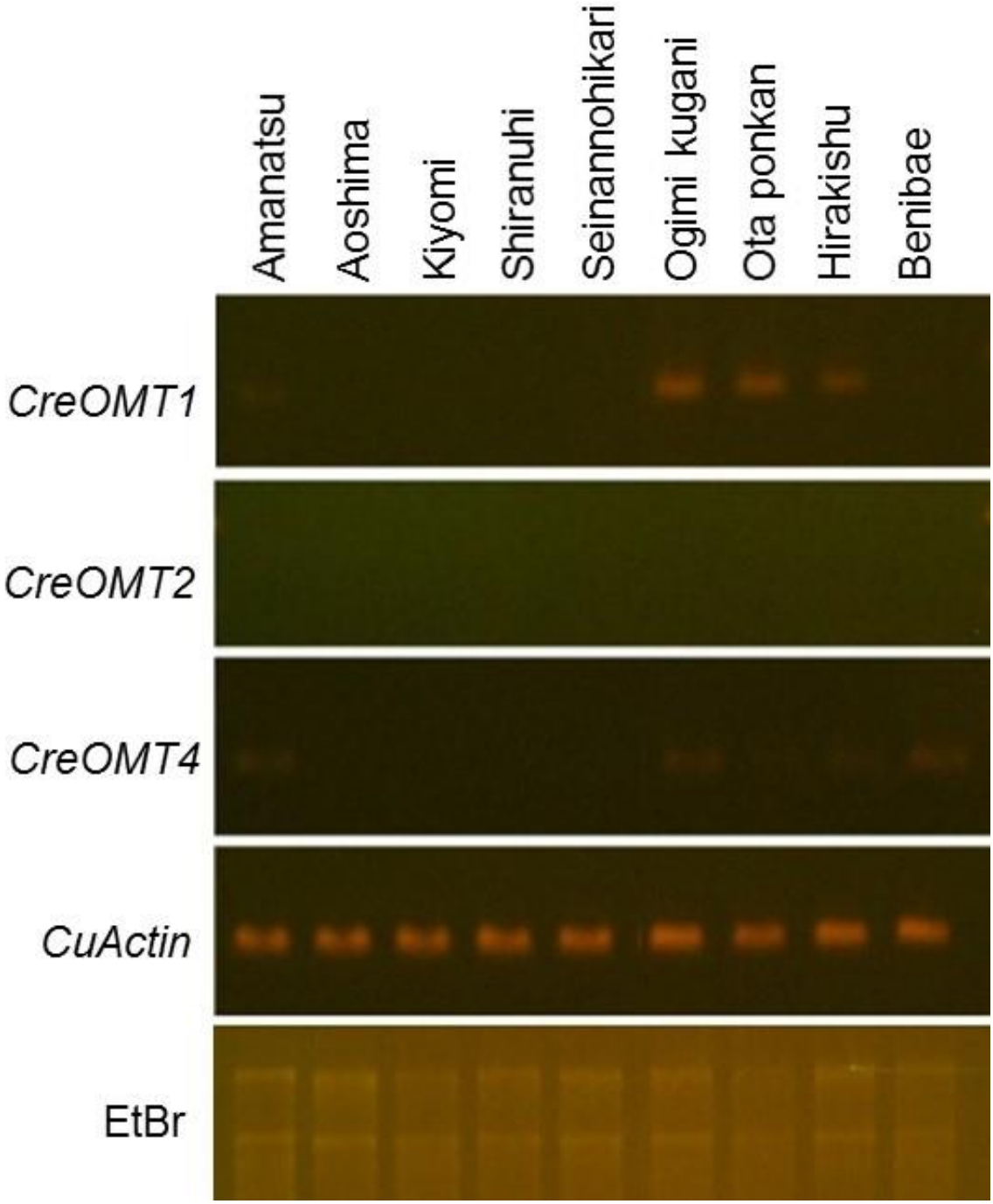


Fig. 2-7. Expression of *CreOMT1*, *CreOMT2* and *CreOMT4* genes in leaves of nine citrus cultivars such as 'Amanatsu', 'Aoshima', 'Kiyomi' 'Shiranuhi', 'Seinannohikari', 'Ogimi kugani', 'Ota' ponkan, 'Hirakishu' and 'Benibae' by semi-quantitative RT-PCR. The citrus actin (*C. unshiu*, CuActin) gene was used as a reference gene. Amplified PCR products were separated by a 2% agarose gel.

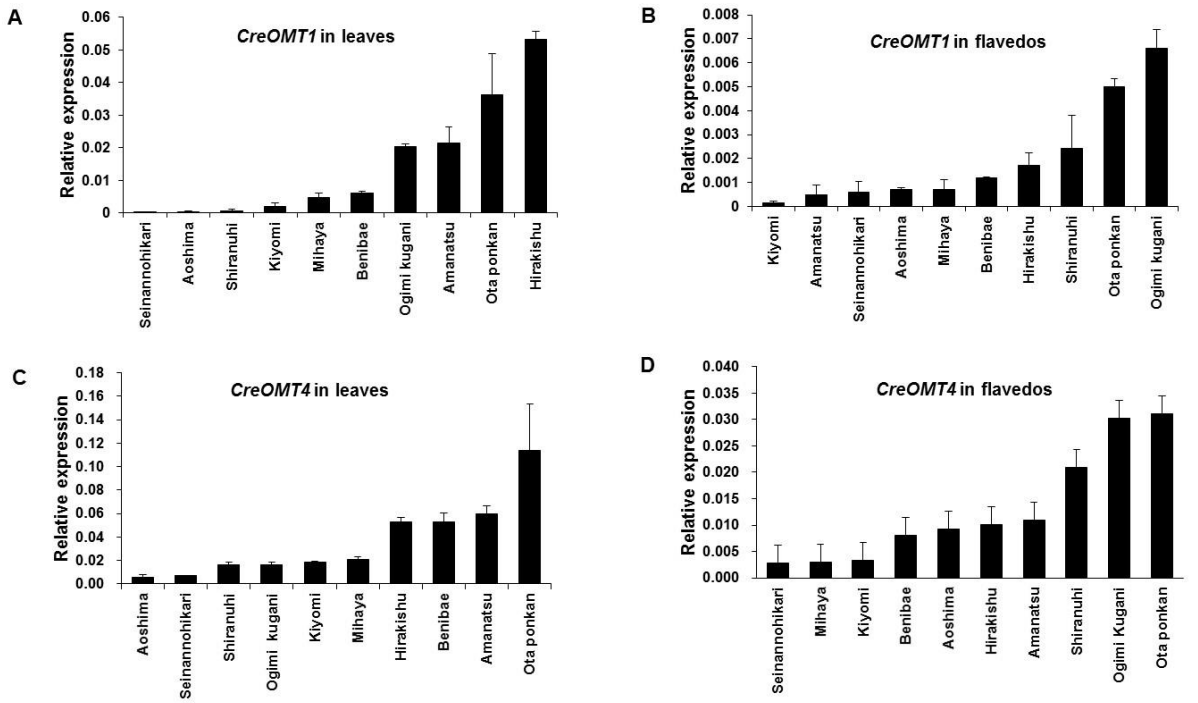


Fig. 2-8. Expression profiles of *CreOMT1* and *CreOMT4* in the leaves and flavedos of 10 citrus cultivars, ‘Amanatsu’, ‘Aoshima’, ‘Benibae’, ‘Hirakishu’, ‘Kiyomi’, ‘Mihaya’, ‘Ogimi kuganii’, ‘Ota’ Ponkan, ‘Seinannohikari’, and ‘Shiranuhi’.

(A) The expression of *CreOMT1* in leaves. (B) The expression of *CreOMT1* in flavedos. (C) The expression of *CreOMT4* in leaves. (D) The expression of *CreOMT4* in flavedos. The citrus actin gene (*CuActin*) was used as a reference gene. The values are means ± SD of the results from three technical replicates per cultivar.

