The Horticulture Journal *Preview*
doi: 10.2503/hortj.UTD-146

The Japanese Society for Horticultural Science
http://www.jshs.jp/

Accumulation of Polymethoxyflavones and *O*-methyltransferase Gene Expression in Various Citrus Cultivars

Fatema Tuz Zohra[1], Shotaro Takematsu[2], Yuri Itami[3] and Nobuhiro Kotoda[1,2,3]*

[1]*The United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan*
[2]*Faculty of Agriculture, Saga University, Saga 840-8502, Japan*
[3]*Graduate School of Agriculture, Saga University, Saga 840-8502, Japan*

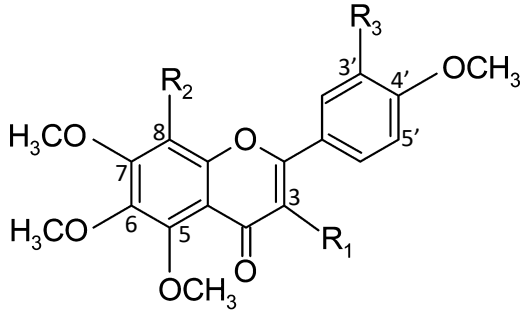
Polymethoxyflavones (PMFs) are naturally occurring *O*-methylated flavones predominantly accumulated in different citrus tissues that show numerous human-health promoting activities. *O*-methyltransferase (OMT) family members are involved in the biosynthesis of PMFs in plant species including citrus. Although the distribution of PMFs has been determined in citrus cultivars, the relation between the accumulation of PMFs and the related *OMT* gene expression in leaves and flavedos (exocarps) of citrus cultivars remains to be clarified. In this study, we investigated the accumulation of nobiletin and tangeretin in the leaves and flavedos of various citrus cultivars widely grown in Japan. As expected, the accumulation of nobiletin and tangeretin varied among the citrus cultivars and the two tissues, and nobiletin and tangeretin accumulated at higher levels in flavedos than in leaves. In particular, we clearly demonstrated that the nobiletin content showed a significant and positive correlation ($r = 0.824$, $P = 0.00182$) between leaves and flavedos. Based on these results, the concentration of PMFs in leaves could be utilized as an early selection marker for seedlings in the juvenile phase that are expected to accumulate a higher amount of PMFs in fruit, resulting in a shortening of the breeding period. We also identified two novel candidate genes, *CreOMT1* and *CreOMT4*, putatively involved in PMF biosynthesis in citrus. Notably, the expression of *CreOMT1* showed a significant correlation ($r = 0.700$, $P = 0.0243$) with the nobiletin content in the flavedos of 10 citrus cultivars. Our results provide valuable information for developing new citrus cultivars that could accumulate higher PMFs and insight into elucidating the PMF biosynthetic pathway in citrus in the future.

Key Words: flavedo, flavone, HPLC, nobiletin, tangeretin.

Introduction

Citrus species are some 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). Polymethoxyflavones (PMFs) are naturally occurring *O*-methylated flavonoid compounds found exclusively in citrus that belong to the Rutaceae family, as well as in plant species in the Lamiaceae and Asteraceae families (Fig. 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 PMFs 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 juice production and other industries and citrus peel byproducts are utilized as a source of functional ingredients (Braddock, 1999; Manthey and Grohmann, 2001;

Received; September 25, 2019. Accepted; November 25, 2019.
First Published Online in J-STAGE on February 15, 2020.
This work was financially supported by the Cabinet Office, Government of Japan, Cross-ministerial Strategic Innovation Promotion Program (SIP), "Technologies for creating next-generation agriculture, forestry and fisheries" (funding agency: Bio-oriented Technology Research Advancement Institution, NARO), Ichimura Foundation for New Technology, and Yanmar Environmental Sustainability Support Association (YESSA).
Part of this study was presented at the Autumn Annual Meeting of The Japanese Society for Horticultural Science, 2018.
* Corresponding author (E-mail: koto@cc.saga-u.ac.jp or nobu_fruits@yahoo.co.jp).



Compound	R_1	R_2	R_3	Total no. of -OCH_3
Sinensetin	H	H	OCH_3	5
Tangeretin	H	OCH_3	H	5
Nobiletin	H	OCH_3	OCH_3	6
Heptamethoxyflavone	OCH_3	OCH_3	OCH_3	7

Fig. 1. Chemical structures of polymethoxyflavones found in citrus.

Rafiq et al., 2018). Nobiletin and tangeretin are two major PMFs that are particularly abundant in citrus peels (Choi et al., 2007; Inafuku-Teramoto et al., 2011; Nogata et al., 2006). Recently, citrus PMFs have attracted great interest due to their protective effects against memory impairment in Alzheimer's disease (Braidy et al., 2017; Kimura et al., 2018; Onozuka et al., 2008), as well as their 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).

In plants, *O*-methyltransferase (OMT) is considered a major methyltransferase group. 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 PMFs, the hydroxy group(s) is replaced with a methoxy group(s). Citrus PMFs are a special group of *O*-methylated flavonoids that have many methoxy groups in their parent molecules. OMT proteins that are encoded by the *OMT* gene family transfer a methyl group from SAM to the hydroxy group, and they are considered to be involved in the biosynthesis of PMFs (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 is known to accumulate nobiletin in its fruit peel (Itoh et al., 2016). Moreover, 58 *OMT* genes were identified from the entire *C. sinensis* genome (Liu et al., 2016). However, little is known about the distribution of PMF accumulation, the genes responsible for PMF biosynthesis, or their expression in the leaves and flavedos (exocarps) 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*, was possibly related to the accumulation of PMFs. Identifying the *OMT* genes related to PMF biosynthesis and elucidating their functions in relation to PMF accumulation in leaves and flavedos could play an important role in elucidating the detailed mechanism of PMF accumulation in citrus.

Materials and Methods

Plant materials

We preliminarily screened 121 citrus accessions, including wild citrus plants, for PMF accumulation in mature leaves (data not shown). According to the PMF accumulation, we classified them into three categories, that is, high, medium and low PMF-accumulating plants. From these, we selected 11 citrus cultivars from the high, medium and low group for this study. Tissue samples (leaves and flavedos) of 11 citrus cultivars, 'Amanatsu' (*C. 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. 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 PMFs, respectively. The mature leaves were collected randomly, whereas flavedos were collected from 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.

