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

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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 of citrus fruits 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;
Nobiletin and tangeretin are two major PMFs that are particularly abundant in citrus peel (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., 2017; Kimura et al., 2018; Onozuka et al., 2008).

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 Shikuwasha (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’ (‘Tsonozozomi’ × ‘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-methylation 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).

**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

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**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 | Ciclev10017524m |
| CcOMT9 | Ciclev10015993m |
| CcOMT10 | Ciclev10017872m |
| CcOMT11 | Ciclev10017930m |
| CcOMT12 | Ciclev10018226m |
| CcOMT13 | Ciclev10015685m |
| CcOMT14 | Ciclev10017683m |
| CcOMT15 | Ciclev10017585m |
| CcOMT16 | Ciclev10017649m |
Hordeum vulgare (CA454616), corn [Zea mays (DR811764)], Madagascar periwinkle [Catharanthus roseus (AY127568)], mango [Mangifera indica (KP992176)], 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 micro-liter 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 kugani’, ‘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 kugani’, 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 kugani’, ‘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 kugani’, 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 kugani’, ‘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. 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 \[Mf4’-OMT (KP993796), peppermint \[Mp3’-OMT (AY337458), Mp7-OMT (AY337458), Mp7-OMT1 (AY337458), Mp8-OMT (AY337458), rice \[Os3’-OMT1 (DQ288259), Os3’-OMT2 (DQ530257), Shiikuwasha \[CdFOMT3 (LC126059), vanilla \[VanOMT2 (DQ400399), VanOMT3 (DQ400400), and wheat \[Ta3’4’-OMT (DQ223971), together with 16 CreOMT candidate genes from Clementin and three CreOMT genes \[CreOMT1 (LC507211), CreOMT2 (LC507212), CreOMT4 (LC507213)] isolated from ‘Yoshida’ Ponkan.](image-url)
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’. CreOMT4 was expressed in ‘Benibae’, ‘Mihaya’, ‘Amanatsu’, and ‘Kiyomi’, and ‘Seinannohikari’, ‘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. 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 (CAA4616)], 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. 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).
The OMT multigene family has been identified in many plant species, including alfalfa (Maxwell et al., 1993), American golden saxifrage (Gauthier et al., 1998), barley (Christensen et al., 1998; Zhou et al., 2008), Madagascar periwinkle (Cacace et al., 2003), corn (Zhou et al., 2004), 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 (CreOMT2)* 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 (MpOMT2)* 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 kugani’, 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 mature-
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 (Citrus × hystrix DC. ‘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.

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