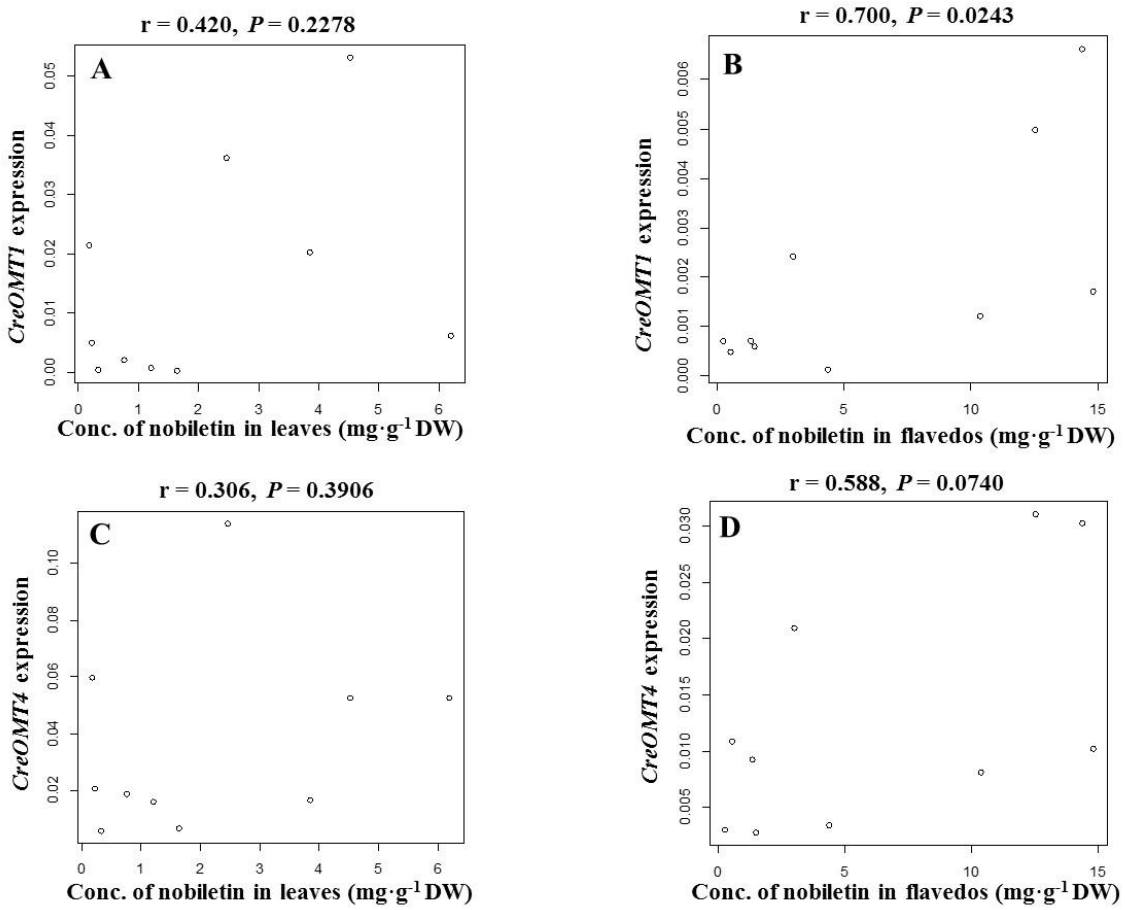


Fig. 2-9. Correlation of the nobiletin content and the *CreOMT* gene expression.

The citrus cultivars used in this analysis were the same as those used in the gene expression experiment. Scatter plot of (A) nobiletin content and *CreOMT1* expression in leaves, (B) nobiletin content and *CreOMT1* expression in flavedos, (C) nobiletin content and *CreOMT4* expression in leaves, (D) nobiletin content and *CreOMT4* expression in flavedos. Displaying a scatter plot and statistical analysis was performed using R-3.2.0 (R Core Team, 2015).

Chapter 3: Relationship between the limonoid content in different parts of the sour orange (*Citrus aurantium* L.) and the ligand activity of a bile acid receptor, TGR5

3.1 Introduction

Limonoids represent a group of highly oxygenated triterpenoids and are mostly found in the Rutaceae and Meliaceae families (Hasegawa et al., 1999). Citrus plant tissues and organs such as the stem, leaf, seed, and fruit (peel and juice sac) accumulate limonoids at a significant level as aglycones or glucosides (Gualdani et al., 2016; Herman et al., 1989; Li et al., 2014; Rouseff and Nagy, 1982). The major neutral limonoids of citrus are limonin, nomilin, obacunone, and deacetylnomilin. Thirty-seven limonoid aglycones and 17 limonoid glucosides have been identified in the *Citrus* species and its hybrids (Jayaprakasha et al., 2008; Patil et al., 2009). Limonoid aglycones are found predominantly in citrus seeds and peels, while limonoid glucosides are the major components in the juice sac (Hasegawa et al., 1980, 1989; Russo et al., 2016; Vikram et al., 2007). Limonin and nomilin are the two major limonoids in citrus and responsible for the bitter taste in citrus fruits (Dea et al., 2013; Endo et al., 2002; Hasegawa et al., 1973; Higby, 1938). Many studies have demonstrated the significant biological and physiological impacts of limonoids on human health, such as anti-cancer (Jacob et al., 2000; Lam et al., 1994; Miller et al., 2004) and anti-microbial (Chowdhury et al., 2003; Govindachari et al., 2000) activities. TGR5 (Takeda G protein-coupled receptor 5), also known as a novel G protein-coupled bile acid receptor 1 (GPBAR1), is a vital member of the membrane-bound G protein-coupled receptor (GPCR) family (Kawamata et al., 2003; Maruyama et al., 2002). TGR5 is expressed in various tissues and organs throughout the body, such as the heart, liver, lung, spleen, kidney, placenta stomach, gallbladder, intestine, brown adipose tissue, and endocrine glands and is recognized and activated by the binding of bile acids (BAs) as its endogenous ligands (Duboc et al., 2014; Kawamata et al., 2003; Maruyama et al., 2002; Pols et al., 2011). In recent years, TGR5 has attracted the attention of researchers as an important target for the potential treatment of several metabolic disorders (Tiwari and Maiti, 2009; van Nierop et al., 2017). A number of novel TGR5 agonists have been found in many plant species

including olive and citrus and their biological activities have been investigated in detail (Genet et al., 2010; Horiba et al., 2015; Ono et al., 2011; Sato et al., 2007). Recent studies have indicated that a nomilin binds to the bile acid receptor TGR5 and exhibits TGR5 activity as an agonist (Ono et al., 2011; Sasaki et al., 2017). In addition, TGR5 was found to be involved in glucose uptake into cells and in the remodeling of energy-storing white fat into energy expending beige fat, suggesting its potential anti-obesity and anti-hyperglycemic effects (Lo et al., 2016; Velazquez-Villegas et al., 2018). The seed extracts of seven citrus plants were preliminarily screened for TGR5 ligand activity and found the sour orange to have the highest activity. Several previous studies revealed the content of limonoids in sour orange fruit and seed extracts (Bennett et al., 1991; Dandekar et al., 2008; Matsumoto et al., 2008; Miyake et al., 1992; Ozaki et al., 1991; Vikram et al., 2007). However, no studies have considered the relationship between limonoid content and TGR5 ligand activity in citrus extracts. The aim of this study was to clarify the relationship between the content of four major limonoids and TGR5 ligand activity in the sour orange fruit by using high-performance liquid chromatography (HPLC) and a luciferase assay system.

3.2 Materials and Methods

3.2.1 Plant materials

The sour orange (*Citrus aurantium* L.) fruit, which was introduced by Italy, used in this study was collected in February 2017 and April 2019 from the field of the Faculty of Agriculture at Saga University (Fig. 3-1). The fruit was separated into seed, juice sac, and peel. Alternatively, the single seed was divided into the seed coat and cotyledon. The sour orange seeds without seed coats were germinated on a glass petri dish containing moistened blotting paper and grown under dark conditions at 25°C in a growth chamber. Ten-day-old germinated seeds were transplanted from the petri dish to moistened potting soil composed of vermiculite and grown

in a greenhouse under natural light conditions. In this study, the peel, juice sac, seed, seed coat, cotyledon, germinated seed, and 2- and 4-week-old seedlings were used as plant materials.

3.2.2 Preparation of plant extracts

The freeze-dried tissue samples were ground to a fine powder with a multi-bead shocker (Yasui Kikai Co., Osaka, Japan). The powder was subjected to solvent extraction prior to HPLC analysis. Two grams of freeze-dried powder of the seed, peel, and juice sac (sampled in February 2017) was placed in a Soxhlet extractor (TGK Co., Ltd., Tokyo, Japan) and extracted with 100 mL hexane at 60–70°C for 2 h. Then it was further extracted with 100 mL of ethyl acetate at the same temperature as above for 4 h. The upper phase was transferred to a flask, and 25 mL of methanol was added to the residues. After ultrasonication for 10 min, the extracts were kept at room temperature for 1 h, followed by filtration. The methanol extraction procedure was repeated twice. The solvent was completely evaporated with a rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 100 rpm, 40°C and diluted with appropriate solvents to be used for HPLC analysis. For the intact seed, cotyledon, seed coat, germinated seed (10 days under dark conditions at 25°C after sowing), and 2- and 4-week-old seedlings (derived from the seeds sampled in April 2019), 1 g of freeze-dried powder was extracted with 10 mL of 70% acetone three times. A total of 30 mL of the extract was evaporated with a rotary evaporator (Tokyo Rikakikai Co., Ltd.) at 40°C under low pressure to remove the acetone. The residual water was transferred into a tube. After 10 mL of ethyl acetate was added, the tube was vortexed and centrifuged at 500 rpm for 10 min, and then the ethyl acetate layer was removed to a new tube (this step was repeated thrice). Sodium sulfate was added to the ethyl acetate layer, followed by filtration. Then the ethyl acetate was evaporated to dryness with a rotary evaporator (Tokyo Rikakikai Co., Ltd.). Each concentrated extract derived from 1 g of freeze-dried powder was dissolved in 1 mL of dimethyl sulfoxide (DMSO) as a stock solution for an HPLC analysis and a luciferase assay.