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

Tissue samples were freeze-dried 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 were added to 500 µL of 80% methanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and mixed by vortexing, then extracted using an ultrasonic cleaner US-3R (AS ONE Corp., Osaka, Japan) for 10 min. The mixture was then 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 vortexing, and centrifuged at 10000 rpm for 1 min at r.t. (this step was repeated twice). Finally, the extracted supernatant was adjusted up to 2.5 mL by adding 80% methanol, 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.

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 Corp., 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, and 87.5% A from 21 to 30 min. The flow rate was 1.2 mL·min^{-1}, and the UV was measured at 254 nm. The column oven temperature was set at 40°C and the sample injection volume was 5.0 µL. The analysis time was 30 min per sample.

Identification of OMT genes

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. The 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 1).

Table 1. Identified *OMT*-related genes from the latest *C. clementina* genome database (ver. 1.0) 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

Isolation of OMT genes from 'Yoshida' Ponkan

Leaves of 'Yoshida' Ponkan were used to isolate the cDNA of three *OMT* genes corresponding to *CcOMT1*, *CcOMT2*, and *CcOMT4*. The cDNA was amplified by PCR using KOD FX (TOYOBO Co., Ltd., Osaka, Japan) from the RT products. Each amplified PCR product was cloned into a pGEM®-T Easy vector (Promega, Madison, WI, USA) after an adenine nucleotide addition to the products. The isolated genes were designated as *CreOMT1* (accession no. LC507211), *CreOMT2* (acc. no. LC507212), and *CreOMT4* (acc. no. LC507213), respectively. To obtain the genomic DNA of *CreOMT1*, *CreOMT2*, and *CreOMT4*, the same primer sets as those for cDNA isolation were used with the total DNA of 'Yoshida' Ponkan as a template. Sequences were confirmed using an ABI 3130xl DNA sequencer (Life Technologies Corp., Carlsbad, CA, USA). The primer sets used in gene cloning are listed in Table 2.

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 multiple sequence alignment program ver. 2.0.5 (Jeanmougin et al., 1998) and the 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)], barley

Table 2. Primer sequences used in gene cloning, semi-qRT-PCR and qRT-PCR.

Gene	Primer	Oligonucleotide sequence (5′→3′)
Cloning		
CreOMT1	Ccl_7OMT1_F1	TAACCCTACTTAGCCTAATAA
	Ccl_7OMT1_R1	CTCTGGTGGCCCTAGGGCCA
CreOMT2	Ccl_7OMT2_F1	TATCAATTAACCCTACTTAGCC
	Ccl_7OMT2_R1	GCCCTAGGATCGAGAAACAA
CreOMT4	Ccl_7OMT4_F1	CTCGCTTCTTGTTAATGACAG
	Ccl_7OMT4_R1	TATTGGGAAGACACAGGATC
Semi-qRT-PCR/qRT-PCR		
CreOMT1	qPCR_7OMT-1_F	TGTTGTACTCAAGTGGATATTG
	qPCR_7OMT-1_R	TTCAGTAGACTCATCATTCCC
CreOMT2	qPCR_7OMT-2_F	GGTTCTATTCAAGTGGATATTA
	qPCR_7OMT-2_R	TTTCAATAGACTCAGAATCCCT
CreOMT4	qPCR_7OMT-4_F	TACTCAAGTGGGTTCTGCATA
	qPCR_7OMT-4_R	GGTTGAGTCCTTGTCTATGC
Reference gene		
CuActin	CuActin_F (-86 > -66)	GAGCGATAGAGAGAATCGACA
	CuActin_R (-1 < 19)	TATCCTCAGCATCGGCCATT

[*Hordeum vulgare* (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, multiple sequence alignment was conducted for CreOMT1, CreOMT2, and CreOMT4 with three other OMT proteins from barley (CAA54616), Madagascar periwinkle (AY127568), and peppermint (AY337459).

RNA isolation

Total RNA was isolated from the leaves of 10 citrus cultivars ('Amanatsu', 'Aoshima', 'Benibae', 'Hirakishu', 'Kiyomi', 'Mihaya', 'Ogimi kuganii', 'Ota' Ponkan, 'Seinannohikari', and 'Shiranuhi') using a CTAB-based method and from flavedos using an SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Isolated total RNA was purified with an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). 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·μL^{-1}.

Expression analysis of citrus OMT genes

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 instructions. 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). *CuActin* (*C. unshiu* actin) was used as an internal control. The RT-PCR products were separated by 2% agarose gel.

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 15 s, 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.

Statistical analysis

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

Results

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

We investigated the accumulation of PMFs, nobiletin and tangeretin, in the leaves and flavedos of 11 citrus cultivars as shown in Figure 2. We found that nobiletin and tangeretin were widely distributed among the citrus cultivars and varied in the degree of accumulation in the both tissues. Nobiletin and tangeretin were accumulated at higher levels in flavedos (Fig. 2C, D) than in leaves (Fig. 2A, B).

In the leaves, a higher level of nobiletin was found in 'Benibae' (6.19 mg·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·g^{-1} DW), followed by 'Shiranuhi', 'Kiyomi', 'Aoshima', 'Mihaya', and 'Amanatsu' (Fig. 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, while a low tangeretin content was observed in 'Seinannohikari' (0.43 mg·g^{-1} DW), followed by 'Kiyomi', 'Shiranuhi', 'Aoshima', 'Amanatsu', and 'Mihaya' (Fig. 2B).

In the flavedos, nobiletin was highly accumulated in 'Hirakishu' (14.81 mg·g^{-1} DW), followed by 'Ogimi kuganii', 'Ota' Ponkan, 'Benibae', and 'Yoshida' Ponkan, while a lower amount of nobiletin was accumulated in 'Kiyomi' (4.38 mg·g^{-1} DW), followed by 'Shiranuhi', 'Seinannohikari', 'Aoshima', 'Amanatsu', and 'Mihaya' (Fig. 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. 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 contents were 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 contents were found in 'Mihaya' (0.17 mg·g^{-1} DW and 0.09 mg·g^{-1} DW, respectively).

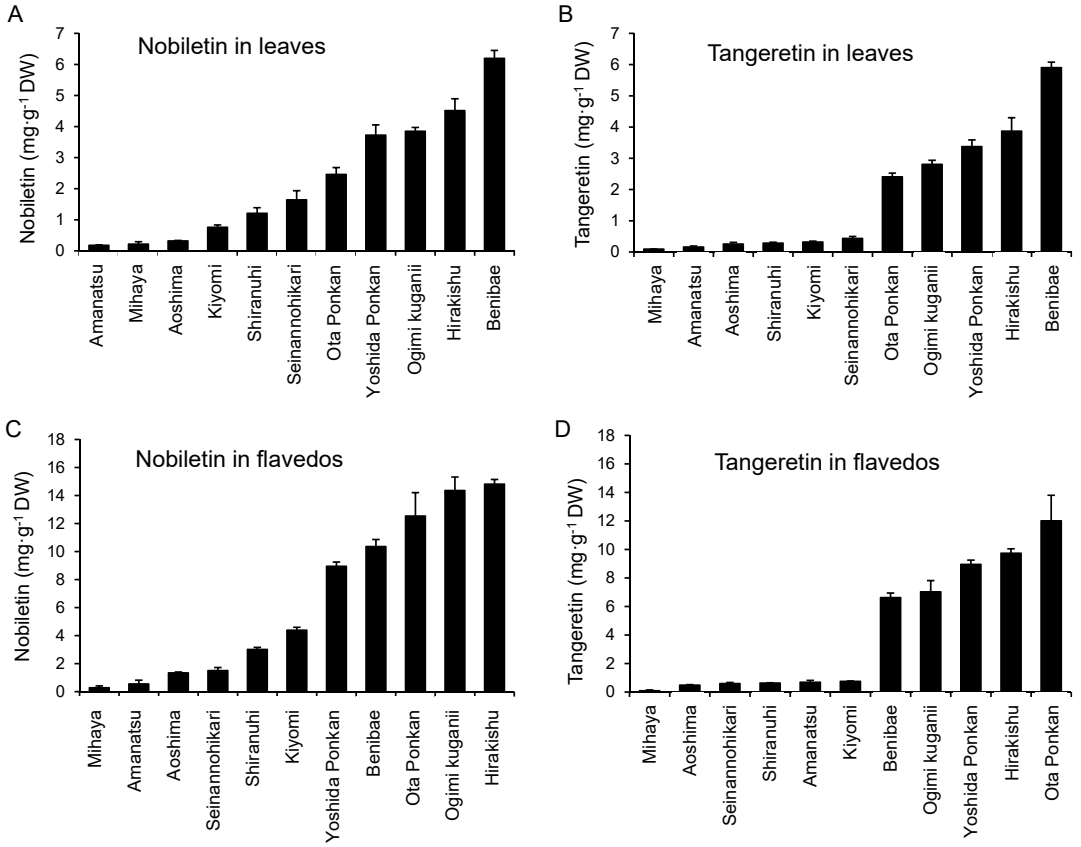


Fig. 2. Accumulation of nobiletin and tangeretin in the leaves and flavedos of 11 citrus cultivars: 'Amanatsu', 'Aoshima', 'Benibae', 'Hirakishu', 'Kiyomi', 'Mihaya', 'Ogimi kuganii', 'Ota' Ponkan, 'Seinannohikari', 'Shiranuhi', and 'Yoshida' Ponkan. (A) The nobiletin content in leaves. (B) The tangeretin content in leaves. (C) The nobiletin content in flavedos. (D) The tangeretin content in flavedos. The values are means ± SD of the results from three biological replicates per cultivar.

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

To evaluate the distribution of nobiletin content in both the leaves and flavedos of 11 citrus cultivars, we performed 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. 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. S1).

Isolation of OMT genes from 'Yoshida' Ponkan

We found 16 *OMT*-related genes in the *C. clementina* genome database (ver. 1.0) by conducting a BLAST search (Table 1). To infer the function of *OMT* genes in citrus, we isolated the cDNA of 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*), using three isolated genes from 'Yoshida' Ponkan [assigned as *CreOMT*: *CreOMT1* (LC507211), *CreOMT2* (LC507212), and *CreOMT4* (LC507213)] and 18 previously reported *OMT* genes from other plant species, including *Hv7-OMT* of barley, we constructed a phylogenetic tree based on the deduced amino acid sequences (Fig. 4). 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 were 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'-positions of flavonoid, respectively (Cacace et al., 2003; Willits et al., 2004). The identity levels (similarity) between CreOMT1 and CreOMT2, CreOMT1 and CreOMT4, and CreOMT2 and CreOMT4 were 94.5% (96.4%), 54.1% (70.8%), and 54.4% (71.4%), respectively. CreOMT1 and CreOMT2 was 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. 5). These motifs (motif I to V) are the residues found in SAM-dependent *O*-methyltransferase.

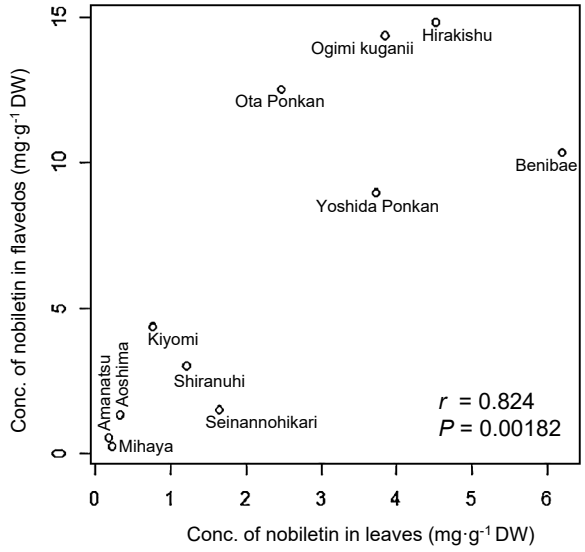


Fig. 3. Correlation of nobiletin content between the leaves and flavedos of 11 citrus cultivars: 'Amanatsu', 'Aoshima', 'Benibae', 'Hirakishu', 'Kiyomi', 'Mihaya', 'Ogimi kuganii', 'Ota' Ponkan, 'Seinannohikari', 'Shiranuhi', and 'Yoshida' Ponkan. Statistical analysis was performed using R-3.2.0 (R Core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

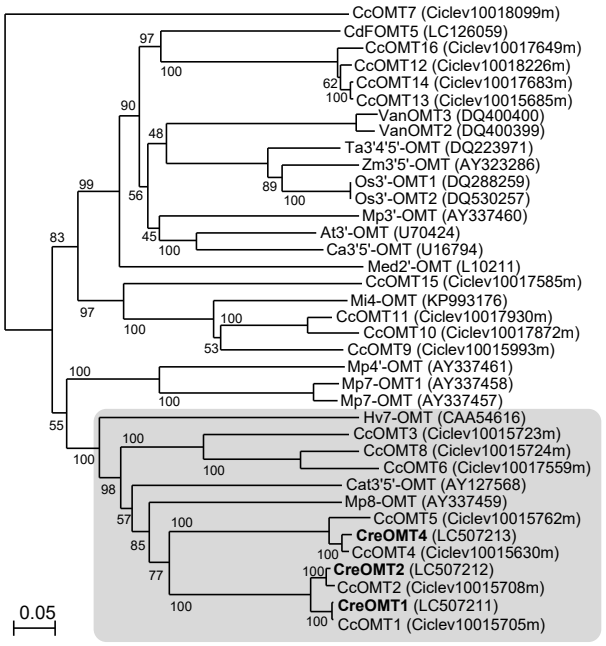


Fig. 4. Phylogenetic analysis of *OMT* genes of citrus cultivars together with other plant species. The tree was constructed by the neighbor-joining (N-J) method and 100 bootstrap trials for the deduced amino acid sequences of the *OMT* genes from alfalfa [*Med2'-OMT* (L10211)], *Arabidopsis* [*At3'-OMT* (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)], rice [*Os3'-OMT1* (DQ288259), *Os3'-OMT2* (DQ530257)], Shiikuwasha [*CdFOMT5* (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.