3.2.3 Quantitative analysis of limonoids using HPLC

Quantitative analysis of limonoids was performed using an HPLC system (JASCO Corp., Tokyo, Japan) with a UV detector (UV-1570; JASCO Corp.) and an Inertsil ODS-3 column (3 μm, 4.6 × 100 mm; GL Science, Tokyo, Japan) at 40°C The mobile phases were 10% acetonitrile containing 3 mM phosphoric acid (solvent A) and 50% acetonitrile containing 3 mM phosphoric acid (solvent B). The elution of a binary solvent was conducted in a gradient fashion, starting at 100/0 (A/B), changing to 15/85 (A/B) for 50 min, and maintaining at 0/100 (A/B) for 5 min, with a flow rate of 1.2 $mL \cdot min^{-1}$. Five microliters of each sample were injected, and absorbance of 210 nm was measured using the UV detector. Quantitative determinations were carried out based on the 25, 50, and 100 ppm standards of limonin, nomilin, obacunone, and limonin glucoside using a chromatography data system (ChromNAV 1.0; JASCO Corp.).

3.2.4 Cell culture

CHO (Chinese hamster ovary) cells were cultured in MEMα (alpha-modified minimum essential medium) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS, Gibco®; Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured in an incubator (MCO-5AC; SANYO Electric Co., Ltd., Osaka, Japan) set at 37°C in a humidified atmosphere of 5% CO2. Cells were sub-cultured every 3 days.

3.2.5 Assay for TGR5 ligand activity

To measure TGR5 ligand activity, a luciferase assay system was used in this study. p3×FLAG-hTGR5 was constructed by cloning a polymerase chain reaction (PCR) fragment of human TGR5 cDNA derived from IRAK064N14 (RIKEN Gene Bank, Tsukuba, Japan) into a p3×FLAG-CMV vector (Sigma Aldrich). First, TGR5 genes (p3×FLAG-hTGR5), CRE reporter genes [pGL4.29 (luc2P/CRE/Hygro); Promega Corp., Madison, WI, USA], and polyethylenimine max (Polysciences Inc., Warrington, PA, USA) were placed in a 96-well

plate. Second, CHO cells were plated at a density of 6 × 104 cells/well in 96-well plate for transfection. After a 4 h incubation of the CHO cells, the medium was replaced with MEMα containing 10% charcoal-stripped FBS. Samples [stock solutions for each extract, positive controls, and dimethyl sulfoxide (DMSO)] diluted with the medium (1:250) were added at a volume of 100 μL/well 24 h after transfection. Five hours after the samples were added, a solution of Bright-GloTM (Promega Corp.) was added to each well of the plate. Chenodeoxycholic acid (CDC, one of the bile acids), limonin, and nomilin (Sigma Aldrich) were used as positive controls at a final concentration of 50 μM. TGR5 ligand activity was measured by detecting luciferase activity through luminescence with a luminometer (Luminoskan Ascent FL; Thermo Fisher Scientific Inc.). In the assay of the extracts, the stock solution (each sample derived from 1 g of starting materials was dissolved in 1 mL of DMSO) was finally diluted 250 times in the medium as a 1/250 diluted sample (a sample derived from 4 mg of starting materials per mL). The sample solution was further diluted in the medium and used for the dose-dependent assay as 1/1250, 1/2500, and 1/5000 diluted samples.

3.2.6 Statistical analysis

Data were analyzed using a one-way ANOVA and the differences were contrasted using Tukey's multiple comparison test. The relationship between the limonoid content and TGR5 ligand activity was investigated using Pearson's correlation analysis. All statistical analyses were performed at a significant level of $P < 0.05$ using R-3.2.0 (R Core Team, 2015).

3.3 Results

3.3.1 Limonoid content in the sour orange

HPLC was used to determine the limonoid content in the sour orange seed, peel, and juice sac extracted by three solvents, hexane, ethyl acetate, and methanol. As shown in Figure 3-2, four limonoids (limonin, nomilin, obacunone, and limonin glucoside) were found in the seed and peel, while only two limonoids (nomilin and limonin glucoside) were found in the juice

sac. The total concentration of four limonoids was the highest in the ethyl acetate extract of the seed (1201 $\mu g \cdot g^{-1}$ DW), followed by the methanol extract of the seed (627 $\mu g \cdot g^{-1}$ DW) and the ethyl acetate extract of the peel (229 $\mu g \cdot g^{-1}$ DW) (Table 3-1, Fig. 3-2). In seeds, the total limonin, nomilin, obacunone, and limonin glucoside content was 949, 872, 40, and 161 $\mu g \cdot g^{-1}$ DW, respectively (Table 3-1, Fig. 3-2).

3.3.2 *Correlation of limonoid content and TGR5 ligand activity*

TGR5 ligand activity in the extract from the seed, peel, and juice sac was measured using a luciferase assay system. As a result, the ethyl acetate extract of the seed showed a level of TGR5 ligand activity (the relative value of luminescence = 3.3) similar to that (the relative value of luminescence = 3.1) of 50 μM CDC and nomilin (Fig. 3-3A). However, the final concentration of the total limonoids (limonin, nomilin, and obacunone) in the extract was 9.9 μM (5.6, 3.9, and 0.4 μM, respectively), which was much lower than 50 μM nomilin control (Table 3-1, Fig. 3-3A). The hexane and methanol extract of the seed and the hexane extract of the peel also showed significantly higher activity than the blank (DMSO). In addition, the effect of dose-dependence was observed in the assay in which the concentration of the seed extract was changed from 1/250 to 1/5000 of the first stock solution of the samples (Fig. 3-3B). A positive correlation between TGR5 ligand activity and limonoid content was demonstrated because the order of the three extracts in the TGR5 ligand activity was almost the same as that of the limonoid content detected by HPLC (Fig. 3-4). Scatter plots between the limonoid content (limonin, nomilin, obacunone, limonin glucoside, and total limonoids) and luciferase activity measured for each extract showed a positive correlation with a high correlation coefficient of 0.867 (Fig. 3-4B), 0.808 (Fig. 3-4E), and 0.787 (Fig. 3-4A) for nomilin, total limonoids, and limonin, respectively.

3.3.3 Limonoid content and TGR5 ligand activity in cotyledons and seed coats

Seeds were investigated in more detail by separating them into the cotyledon (seed without the seed coat) and the seed coat. The intact seed and cotyledon of the sour orange contained two major limonoids, limonin and nomilin, whereas the seed coat contained those limonoids less than the intact seed and cotyledon (Fig. 3-5A). The limonin and nomilin contents varied depending on the tissues and showed a significant difference between them. The highest level of limonin was determined in the cotyledon (1090 $\mu g \cdot g^{-1}$ DW), followed by the intact seed (720 $\mu g \cdot g^{-1}$ DW) and seed coat (300 $\mu g \cdot g^{-1}$ DW). The nomilin concentration was significantly higher in the cotyledon (770 $\mu g \cdot g^{-1}$ DW) than in the intact seed (270 $\mu g \cdot g^{-1}$ DW) and seed coat (very small amount). The TGR5 ligand activity of the intact seed–, cotyledon–, and seed coat–derived extract was measured using a luciferase assay system (Fig. 3-5B). The extracts obtained from the intact seed, cotyledon and seed coat showed significantly higher luciferase activity than the blank (DMSO). The cotyledon extract showed the maximum luciferase activity, as compared to the intact seed and the seed coat, whereas the intact seed and seed coat extract showed a similar luciferase activity (Fig. 3-5B).

3.3.4 Limonoid content and TGR5 ligand activity in the early developmental stages of seedlings

Limonoid content was also determined by HPLC in the germinated seed (10 days under dark conditions at 25°C after sowing) and 2- and 4-week-old sour orange seedlings (Fig. 3-6A). Limonin and nomilin were present in significant amounts in all of the tissues. Limonin content was found to be highest (1460 $\mu g \cdot g^{-1}$ DW) in the germinated seed, followed by 2-week-old (710 $\mu g \cdot g^{-1}$ DW) and 4-week-old (620 $\mu g \cdot g^{-1}$ DW) seedlings. The nomilin concentrations were similar in the germinated seed (450 $\mu g \cdot g^{-1}$ DW), 2-week-old (530 $\mu g \cdot g^{-1}$ DW), and 4-week-old (620 $\mu g \cdot g^{-1}$ DW) seedlings. TGR5 ligand activity was also measured for the extract of the germinated seed, 2- and 4-week-old seedlings using a luciferase assay system. All extracts exhibited significant activity for TGR5 (Fig. 3-6B). As expected, the germinated seed extract

exhibited higher ligand activity, followed by the extract derived from the 2- and 4-week-old seedlings.