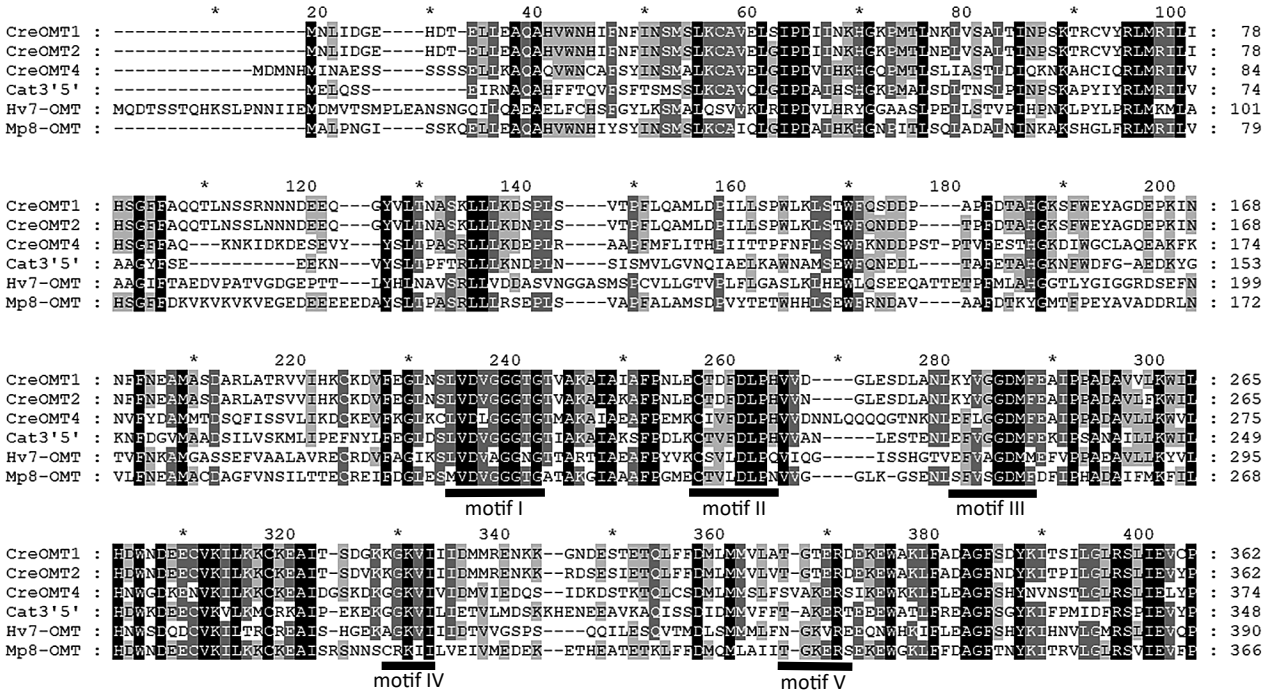


Fig. 5. Alignment of OMT proteins derived from 'Yoshida' Ponkan (*CreOMT1*, *CreOMT2* and *CreOMT4*) and other plant species, Madagascar periwinkle [*Cat3'5'-OMT* (AY127568)], barley [*Hv7-OMT* (CAA54616)], and peppermint [*Mp8-OMT* (AY337458)]. Putative amino acid sequences were aligned using the ClustalX2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

Expression analysis of CreOMT1, CreOMT2, and CreOMT4

To clarify whether or not *CreOMT1*, *CreOMT2*, and *CreOMT4* were expressed in the leaves of nine citrus cultivars in which PMFs accumulate, we analyzed the expression of these genes by semi-qRT-PCR (Fig. S2). *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 *CreOMT2* expression was detected in any of the investigated cultivars. *CreOMT4* was expressed in 'Benibae', 'Hirakishu', 'Ota' Ponkan, 'Ogimi kuganii', and 'Amanatsu', but was not expressed in 'Seinannohikari', 'Shiranuhi', 'Kiyomi', or 'Aoshima'.

To further confirm the transcripts of *CreOMT1* and *CreOMT4* in the leaves and flavedos of 10 citrus cultivars, we performed qRT-PCR. The transcripts of *CreOMT1* and *CreOMT4* were detectable in the two tissues of all citrus cultivars (Fig. 6). 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. 6A). In contrast, the transcripts of *CreOMT1* were relatively abundant in the flavedos of 'Ogimi kuganii', 'Ota' Ponkan, 'Shiranuhi', and 'Hirakishu', with only slight accumulation of transcripts in 'Benibae', 'Mihaya', 'Aoshima', 'Seinannohikari', 'Amanatsu', and 'Kiyomi' (Fig. 6B). 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. 6C). In flavedos, *CreOMT4* was expressed at higher levels in 'Ota' Ponkan, 'Ogimi kuganii', and 'Shiranuhi', whereas lower levels of transcripts were detected in 'Kiyomi', 'Mihaya', and 'Seinannohikari' (Fig. 6D).

To investigate the correlation between nobiletin content and *CreOMT* gene expression, we conducted correlation analysis as shown in Figure 7. The result showed a significant correlation of the nobiletin content and *CreOMT1* expression in the flavedos ($r = 0.700$, $P = 0.0243$; Fig. 7B), with an apparent correlation between the nobiletin content and *CreOMT4* in the flavedos ($r = 0.588$, $P = 0.0740$; Fig. 7D), the nobiletin content and *CreOMT1* in the leaves ($r = 0.420$, $P = 0.2278$; Fig. 7A), and the nobiletin content and *CreOMT4* in the leaves ($r = 0.306$, $P = 0.3906$; Fig. 7C).

Discussion

In 11 citrus cultivars tested, the nobiletin and tangeretin contents were generally higher in flavedos than in leaves, except for nobiletin in 'Seinannohikari' and tangeretin in 'Mihaya' (Fig. 2). Similarly, a higher accumulation of PMFs in flavedos/peels has been observed in various citrus cultivars as compared to other tissues such as the fruit, juice vesicle, albedo, and seg-

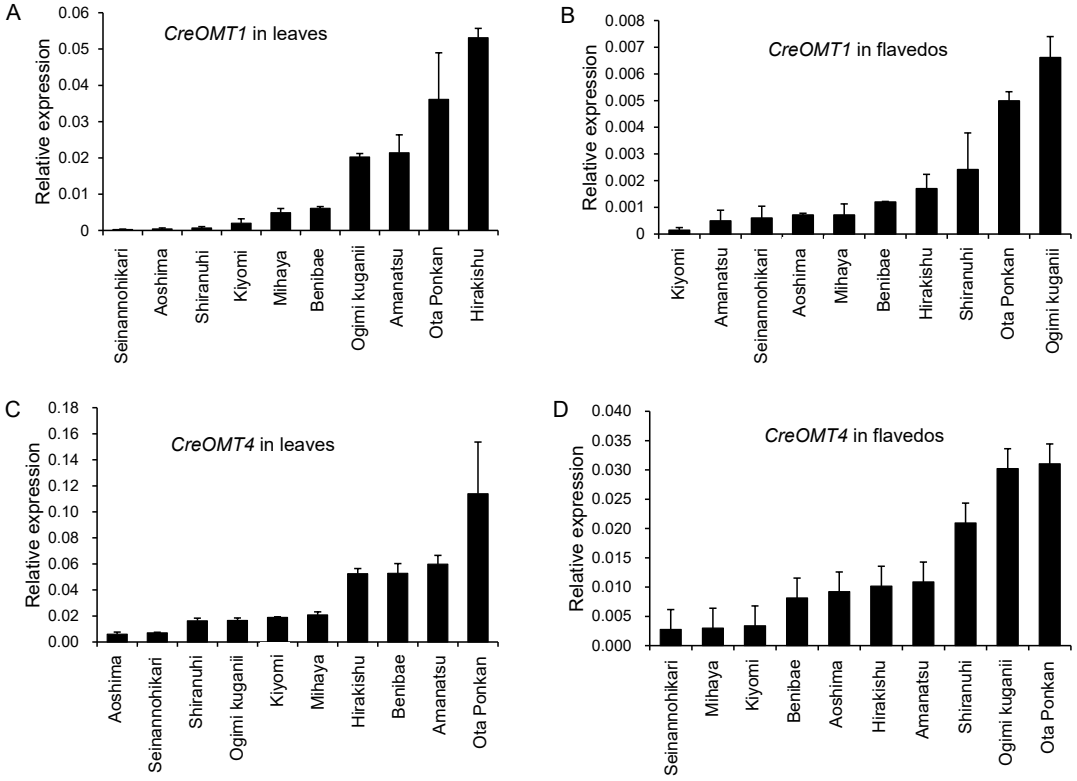


Fig. 6. Expression profiles of *CreOMT1* and *CreOMT4* in the leaves and flavedos of 10 citrus cultivars: 'Amanatsu', 'Aoshima', 'Benibae', 'Hirakishu', 'Kiyomi', 'Mihaya', 'Ogimi kuganii', 'Ota' Ponkan, 'Seinannohikari', and 'Shiranuhi'. (A) The expression of *CreOMT1* in leaves. (B) The expression 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.

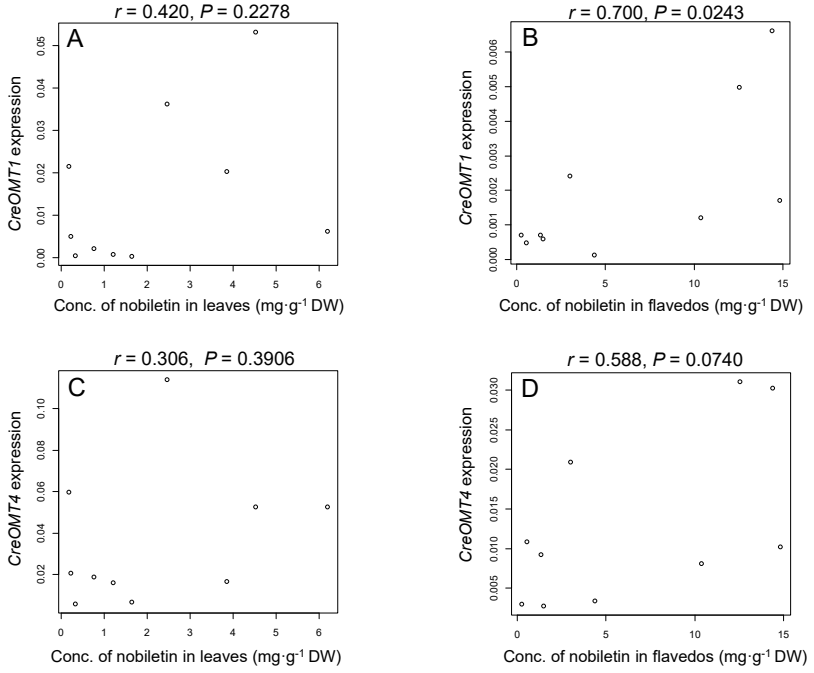


Fig. 7. Correlation of the nobiletin content and the *CreOMT* gene expression. The citrus cultivars used in this analysis were the same as those used in the gene expression experiment. Scatter plot of (A) nobiletin content and *CreOMT1* expression in leaves, (B) nobiletin content and *CreOMT1* expression in flavedos, (C) nobiletin content and *CreOMT4* expression in leaves, (D) nobiletin content and *CreOMT4* expression in flavedos. Displaying scatter plot and statistical analysis were performed using R-3.2.0 (R Core Team, 2015).

ment epidermis (Nogata et al., 2006). Of the 11 cultivars, five ('Benibae', 'Hirakishu', 'Ogimi kuganii', 'Ota' Ponkan, and 'Yodhida' Ponkan) accumulated a higher level of PMFs. Because the parents of 'Benibae' are HF No. 9 ('Hayashi' Satsuma mandarin × 'Fukuhara' orange) and 'Encore', the high content of PMFs in 'Benibae' may be derived from 'Encore', which accumulated 3.73 $mg \cdot g^{-1}$ DW of nobiletin in its 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. 2A, B). The flavedos also had more nobiletin than tangeretin, except for 'Amanatsu' (Fig. 2C, D). These results indicated that nobiletin tended to accumulate at higher levels than tangeretin and that there was a significant correlation between the accumulation of nobiletin and tangeretin in leaves ($r = 0.978$, $P < 0.000001$; Fig. S1A) and flavedos ($r = 0.913$, $P < 0.0001$; Fig. S1B) in the citrus cultivars 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 may be due to the fact that nobiletin has six methoxy groups and tangeretin has five, and so the structural difference is only one methoxy group (Fig. 1), suggesting that their biosynthetic pathways are 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. 3). Similarly, tangeretin content was shown to be positively correlated in the leaves and flavedos (data not shown). These results suggest that a set of genes that synthesize PMFs is present in both leaves and flavedos. In a 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 PMFs in fruit, resulting in a shortening of the breeding period.