3.4 Discussion

3.4.1 Limonoid content in the sour orange

Distribution of major limonoids (limonin, nomilin, obacunone, and limonin glucoside) was confirmed in the sour orange fruit by extracting those compounds with three different solvents. As a whole, those four limonoids accumulated mainly in the seeds (2022 $\mu g \cdot g^{-1}$ DW), followed in order by peels (443 $\mu g \cdot g^{-1}$ DW) and juice sacs (83 $\mu g \cdot g^{-1}$ DW) (Fig. 3-2). These results were consistent with those of previous reports showing that the distribution of limonoids in citrus fruit is tissue-specific, as the concentration was different in each tissue and that the seed is the source of abundant limonoids in fruit (Hasegawa et al., 1980; Miyake et al., 1992; Ohta and Hasegawa, 1995; Rouseff and Nagy, 1982; Sun et al., 2005; Wang et al., 2016). In the sour orange seeds used in this study, the concentration of limonin, nomilin, obacunone, and limonin glucoside was 949, 872, 40, and 161 $\mu g \cdot g^{-1}$ DW, respectively (Table 3-1, Fig. 3-2). Rouseff and Nagy (1982) also reported the concentration of limonin, nomilin, and obacunone as 1256, 242, and 104 $\mu g \cdot g^{-1}$ DW, respectively, in the common sour orange. Likewise, Miyake et al. (1992) reported a concentration of obacunone (32 $\mu g \cdot g^{-1}$ DW) in sour orange seeds that was similar to in this study, although those of limonin, nomilin, and limonin glucoside (2470, 178, and 610 $\mu g \cdot g^{-1}$ DW, respectively) were different from those in this study. On the other hand, Vikram et al. (2007) detected no nomilin in the sour orange seed extract. Considering the report that the limonoid content depends on the maturation stage or cultivar (Matsumoto et al., 2008; Sun et al., 2005; Wang et al., 2016), inconsistencies in the reported limonoid concentration seem to be due to differences in the season of sampling, cultivar/strain of the sour orange, or method of sample preparation.

3.4.2 Correlation of limonoid content and TGR5 ligand activity

The ligand activity of TGR5 was significantly higher in the extract from seeds, which contained a higher concentration of limonoids, than in those from other organs, except for the hexane extract from the peels (Fig. 3-3A). All of the samples that showed a value significantly higher than blank (DMSO) contained nomilin to some extent (Fig. 3-2 and 3-3A). The effect of dose-dependence on ligand activity indicated that the extract surely contained compounds that highly activated TGR5 (Fig. 3-3B). It is noteworthy that the correlation coefficient between TGR5 ligand activity and the nomilin content was the highest among the four limonoids analyzed in this study (Fig. 3-4B). On the other hand, there was no correlation (r = 0.006) between the TGR5 ligand activity and limonin glucoside content, suggesting that limonin glucoside did not contribute to the TGR5 receptor activation (Fig. 3-4D). These results revealed that nomilin in the sour orange extracts effectively activated the TGR5 receptor. These results are consistent with the report of Ono et al. (2011), wherein the TGR5 ligand activity of nomilin was higher than that of limonin or obacunone. However, the ethyl acetate extract of the seed showed activity equivalent to that of a positive control of 50 μM nomilin in spite of the fact that the final limonoid concentration (limonin: 5.6 μM; nomilin: 3.9 μM; obacunone: 0.4 μM) derived from the ethyl acetate extract in the assay was much lower than 50 μM (Fig. 3-2 and 18). This result suggests that there might be some compounds with higher TGR5 ligand activity than nomilin, which was expected to be a major compound for activating TGR5 in the extracts.

3.4.3 Limonoid content and TGR5 ligand activity in germinating seeds and seedlings

Because higher TGR5 ligand activity was found in the seed extract, here investigated both the limonoid content and the TGR5 ligand activity in more detail in the seeds and seedlings. It was found that limonin and nomilin accumulated more in the cotyledons than in the seed coats (Fig. 3-5A). As expected, the TGR5 ligand activity of the cotyledons was higher than that of the seed coats. However, the TGR5 ligand activity of the seed coats showed a level similar to that of the intact seeds, suggesting that some compounds contained in the seed coats activate

the TGR5 receptor in addition to nomilin because only a trace amount of nomilin was detected in the seed coats (Fig. 3-5). Germinated seeds (10 days under dark conditions after sowing) had a higher limonin content (1460 $\mu g \cdot g^{-1}$ DW) than that (1097 $\mu g \cdot g^{-1}$ DW) in cotyledons (seeds without seed coats) but a lower nomilin content (459 $\mu g \cdot g^{-1}$ DW) than that (771 $\mu g \cdot g^{-1}$ DW) in cotyledons (Fig. 3-6A). The total limonoid content (limonin + nomilin) before (1868 $\mu g \cdot g^{-1}$DW in cotyledon) and after (1919 $\mu g \cdot g^{-1}$DW in the germinated seed) germination was not greatly changed. This could be due to the metabolism of nomilin to limonin through ichangin on the sour orange biosynthetic pathway (Miyake et al., 1992). In constrast, Ariza et al. (2015) reported that a higher amount of limonoids, such as limonin, nomilin, and ichangin, were present in the germinating seeds than in the dormant seeds of *Citrus aurantium.* After germination (2 and 4 weeks after transplanting to pots in the greenhouse), the limonin content decreased gradually, whereas the nomilin content did not vary significantly from germination to 4 weeks after transplanting (Fig. 3-6A). As expected, the TGR5 ligand activity was higher in the germination stage, followed by that in 2- and 4-week-old seedlings (Fig. 3-6B). The differences in limonin content would reflect those in TGR5 activity because the nomilin content was almost the same among the three samples. Bennett and Hasegawa (1980) reported that sour orange seeds contained limonoids other than the four examined in this study and that they contained a higher amount of ichangin and isolimonic acid. Recently, Sasaki et al. (2017) constructed an hTGR5–nomilin binding model and demonstrated that four hydrophilic hydrogen-bonding interactions occurred between the oxygen atoms of nomilin and the amino acid residues of hTGR5. Based on their results and in this study, novel agonists with higher activity for TGR5 could be found in citrus fruits, such as the sour orange, by considering compounds with a structure similar in part to that of nomilin.

3.5 Conclusion

This study revealed the content of four kinds of limonoids and theTGR5 ligand activity in extracts obtained via the three different solvents, from the seed, peel, and juice sac of the sour orange. In addition, this study confirmed a high correlation between the nomilin content and TGR5 ligand activity, although the activity could not be explained by the nomilin content in the seed extract alone. Thus, the sour orange seeds might contain other components with a TGR5 ligand activity higher than that of nomilin. The seed extracts of citrus fruits, such as the sour orange, might be a potential source of compounds that prevent obesity and metabolic disorders. To the best of our knowledge, this is the first study to report on the TGR5 activity in crude extracts from citrus tissues. In a future study, it will be necessary to comprehensively investigate the citrus seed extract for unidentified agonists for the TGR5 receptor.

Table 3-1. Distribution of four limonoids in the sour orange fruit (μg·g^{-1} DW).

tissue	extraction solvent	limonin	nomilin	obacunone	limonin glucoside	total by solvent
seed	hexane	78	115	–	–	193
	ethyl acetate	660	501	40	–	1201
	methanol	210	256	–	161	627
	total by compound	949	872	40	161	2021
peel	hexane	86	62	–	–	148
	ethyl acetate	86	0	13	131	229
	methanol	53	0	12	–	65
	total by compound	225	62	25	131	442
juice sac	hexane	–	48	–	–	48
	ethyl acetate	–	–	–	35	35
	methanol	–	–	–	–	–
	total by compound	–	48	–	35	83

Fig. 3-1. The sour orange fruit used in this study. Scale bar, 3 cm.

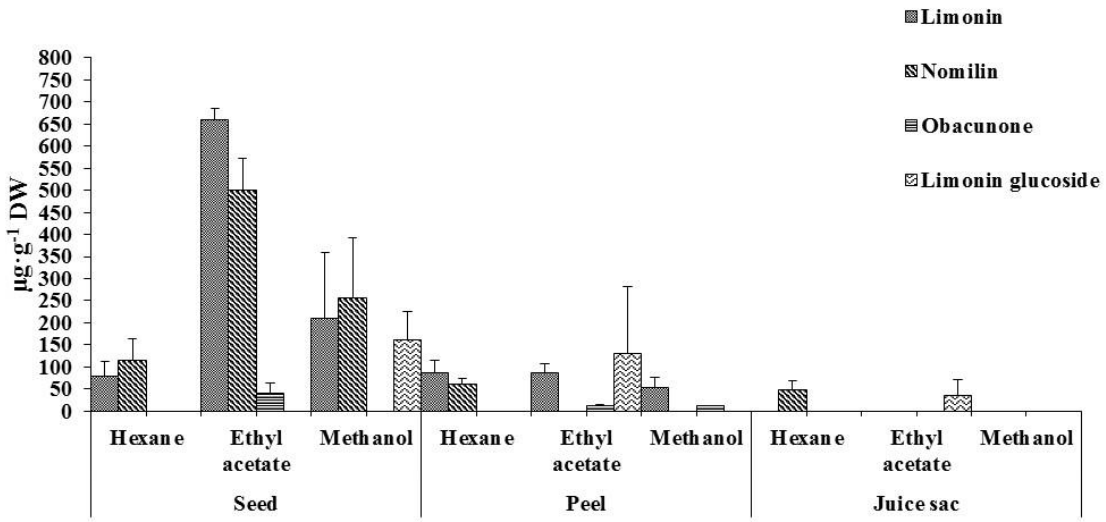


Fig. 3-2. The concentration of four limonoids (limonin, nomilin, obacunone, and limonin glucoside) extracted from the seed, peel, and juice sac of the sour orange using three different solvents (hexane, ethyl acetate, and methanol). All of the tissues were freeze-dried before extraction. Each extract derived from 1 g of the freeze-dried sample was dissolved in 1 mL of DMSO, and then a 1/250 dilution of the first prepared extract was used for the assay. The values are the mean of results from three biological replicates. The error bars indicate the standard deviation (SD).

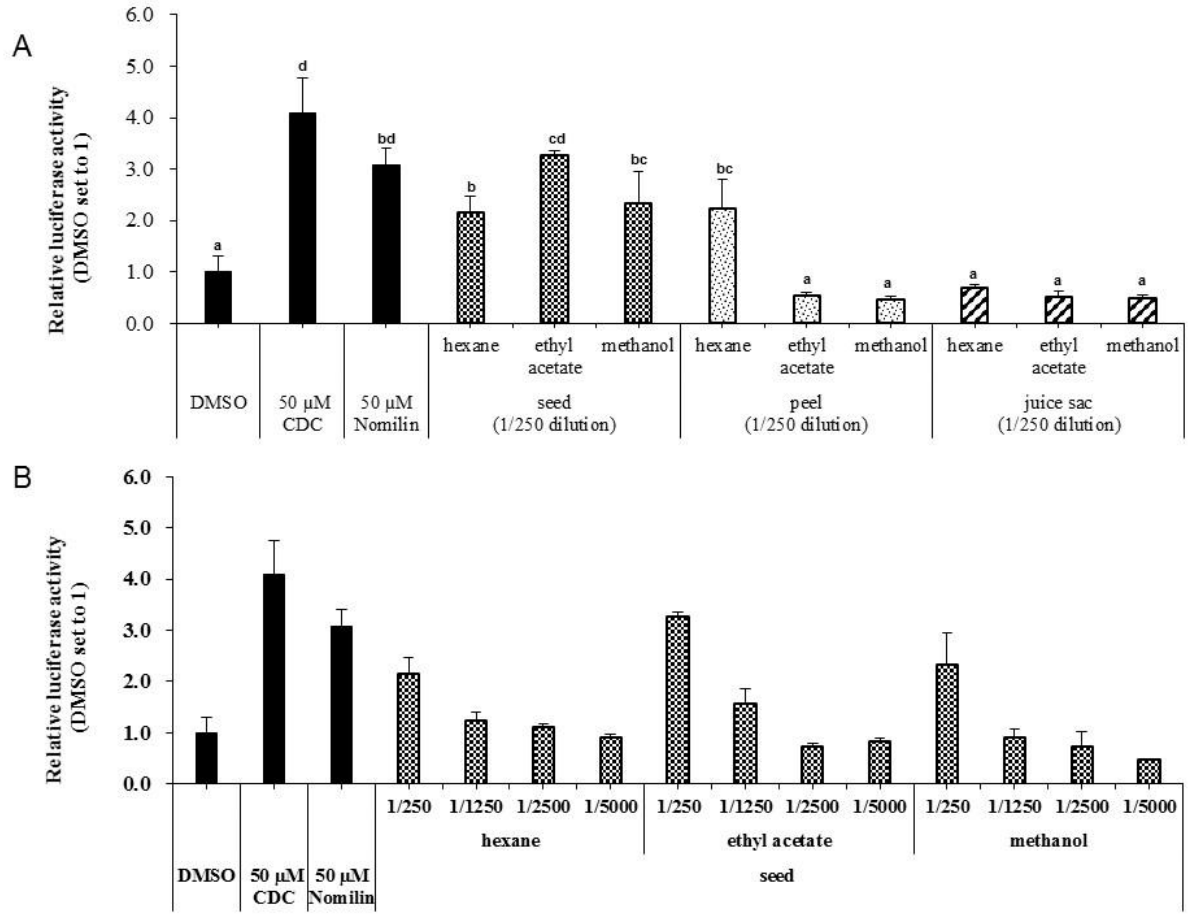


Fig. 3-3. TGR5 ligand activity assay.

(A) Luciferase assay for TGR5 ligand activity in extracts from the seed, peel, and juice sac of the sour orange. CDC (50 μM), nomilin (50 μM), and each extract (hexane, ethyl acetate, and methanol) of the sour orange seed, peel, and juice sac (1/250 dilution of the first prepared extract) were subjected to the assay. Different letters at the top of each bar indicate significant differences among the samples contrasted by Tukey's multiple comparison test ($P < 0.05$). (B) Luciferase assay for TGR5 ligand activity in different dilutions of the seed extract of sour oranges. Different dilutions (1/250, 1/1250, 1/2500, 1/5000) of the first prepared extract (hexane, ethyl acetate, methanol) of sour orange seeds were subjected to the assay. The values were expressed as a relative luciferase activity (DMSO used as a blank was set to 1). Data are represented as the mean ± SD of five technical replicates.

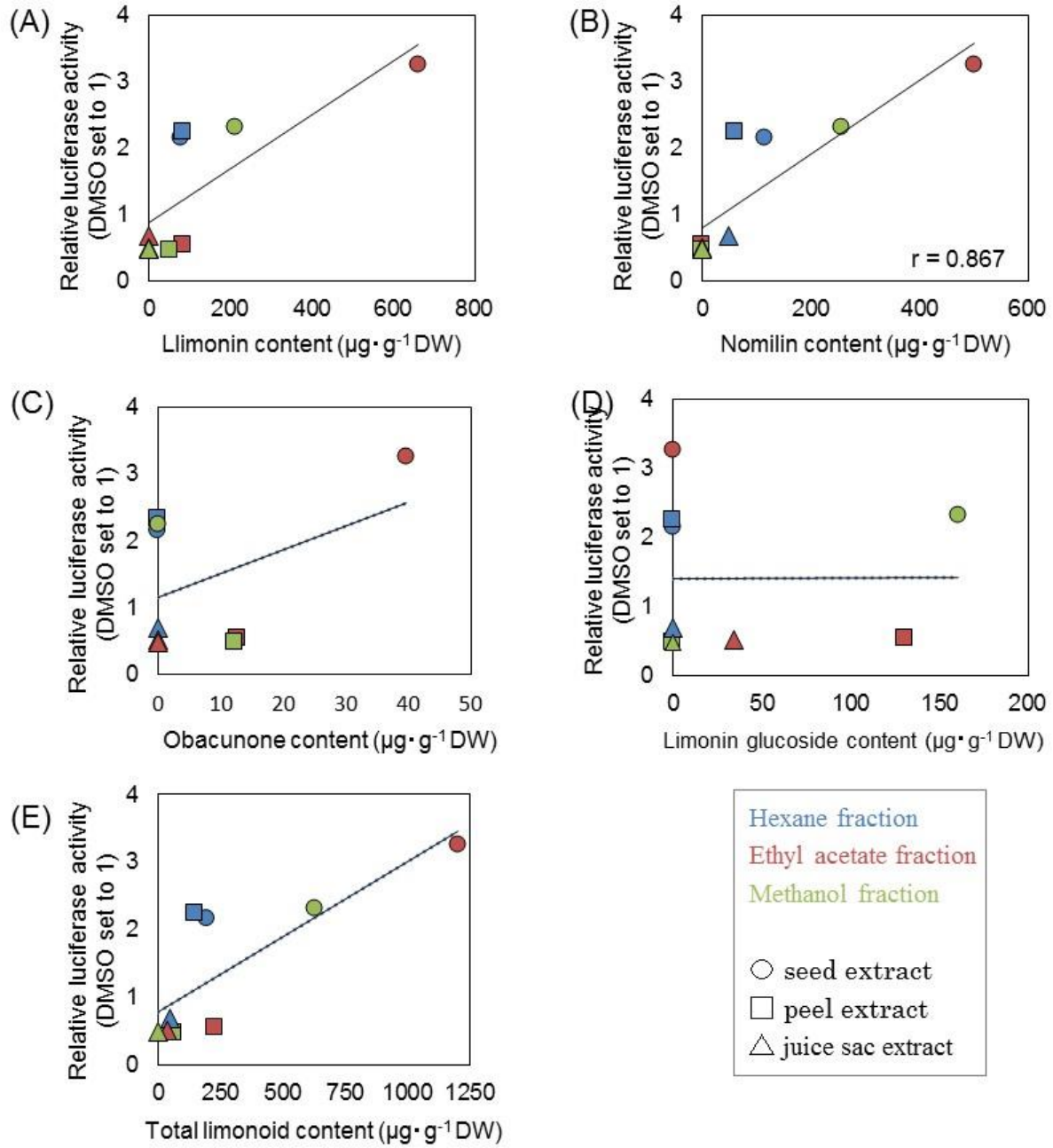


Fig. 3-4. Scatter plots between the limonoid content (μg·g^{-1} DW) and the values expressed as relative luciferase activity (DMSO was set to 1).