The OMT multigene family has 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 (Wang et al., 2018; Zhou et al., 2006). Recently, five *OMT* genes for methylation of flavones were isolated from Shiikuwasha (Itoh et al., 2016). In this study, we identified 16 putative *OMT* genes 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 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'-positions of flavonols and in their biosynthesis (Cacace et al., 2003). In peppermint, *Mp8-OMT* (*Mp*OMT2) was 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 may be involved in the biosynthesis of nobiletin and tangeretin, which are methylated at C7 and C8, respectively (Fig. 1). Especially, CreOMT1 and CreOMT4 may 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. 5). Basically, consensus sequence motifs I to IV are characteristic of SAM-dependent *O*-methyltransferase, which transfers a methyl group from SAM to a hydroxy group; 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* could be expected to be involved in the biosynthesis of PMFs in citrus.

We investigated the expression of *CreOMT1*, *CreOMT2*, and *CreOMT4* in the leaves of nine citrus cultivars by semi-qRT-PCR. The results demonstrated that *CreOMT1* and *CreOMT4* were expressed in 'Benibae', 'Hirakishu', 'Ota' Ponkan, 'Ogimi kuganii', and 'Amanatsu' (Fig. S2). This result reflected the accumulations of nobiletin and tangeretin in the leaves of those cultivars, except for 'Amanatsu'. In contrast, no *CreOMT2* expression was detected in any of the investigated cultivars, suggesting that *CreOMT2* may not be involved in the biosynthesis of PMFs in the leaves of citrus cultivars. Therefore, we selected two *OMT* genes, *CreOMT1* and *CreOMT4*, for further expression analysis of 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 for the transcripts of *CreOMT1* and *CreOMT4* in leaves to be much higher than those in flavedos in the same cultivars (Fig. 6). These results suggest differences in the state of the RNA of evergreen leaves and senescent flavedos (exocarps) in the matura-

tion season. Flavedos almost cease growing in January, and RNA synthesis is not thought to be very active. Because we confirmed seasonal changes in the accumulation of PMFs 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 PMFs may vary with developmental stage, although sufficient PMFs had accumulated in the tissues.

The transcripts of *CreOMT1* and *CreOMT4* were associated with the accumulation of PMFs in leaves and flavedos of the cultivars studied (Figs. 2 and 6). The expression of *CreOMT1* and *CreOMT4* tended to be more related to the accumulation of nobiletin in flavedos than in leaves (Fig. 7). Notably, there was a significant correlation ($r = 0.700$, $P = 0.0243$) between the *CreOMT1* expression and the nobiletin content in flavedos (Fig. 7B). In leaves, however, *CreOMT1* was expressed relatively highly in 'Amanatsu', which accumulated a lower level of PMFs, to the same degree as in 'Ogimi kuganii', which had a higher level of PMFs. 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 PMFs 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' may have inherited a part of the gene sets related to the biosynthesis of PMFs from a PMF-accumulating cultivar, Kishu-mikan (*C. kinokuni* hort. ex Tanaka), which could be a pollen parent of 'Amanatsu' (Shimizu et al., 2016). In 'Shiranuhi', on the other hand, the higher expression of those *OMT* genes may 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*.

In this study, we clarified the accumulation of two PMFs, nobiletin and tangeretin, in the leaves and flavedos of various citrus cultivars grown in Japan and found a positive correlation in the accumulation of PMFs between leaves and flavedos. Based on these results, the concentration of PMFs in leaves could be utilized as an early selection marker for seedlings in the juvenile phase as these are expected to accumulate a higher amount of PMFs in fruit, resulting in a shortening of the breeding period. In addition, two candidate genes responsible for *O*-methylation in the biosynthesis of PMFs were identified. Notably, the expression of *CreOMT1* showed a significant correlation with the accumulation of nobiletin in the citrus cultivars used in this study. Our results will be useful in breeding new cultivars that accumulate higher levels of PMFs and also for elucidating the PMF biosynthetic pathway in citrus in the future.

Acknowledgements

We are grateful to the staff of Analytical Research Center for Experimental Sciences, Saga University, for technical assistance.

Literature Cited

Berim, A. and D. R. Gang. 2016. Methoxylated flavones: occurrence, importance, biosynthesis. Phytochem. Rev. 15: 363–390.

Braddock, R. J. 1999. Handbook of citrus by-products and processing technology. John Wiley & Sons, Inc., New York.

Braidy, N., S. Behzad, S. Habtemariam, T. Ahmed, M. Daglia, S. M. Nabavi, E. Sobarzo-Sanchez and S. F. Nabavi. 2017. Neuroprotective effects of citrus fruit-derived flavonoids, nobiletin and tangeretin in Alzheimer's and Parkinson's disease. CNS Neurol. Disord. Drug Targets 16: 387–397.

Byeon, Y., G.-H. Choi, H. Y. Lee and K. Back. 2015. Melatonin biosynthesis requires *N*-acetylserotonin methyltransferase activity of caffeic acid *O*-methyltransferase in rice. J. Exp. Bot. 66: 6917–6925.

Cacace, S., G. Schröder, E. Wehinger, D. Strack, J. Schmidt and J. Schröder. 2003. A flavonol *O*-methyltransferase from *Catharanthus roseus* performing two sequential methylations. Phytochemistry 62: 127–137.

Chidley, H. G., P. S. Oak, A. B. Deshpande, K. H. Pujari, A. P. Giri and V. S. Gupta. 2016. Molecular cloning and characterization of *O*-methyltransferase from mango fruit (*Mangifera indica* cv. Alphonso). Mol. Biotechnol. 58: 340–350.

Choi, S.-Y., H.-C. Ko, S.-Y. Ko, J.-H. Hwang, J.-G. Park, S.-H. Kang, S.-H. Han, S.-H. Yun and S.-J. Kim. 2007. Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits. Biol. Pharm. Bull. 30: 772–778.