(A) Limonin, (B) nomilin, (C) obacunone, (D) limonin glucoside, and (E) total limonoids. Each panel shows the plot of the values. The correlation coefficient was calculated using Pearson's correlation analysis.

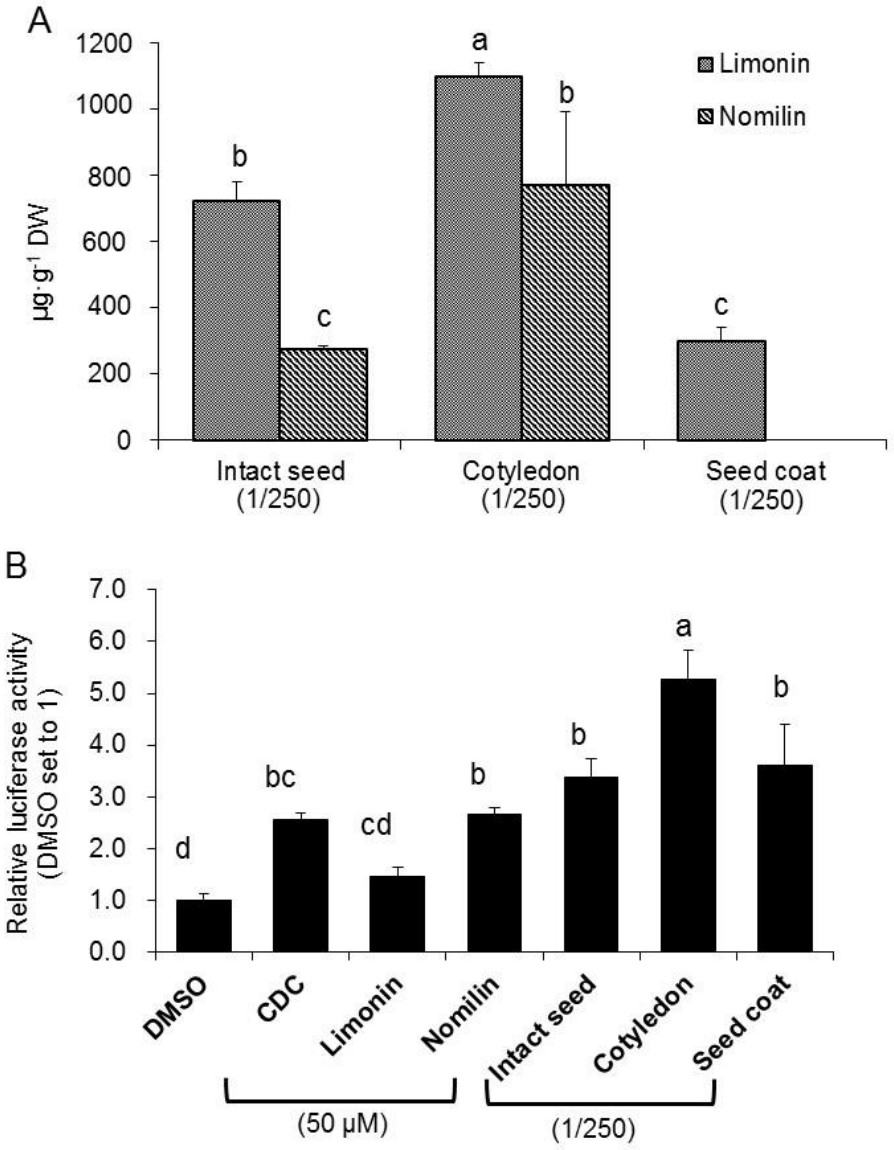


Fig. 3-5. Limonoid content and TGR5 ligand activity in seeds.

(A) The concentration of limonin and nomilin in extracts from the intact seed, cotyledon (seed without seed coat), and seed coat of the sour orange; tr. = trace amount. (B) Luciferase assay for TGR5 ligand activity in extracts from the intact seed, cotyledon, and seed coat of the sour orange. CDC (50 μM), limonin (50 μM), nomilin (50 μM), and extracts (1/250 dilution of the first prepared extracts) from intact seed, cotyledon, and seed coat of the sour orange were subjected to the assay. The values were expressed as a relative luciferase activity (DMSO was set to 1). Data are represented as the mean ± SD of three biological (A) and five technical (B) replicates. Different letters on each bar indicate significant differences among the samples contrasted by Tukey's multiple comparison test ($P < 0.05$).

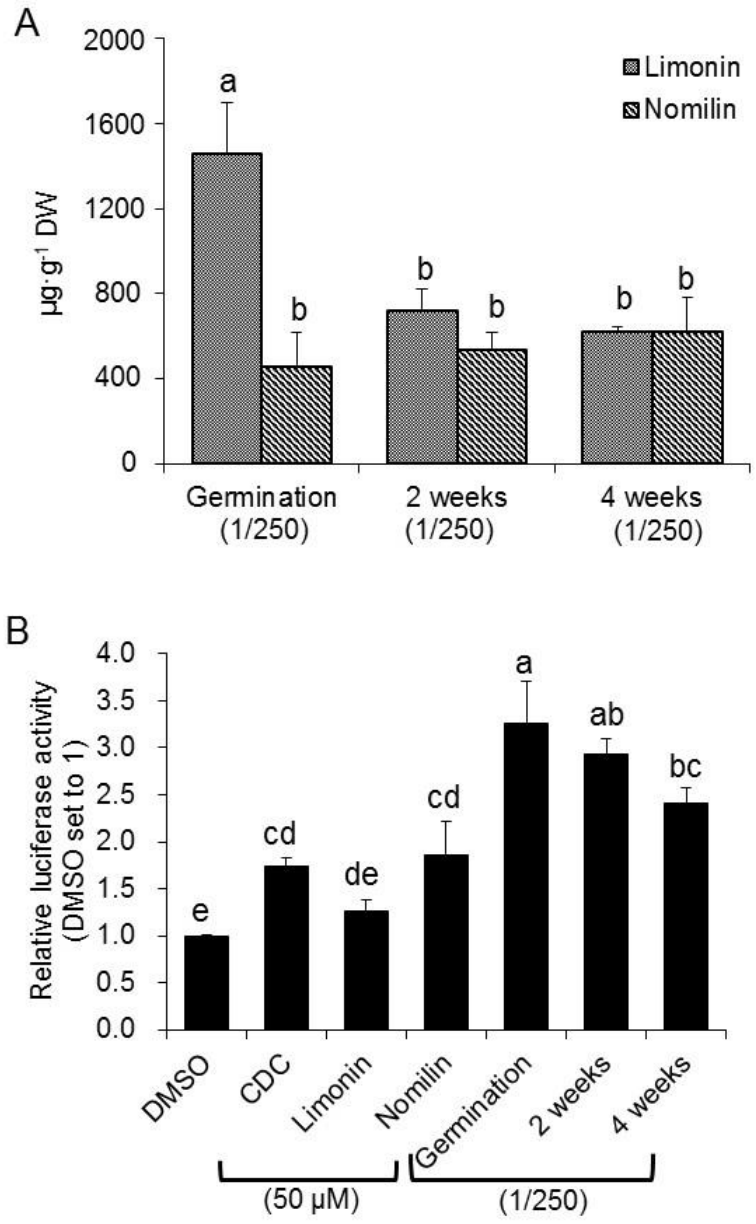


Fig. 3-6. Limonoid content and TGR5 ligand activity in the tissues after germination.

(A) The concentration of limonin and nomilin extracted from the germinated seed (10 days under dark conditions at 25°C after germination) and 2- and 4-week-old seedlings (germinated seeds were transplanted into pots containing vermiculite in the greenhouse) of the sour orange. (B) Luciferase assay for TGR5 ligand activity in extracts from the germinated seed and 2- and 4- week-old seedlings of the sour orange. The values were expressed as a relative luciferase activity (DMSO was set to 1). CDC (50 μM), limonin (50 μM), nomilin (50 μM), and extracts (1/250 dilution of the first prepared extracts) from the germinated seed and 2- and 4-week-old seedlings were subjected to the assay. Data are represented as the mean ± SD of three biological (A) and five technical (B) replicates. Different letters at the top of each bar indicate significant differences among the samples contrasted by Tukey's multiple comparison test ($P < 0.05$).

Chapter 4: General Discussion

Citrus is an evergreen and one of the most cultivated fruits in the world. Many citrus species are considered to be rich in secondary metabolites especially polymethoxyflavones (PMFs) and limonoids that play various important roles in human health due to their functional properties (Hasegawa et al., 1999; Patil et al., 2006; Tripoli et al., 2007). PMFs show the protective effects against memory impairment of Alzheimer`s disease (Braidy et al., 2017; Kimura et al., 2018) as well as anti-tumor, anti-hyperglycemia, anti-obesity and anti-neuroinflammation and insulin resistance activities (Ho and Kuo, 2014; Kawaii et al., 1999; Lee et al., 2010, 2013; Minagawa et al., 2001; Miyata et al., 2008; Onda et al., 2013; Surichan et al., 2018; Wang et al., 2014). On the other hand, many studies have demonstrated the significant biological and physiological impacts of limonoids on human health, such as anti-cancer (Jacob et al., 2000; Lam et al., 1994; Miller et al., 2004) and anti-microbial (Chowdhury et al., 2003; Govindachari et al., 2000) activities. Both important metabolites, PMFs and limonoids, are present in the pulp, seed, leaf, pressed oil and fruit (peel and juice sac) at significant levels (Guldani et al., 2016; Herman et al., 1989; Patil et al., 2006; Rouseff and Nagy, 1982; Tripoli et al., 2007). Because of their functional activities on human health, citrus PMFs and limonoids are of great interest to the citrus researcher in recent years. To increase the PMF content in various citrus cultivars for future citrus breeding, it is necessary to investigate the high, medium and low PMFs accumulated cultivars and identify the PMFs biosynthesis-related OMT genes in various citrus tissues such as leaves and flavedos. Also, to investigate the biological activity of citrus limonoids, it is essential to determine the limonoid content in various citrus tissues of popular citrus cultivars.

The present study aimed to provide knowledge about the PMF content in various citrus cultivars in leaves and flavedos and their correlation and identify the PMF biosynthesis-related OMT genes and their expression in both tissue; furthermore, determine the limonoid content

in various citrus tissues and investigate their biological activities using a luciferase assay system.

In the present study (Chapter 2), it was observed that two major PMFs, nobiletin and tangeretin, accumulated at higher levels in flavedos than in leaves of 11 citrus cultivars, except for nobiletin content in 'Seinannohikari' and tangeretin content in 'Mihaya' (Fig. 2-2). Nogata et al. (2006) also reported the higher PMF accumulation in flavedos/peels of various citrus cultivars compared to the fruit, juice sac, albedo, and segment epidermis. Of the 11 citrus cultivars tested, 'Benibae' accumulated the highest level of PMF compared to the others tested. One possible explanation is that 'Benibae' was originated from the parents HF No.9 ('Hayashi' Satsuma mandarin × 'Fukuhara' orange) and 'Encore'. There is a possibility that the higher PMF accumulation in 'Benibae' could be this phenotypic trait derived from parent 'Encore', which accumulated 3.73 $mg \cdot g^{-1}$ DW of nobiletin in leaves (Laboratory of Fruit Sciences, Saga University, unpublished result).

In general, nobiletin tended to accumulate more than tangeretin in leaves, confirming a significant correlation between the accumulation of nobiletin and tangeretin in leaves ($r = 0.978$, $P < 0.000001$; Figure 2-4A) and flavedos ($r = 0.913$, $P < 0.0001$; Fig. 2-4B) in the citrus tested. Nogata et al. (2006) comprehensively investigated flavonoids, including PMFs, in the flavedos of 45 citrus cultivars, and similar results were obtained in about 76% of 21 cultivars with some nobiletin content (> 0.2 $mg \cdot g^{-1}$ FW). These results suggest that higher nobiletin content in leaves and flavedos may be due to their structural difference of the methoxy group. The nobiletin has six methoxy groups and tangeretin has five, and the structural difference is only one methoxy group (Fig. 2-1), suggesting that their biosynthetic pathway could be similar. There was a positive correlation ($r = 0.824$, $P = 0.00182$) of nobiletin content between leaves and flavedos (Fig. 2-3). Similarly, tangeretin content was shown to be positively correlated in

the leaves (r = 0.978, P < 0.000001) and flavedos (r = 0.913, P < 0.0001). The results of correlation suggest that a set of PMF biosynthesis-related genes could be present in both tissues.

Phylogenetic analysis revealed that *CreOMT1*, *CreOMT2*, and *CreOMT4* were closely related to *OMT* genes such as *Mp8-OMT* (AY337458), *Cat3'5'-OMT* (AY127568), and *Hv7-OMT* (CAA54616). Barley *Hv7-OMT* is involved in the *O*-methylation of the hydroxy group at the 7th position of flavones (Christensen et al., 1998), Madagascar periwinkle *Cat3'5'-OMT* (*CrOMT2*) is involved in the two sequential *O*-methylations of the hydroxy group at the 3'- and 5'-positions of flavonols (Cacace et al., 2003) and peppermint, *Mp8-OMT* (*MpOMT2*) is found to be involved in the *O*-methylation of the hydroxy group at the C8-position of flavones and in their biosynthesis (Willits et al., 2004). Because Hv7-OMT and Mp8-OMT methylate hydroxyflavones at C7 and C8, respectively, these citrus *OMT*-like genes might be involved in the biosynthesis of nobiletin and tangeretin, which are methylated at C7 and C8, respectively (Fig. 2-1). Especially, *CreOMT1* and *CreOMT4* might catalyze the hydroxy group at the 8th position of flavones, considering that they were more related to *Mp8-OMT* than to *Cat3'5'-OMT* or *Hv7-OMT* in the phylogenetic tree.

According to the multiple sequence alignment, *CreOMT1*, *CreOMT2*, and *CreOMT4* showed five consensus sequence motifs (motif I to V) with *Hv7-OMT*, *Cat3'5'-OMT*, and *Mp8-OMT* (Figure 2-6). These consensus sequence motifs I to IV represented a characteristic of SAM-dependent *O*-methyltransferase, which transfers a methyl group from SAM onto a hydroxy group and SAM-dependent methylations are necessary for the flavonoids biosynthesis (Christensen et al., 1998; Gana et al., 2013; Ibrahim et al., 1998; Vidgren et al., 1994; Willits et al., 2004). Thus, it could be concluded that *CreOMT1*, *CreOMT2*, and *CreOMT4* might be involved in the biosynthesis of PMF in citrus. Moreover, among the leaves of nine citrus cultivars, *CreOMT1* and *CreOMT4* were expressed in 'Benibae', 'Hirakishu', 'Ota' Ponkan, 'Ogimi kuganii', and'Amanatsu' (Fig. 2-7). These results reflected the nobiletin and tangeretin

accumulation in the leaves of those cultivars, except for ‘Amanatsu’. On the contrary, *CreOMT2* was not expressed in the leaves of the investigated cultivars. One possible explanation for this results is that *CreOMT2* may not be involved in the biosynthesis of PMFs in the leaves of citrus cultivars investigated. Furthermore, *CreOMT1* and *CreOMT4* showed a higher transcript level in leaves than in flavedos of 10 citrus cultivars (Fig. 2-8). These results suggest the differences in the RNA synthesis between the evergreen leaves and senescent flavedos (exocarps). Flavedos almost cease growing in January, and RNA synthesis is not thought to be very active. Previously, it was confirmed the seasonal changes in the accumulation of PMF in the leaves and flavedos of Ponkan and Shiikuwasha (Kotoda et al., 2017; Yamaguchi et al., 2015), suggest that PMF biosynthetic genes and enzyme expression might be varied according to developmental stages although enough PMF had accumulated in the tissues. The transcripts of *CreOMT1* and *CreOMT4* were apparently associated with the accumulation of PMFs in leaves and flavedos of the cultivars studied (Fig. 2-2 and 2-8). The expression of both genes tended to be more involved in the accumulation of nobiletin in flavedos than in leaves (Fig. 2-9). In leaves, *CreOMT1* showed a relatively higher expression in ‘Amanatsu’, which accumulated a lower level of PMF and the same levels of expression was showed in ‘Ogimi kuganii’, which had a higher level of PMF. However, the *CreOMT1* expression was not sufficient. On the contrary, in flavedos, *CreOMT1* was expressed relatively highly in ‘Shiranuhi’, which was a low PMF-accumulating cultivar. ‘Amanatsu’ accumulated low PMF in both leaves and flavedos but *CreOMT1* and *CreOMT4* were expressed relatively highly, except for the lower expression of *CreOMT1* in flavedos. One of the possible reason for these results is that ‘Amanatsu’ might have inherited a part of the gene sets related to the biosynthesis of PMF from a PMF-accumulating cultivar, Kishu-mikan (*C. kinokuni* hort. ex Tanaka), which would be a pollen parent of ‘Amanatsu’ (Shimizu et al., 2016). Similarly, in ‘Shiranuhi,’ higher expressed *OMT* genes might be derived from the Ponkan genome because

the pollen parent of 'Shiranuhi' is 'Nakano No.3' Ponkan (Matsumoto, 2001). Considering the overall results in Chapter 2, it could suggest that screened citrus cultivars related to PMF accumulation and identified *OMT* genes will play a great impact on a future citrus breeding program to develop the higher PMF accumulating citrus cultivars.