Christensen, A. B., P. L. Gregersen, C. E. Olsen and D. B. Collinge. 1998. A flavonoid 7-*O*-methyltransferase is expressed in barley leaves in response to pathogen attack. Plant Mol. Biol. 36: 219–227.

Gana, R., S. Rao, H. Huang, C. Wu and S. Vasudevan. 2013. Structural and functional studies of S-adenosyl-L-methionine binding proteins: a ligand-centric approach. BMC Struct. Biol. 13: 6. DOI: 10.1186/1472-6807-13-6.

Gauthier, A., P. J. Gulick and R. K. Ibrahim. 1995. The electronic plant gene register: A flavonol 3'/5'-O-methyltransferase cDNA clone (GenBank U16794) from *Chrysosplenium*. Plant Physiol. 108: 1341.

Ho, S.-C. and C.-T. Kuo. 2014. Hesperidin, nobiletin and tangeretin are collectively responsible for the anti-neuroinflammatory capacity of tangerine peel (*Citri reticulatae* pericarpium). Food Chem. Toxicol. 71: 176–182.

Ibrahim, R. K., A. Bruneau and B. Bantignies. 1998. Plant *O*-methyltransferases: molecular analysis, common signature and classification. Plant Mol. Biol. 36: 1–10.

Inafuku-Teramoto, S., R. Suwa, Y. Fukuzawa and Y. Kawamitsu. 2011. Polymethoxyflavones, synephrine and volatile constitution of peels of citrus fruit grown in Okinawa. J. Japan. Soc. Hort. Sci. 80: 214–224.

Itoh, N., C. Iwata and H. Toda. 2016. Molecular cloning and characterization of a flavonoid-*O*-methyltransferase with broad substrate specificity and regioselectivity from *Citrus depressa*. BMC Plant Biol. 16: 180. DOI: 10.1186/s12870-016-0870-9.

Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins and T. J. Gibson. 1998. Multiple sequence alignment with

Clustal X. Trends in Biochem. Sci. 23: 403–405.
Kawaii, S., Y. Tomono, E. Katase, K. Ogawa and M. Yano. 1999. Antiproliferative activity of flavonoids on several cancer cell lines. Biosci. Biotechnol. Biochem. 63: 896–899.
Ke, Z., Y. Yang, S. Tan and Z. Zhou. 2017. Characterization of polymethoxylated flavonoids in the peels of Chinese wild mandarin (*Citrus reticulata* Blanco) by UPLC-Q-TOF-MS/MS. Food Anal. Methods 10: 1328–1338.
Kefford, J. F. and B. V. Chandler. 1970. The chemical constituents of citrus fruits. In: C. O. Chichester, E. M. Mrak and G. F. Stewart (eds.). Advances in food research, Supplement 2. Academic Press, New York and London.
Kim, B.-G., Y. Lee, H.-G. Hur, Y. Lim and J.-H. Ahn. 2006. Flavonoid 3'-*O*-methyltransferase from rice: cDNA cloning, characterization and functional expression. Phytochemistry 67: 387–394.
Kimura, J., K. Shimizu, K. Kajima, A. Yokosuka, Y. Mimaki, N. Oku and Y. Ohizumi. 2018. Nobiletin reduces intracellular and extracellular *β*-amyloid in iPS cell-derived Alzheimer's disease model neurons. Biol. Pharm. Bull. 41: 451–457.
Kotoda, N., A. Fujisawa, M. Yamaguchi, Y. Tominaga and A. Noutomi. 2017. Accumulation of polymethoxyflavones in Ponkan. Mem. Japan Soc. Hort. Sci. Kyushu Br. 25: 70 (In Japanese).
Kotoda, N., S. Matsuo, I. Honda, K. Yano and T. Shimizu. 2016. Isolation and functional analysis of two gibberellin 20-oxidase genes from Satsuma mandarin (*Citrus unshiu* Marc.). Hort. J. 85: 128–140.
Lee, Y.-S., B.-Y. Cha, S.-S. Choi, B.-K. Choi, T. Yonezawa, T. Teruya, K. Nagai and J.-T. Woo. 2013. Nobiletin improves obesity and insulin resistance in high-fat diet-induced obese mice. J. Nutr. Biochem. 24: 156–162.
Lee, Y.-S., B.-Y. Cha, K. Saito, H. Yamakawa, S.-S. Choi, K. Yamaguchi, T. Yonezawa, T. Teruya, K. Nagai and J.-T. Woo. 2010. Nobiletin improves hyperglycemia and insulin resistance in obese diabetic *ob*/*ob* mice. Biochem. Pharmacol. 79: 1674–1683.
Li, H. M., D. Rotter, T. G. Hartman, F. E. Pak, D. Havkin-Frenkel and F. C. Belanger. 2006. Evolution of novel *O*-methyltransferases from the *Vanilla planifolia* caffeic acid *O*-methyltransferase. Plant Mol. Biol. 61: 537–552.
Li, S., M.-H. Pan, C.-Y. Lo, D. Tan, Y. Wang, F. Shahidi and C.-T. Ho. 2009. Chemistry and health effects of polymethoxyflavones and hydroxylated polymethoxyflavones. J. Func. Foods 1: 2–12.
Liu, X., Y. Luo, H. Wu, W. Xi, J. Yu, Q. Zhang and Z. Zhou. 2016. Systematic analysis of *O*-methyltransferase gene family and identification of potential members involved in the formation of *O*-methylated flavonoids in *Citrus*. Gene 575: 458–472.
Lv, X., S. Zhao, Z. Ning, H. Zeng, Y. Shu, O. Tao, C. Xiao, C. Lu and Y. Liu. 2015. *Citrus* fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chem. Cent. J. 9: 68. DOI: 10.1186/s13065-015-0145-9.
Manthey, J. A. and K. Grohmann. 2001. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. J. Agric. Food Chem. 49: 3268–3273.
Matsumoto, R. 2001. 'Shiranuhi,' a late-maturing citrus cultivar. Bull. Natl. Inst. Fruit Tree Sci. 35: 115–120 (In Japanese with English abstract).
Maxwell, C. A., M. J. Harrison and R. A. Dixon. 1993. Molecular characterization and expression of alfalfa isoliquiritigenin 2'-*O*-methyltransferase, an enzyme specifically involved in the biosynthesis of an inducer of *Rhizobium meliloti* nodulation genes. Plant J. 4: 971–981.
Minagawa, A., Y. Otani, T. Kubota, N. Wada, T. Furukawa, K. Kumai, K. Kameyama, Y. Okada, M. Fujii, M. Yano, T. Sato, A. Ito and M. Kitajima. 2001. The citrus flavonoid, nobiletin, inhibits peritoneal dissemination of human gastric carcinoma in SCID mice. Jpn. J. Cancer Res. 92: 1322–1328.
Miyata, Y., T. Sato, K. Imada, A. Dobashi, M. Yano and A. Ito. 2008. A citrus polymethoxyflavonoid, nobiletin, is a novel MEK inhibitor that exhibits antitumor metastasis in human fibrosarcoma HT-1080 cells. Biochem. Biophys. Res. Commun. 366: 168–173.
Nogata, Y., K. Sakamoto, H. Shiratsuchi, T. Ishii, M. Yano and H. Ohta. 2006. Flavonoid composition of fruit tissues of citrus species. Biosci. Biotechnol. Biochem. 70: 178–192.
Onda, K., N. Horike, T. Suzuki and T. Hirano. 2013. Polymethoxyflavonoids tangeretin and nobiletin increase glucose uptake in murine adipocytes. Phytother. Res. 27: 312–316.
Onozuka, H., A. Nakajima, K. Matsuzaki, R.-W. Shin, K. Ogino, D. Saigusa, N. Tetsu, A. Yokosuka, Y. Sashida, Y. Mimaki, T. Yamakuni and Y. Ohizumi. 2008. Nobiletin, a citrus flavonoid, improves memory impairment and A*β* pathology in a transgenic mouse model of Alzheimer's disease. J. Pharmacol. Exp. Ther. 326: 739–744.
Patil, B. S., J. S. Brodbelt, E. G. Miller and N. D. Turner. 2006. Potential health benefits of citrus: An overview. p. 1–16. In: B. S. Patil, J. S. Brodbelt, E. G. Miller and N. D. Turner (eds.). Potential health benefits of citrus. ACS Symp. Series 936. ACS, Washington, D.C.
Patnayak, K. C., S. Rangaswami and T. R. Seshadri. 1942. Chemical investigation of Indian fruits. Part III. A study of the characteristic crystalline components of certain citrus fruits (oranges of the circars). Proc. Indian Acad. Sci. Section A 16: 10–15.
Rafiq, S., R. Kaul, S. A. Sofi, N. Bashir, F. Nazir and G. A. Nayik. 2018. Citrus peel as a source of functional ingredient: A review. J. Saudi Soc. Agri. Sci. 17: 351–358.
R Core Team. 2015. R: A language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria. <http://www.R-project.org/>.
Roje, S. 2006. S-adenosyl-L-methionine: Beyond the universal methyl group donor. Phytochemistry 67: 1686–1698.
Schröder, G., E. Wehinger, R. Lukačin, F. Wellmann, W. Seefelder, W. Schwab and J. Schröder. 2004. Flavonoid methylation: a novel 4'-*O*-methyltransferase from *Catharanthus roseus*, and evidence that partially methylated flavanones are substrates of four different flavonoid dioxygenases. Phytochemistry 65: 1085–1094.
Shimizu, T., A. Kitajima, K. Nonaka, T. Yoshioka, S. Ohta, S. Goto, A. Toyoda, A. Fujiyama, T. Mochizuki, H. Nagasaki, E. Kaminuma and Y. Nakamura. 2016. Hybrid origins of citrus varieties inferred from DNA marker analysis of nuclear and organelle genomes. PLoS ONE 11(11): e0166969. DOI: 10.1371/journal.pone.0166969.
Struck, A. W., M. L. Thompson, L. S. Wong and J. Micklefield. 2012. S-adenosyl-methionine dependent methyltransferases: highly versatile enzymes in biocatalysis, biosynthesis and other biotechnological applications. Chembiochem 13: 2642–2655.
Surichan, S., R. R. Arroo, K. Ruparelia, A. M. Tsatsakis and V. P. Androutsopoulos. 2018. Nobiletin bioactivation in MDA-MB-468 breast cancer cells by cytochrome P450 CYP1 enzymes. Food Chem. Toxicol. 113: 228–235.
Tripoli, E., M. L. Guardia, S. Giammanco, D. D. Majo and M.