In the present study (Chapter 3), the four types of limonoids (limonin, nomilin, obacunone, and limonin glucoside) were determined in the sour orange fruit using three types of extracting solvents. It is noted that limonoids are accumulated mainly in seeds (2022 $\mu g \cdot g^{-1}$ DW), followed in order by peels (443 $\mu g \cdot g^{-1}$ DW) and juice sacs (83 $\mu g \cdot g^{-1}$ DW) (Fig. 3-2). This investigation, consistent with previous studies, demonstrated that distribution of limonoids in citrus fruit is tissue-specific and seed is the rich source of limonoids (Hasegawa et al., 1980; Miyake et al., 1992; Ohta and Hasegawa, 1995; Rouseff and Nagy, 1982; Sun et al., 2005; Wang et al., 2016). Rouseff and Nagy (1982) determined the common sour orange concentration of limonin, nomilin, and obacunone as 1256, 242, and 104 $\mu g \cdot g^{-1}$ DW, respectively. Similarly, Miyake et al. (1992) reported that an obacunone concentration (32 $\mu g \cdot g^{-1}$ DW) of sour orange seeds were well fitted to this investigation. However, the concentration of limonin, nomilin, and limonin glucoside (2470, 178, and 610 $\mu g \cdot g^{-1}$ DW, respectively) was different from this study. Contrastly, Vikram et al. (2007) detected no nomilin in the sour orange seed extract. The limonoid content depends on the maturation stages or cultivar (Matsumoto et al., 2008; Sun et al., 2005; Wang et al., 2016). However, in this study, it was observed that the limonoid content in sour orange also varied due to the sampling season, cultivar/strain and method of sample preparation.

The seeds extract of sour orange contained higher limonoids than other organs, except for the hexane extract from the peels and the ligand activity of TGR5 was significantly higher in the extracts of the seeds (Fig. 3-3A). Samples with high nomilin content showed significantly higher TGR5 ligand activity compared to the blank (DMSO) (Fig. 3-2 and 3-3A). Notably, the

correlation coefficient between TGR5 ligand activity and the nomilin content was the highest among the four limonoids analyzed in this study (Fig. 3-4B). In contrast, no correlation (r = 0.006) was found between the TGR5 ligand activity and limonin glucoside content, suggesting the non-functional roles of limonin glucoside to the TGR5 receptor activation (Fig. 3-4D). In this study, it is revealed that sour orange extracts, nomilin could effectively activate the TGR5 receptor. Similarly, Ono et al. (2011) reported that the TGR5 ligand activity of nomilin was higher than limonin or obacunone. However, in this study, the ethyl acetate extract of the seed showed activity equivalent to that of positive control of 50 μM nomilin although the final limonoid concentration (limonin: 5.6 μM; nomilin: 3.9 μM; obacunone: 0.4 μM) derived from the ethyl acetate extract in the assay was much lower than 50 μM (Fig. 3-2 and 3-3). This result suggest that there might be some compounds involved in the higher TGR5 ligand activity than nomilin, which was expected to be a new and major compound for activating TGR5 in the extracts.

From this study, it was revealed that seed extracts showed higher TGR5 ligand activity. A higher accumulation of limonin and nomilin was determined in the cotyledons than seed coats (Fig. 3-5A). As expected, it was also observed the higher TGR5 ligand activity in the cotyledons than in the seed coats. In this study, seed coats showed a similar level of TGR5 ligand activity to the intact seeds, which suggest that in addition to nomilin seed coats contained some unknown compounds that play a role to activate the TGR5 receptor, because of only a trace amount of nomilin was detected in the seed coats (Fig. 3-5).

A higher limonin content in germinated seeds (1460 $\mu g \cdot g^{-1}$ DW) was determined than cotyledons (1097 $\mu g \cdot g^{-1}$ DW) but nomilin content was lower in germinated seeds (459 $\mu g \cdot g^{-1}$ DW) than cotyledons (771 $\mu g \cdot g^{-1}$ DW) (Fig. 3-6A). However, total limonoid content was similar in cotyledon (1868 $\mu g \cdot g^{-1}$ DW) and germinated seed (1919 $\mu g \cdot g^{-1}$ DW). This close amount of total limonoid could be due to the metabolism of nomilin to limonin through

ichangin in the biosynthetic pathway of sour orange (Miyake et al., 1992). In contrast to this study, Ariza et al. (2015) reported that germinating seeds of *Citrus aurantium* accumulated a higher amount of limonoids, such as limonin, nomilin, and ichangin than dormant seeds. In the seedling (2 weeks), limonin content was drastically decreased, whereas the nomilin content does not significantly change in the seedling (4 weeks) compared to the germinated seeds (Fig. 3-6A). As expected, germinated seeds showed a higher TGR5 ligand activity than seedling (2 and 4 weeks) (Fig. 3-6B). The higher limonin content in germinated seeds would reflect the higher TGR5 activity because the nomilin content was closely similar among the three samples. Bennett and Hasegawa (1980) reported a higher amount of two limonoids, ichangin, and isolimonic acid in sour orange except in this study. Recently, Sasaki et al. (2017) constructed an hTGR5–nomilin binding model and demonstrated that four hydrophilic hydrogen-bonding interactions occurred between the oxygen atoms of nomilin and the amino acid residues of hTGR5. Based on the results of Sasaki et al. (2017) and in this study, novel agonists with higher TGR5 activity could be found in sour orange that similar in the structure of nomilin. From the study of Chapter 3, it could be concluded that studied limonoids content in sour orange and their TGR5 ligand activity, although the activity could not be explained by the nomilin content in the seed extract alone, suggests that the sour orange seeds might contain other components with a TGR5 ligand activity higher than that of nomilin. In the future, it will be necessary to comprehensively investigate the citrus seed extract for unidentified agonists for the TGR5 receptor. The sour orange might be a potential source of compounds that prevent obesity and metabolic disorders.

Taken together, the above findings will provide valuable information in future citrus breeding related to the PMF accumulation and for unidentified agonists for the TGR5 receptor.

Summary

Polymethoxyflavone (PMF) are natural occurring *O*-methylated flavones abundant in different tissues of citrus. On the other hand, limonoids belong to a group of highly oxygenated triterpenoids, and are mostly found in citrus as well. Both metabolites are functionally important for numerous human-health promoting activities.

O-methyltransferases (OMT) family members are possibly involved in the biosynthesis of PMF in plant species including citrus. The content of major PMFs, nobiletin, and tangeretin, was determined in the leaves and flavedos of 10 citrus cultivars that are widely grown in Japan. The accumulation of the PMFs was varied among the citrus cultivars and tissues. Both PMFs were accumulated higher in flavedos than leaves. A significant and positive correlation (r = 0.824, P = 0.00182) was observed in nobiletin content between leaves and flavedos. These investigations suggest that the PMF concentration in leaves could be utilized as an early selection marker for seedlings in the juvenile phase, which are expected to accumulate a higher amount of PMF in fruit, resulting in shortening the breeding period. The expression of two *OMT* genes, *CreOMT1* and *CreOMT4*, showed a close association with nobiletin and tangeretin accumulation in leaves and flavedos, suggesting their possible roles to regulate the PMF biosynthesis in citrus.

On the other hand, recent studies suggested that citrus limonoids bind to a bile acid receptor, TGR5 (Takeda G-protein-coupled receptor 5), and confer anti-obesity and anti-hyperglycemic effects. In this study, the content of limonoids (limonin, nomilin, obacunone, and limonin glucoside) in the sour orange extract was also determined using three different solvents and measured the TGR5 ligand activity for the extracts. The total concentration of the four limonoids was the highest in the extract of ethyl acetate, followed by methanol and hexane in sour orange seeds. Moreover, luciferase assay using CHO cells transfected with a TGR5 confirmed that TGR5 ligand activity in the ethyl acetate extract of the seeds was as high as that in 50 μM nomilin, followed by that in the methanol extract of the seeds. The correlation

coefficient between limonoid content and TGR5 ligand activity showed the highest value (r = 0.867) for nomilin. However, the activity of the extract could not be explained only by the nomilin content because the concentration of nomilin in the extract used for TGR5 assay was 3.9 μM, much lower than that of control (50 μM), suggesting unknown compounds with higher TGR5 ligand activity could be contained in the seed extracts. In addition, the cotyledons or germinated seed extract also showed a higher TGR5 ligand activity. The seed or germinated seed extract of the sour orange might be a potential source of compounds that could prevent obesity and metabolic syndrome.

The findings above will provide valuable information to elucidate the PMF biosynthesis pathway in detail and to develop PMF rich citrus cultivars in future citrus breeding and facilitate the comprehensive investigation of the citrus seed source for unidentified agonists for the TGR5 receptor.

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