Giammanco. 2007. *Citrus* flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chem. 104: 466–479.

Vidgren, J., L. A. Svensson and A. Liljas. 1994. Crystal structure of catechol *O*-methyltransferase. Nature 368: 354–358.

Wang, L., J. Wang, L. Fang, Z. Zheng, D. Zhi, S. Wang, S. Li, C.-T. Ho and H. Zhao. 2014. Anticancer activities of citrus peel polymethoxyflavones related to angiogenesis and others. Biomed. Res. Int. 2014: 453972. DOI: 10.1155/2014/453972.

Wang, M., X. Zhu, K. Wang, C. Lu, M. Luo, T. Shan and Z. Zhang. 2018. A wheat caffeic acid 3-O-methyltransferase TaCOMT-3D positively contributes to both resistance to sharp eyespot disease and stem mechanical strength. Sci. Rep. 8: 6543. DOI: 10.1038/s41598-018-24884-0.

Willits, M. G., M. Giovanni, R. T. N. Prata, C. M. Kramer, V. De Luca, J. C. Steffens and G. Graser. 2004. Bio-fermentation of modified flavonoids: an example of in vivo diversification of secondary metabolites. Phytochemistry 65: 31–41.

Yamaguchi, M., T. Noda, A. Noutomi, H. Takeshita, Y. Tanaka, and N. Kotoda. 2015. Accumulation of polymethoxyflavones in shekwasha 'Katsuyama kugani'. Hort. Res. (Japan) 14 (Suppl. 2): 107 (In Japanese).

Yamamoto, M., N. Nishiguchi, A. Shimada and R. Matsumoto. 2019. Polymethoxylated flavone content of major cultivars and local accessions of citrus cultivated in Kagoshima, Japan. Hort. J. 88: 320–328.

Zhang, H., J. Wang and H. M. Goodman. 1997. An *Arabidopsis* gene encoding a putative 14-3-3-interacting protein, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase. Biochim. Biophys. Acta 1353: 199–202.

Zhou, J.-M., Y. Fukushi, E. Wollenweber and R. K. Ibrahim. 2008. Characterization of two *O*-methyltransferase-like genes in barley and maize. Pharm. Biol. 46: 26–34.

Zhou, J.-M., N. D. Gold, V. J. J. Martin, E. Wollenweber and R. K. Ibrahim. 2006. Sequential *O*-methylation of tricetin by a single gene product in wheat. Biochim. Biophys. Acta 1760: 1115–1124.