RESEARCH PAPER

Characterization of a caffeoyl-CoA O-methyltransferase-like enzyme involved in biosynthesis of polymethoxylated flavones in Citrus reticulata

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Abstract

Polymethoxylated flavones (PMFs), which accumulate exclusively in fruit peel of citrus, play important physiological and pharmacological roles but the genetic basis for the methylation of flavonoids has not been fully elucidated in citrus. Here we characterize a caffeoyl-CoA O-methyltransferase-like enzyme, designated CrOMT1. The expression pattern of CrOMT1 was highly correlated with the concentration of the three major PMFs in two different citrus fruit tissues during fruit maturation. Exposure of fruit to UV-B radiation sharply increased the level of CrOMT1 transcripts and also led to the accumulation of three PMFs. The potential role of CrOMT1 was studied by testing the catalytic activity of recombinant CrOMT1 with numerous possible substrates in vitro. The enzyme could most efficiently methylate flavones with neighboring hydroxy moieties, with high catalytic efficiencies found with 6-OH- and 8-OH-containing compounds, preferences that correspond precisely with the essential methylation sites involved in the synthesis of the three naturally occurring PMFs in Citrus reticulata. This indicates that CrOMT1 is capable of in vitro methylation reactions required to synthesize PMFs in vivo. Furthermore, transient overexpression of CrOMT1 increased levels of the three major PMFs in fruit, indicating that CrOMT1 is likely to play an essential role in the biosynthesis of PMFs in citrus.

Keywords: Citrus, flavonoids, fruit development, nobiletin, O-methyltransferase, polymethoxylated flavones.

Introduction

Flavonoids are a large class of polyphenols ubiquitous in plants. With various modifications (hydroxylation, acylation, glycosylation, methylation, etc.) to the basic skeleton of C₆–C₃–C₆, flavonoids are generally categorized into six groups, namely flavonols, flavones, dihydroflavonols, dihydroflavones, isoflavones, and anthocyanins. Flavonoids with different
structures contribute to pigment formation (Winkel-Shirley, 2001) and phytohormone signaling (Brunetti et al., 2018) in plants, and they also play vital roles in defense against various biotic and abiotic stresses (Winkel-Shirley, 2001; Samanta et al., 2011; Moore et al., 2014; Nakabayashi et al., 2014; Schulz et al., 2015), including defense against herbivores and microorganisms, tolerance against drought and freezing, along with protection against UV-B irradiation (Li et al., 2013). The position of the flavonoid B-ring in vitro active against the meta-position of the flavonoid B-ring in Vanilla planifolia (Widiez et al., 2001; Landry et al., 1995; Rozema et al., 1997; Falcone Ferreyra et al., 2010). Flavonoids have also gained increasing attention in recent years due to their beneficial effects on human health (Chen and Chen, 2013). It has been demonstrated that methylated flavonoids have greater biological activity than those that are unmethylated because methylation enhances their stability and oral bioavailability (Koirala et al., 2016). Polymethoxylated flavones (PMFs) are enriched in the flavedo of citrus fruit, and the three major PMFs nobiletin, tangeretin, and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (5-HPMF) have received increasing attention. These PMFs in citrus have been demonstrated to have potent effects as antioxidants, and as anti-cardiovascular disease and anti-cancer agents, and they also serve as nutraceutical supplements in the regulation of metabolic syndrome (Koirala et al., 2016; Gao et al., 2018). In addition to their pharmacological effects, PMFs are involved in plant defense against several important pathogens, including those causing common diseases in citrus: green mold and Huanglongbing (Berim and Gang, 2016). It is important to explore the biosynthesis of PMFs in citrus given their important physiological and pharmacological roles.

The hydroxy groups of flavonoids are methylated by S-adenosyl-l-methionine (SAM)-dependent O-methyltransferases (OMTs), which are critical enzymes for synthesis of PMFs. Plant OMTs are generally divided into two major subfamilies: the caffeic acid OMT (COMT) subfamily and the caffeoyl-CoA OMT (CCoAOMT) subfamily (Joshi and Chiang, 1998), based on the molecular weight and cation dependency of the proteins. Members of the COMT subfamily do not require cations to function and have a molecular weight ranging from 40 kDa to 43 kDa. Most OMTs involved in the methylation of flavonoids have been demonstrated to belong to this subfamily. Members of the CCoAOMT subfamily are smaller in size (26–30 kDa) and are usually cation dependent. CCoAOMT enzymes have been thought to use caffeoyl-CoA as a substrate and play important roles in the pathway of lignin biosynthesis (Kim et al., 2010). However, a new Mg²⁺-dependent OMT with high sequence similarities to that of CCoAOMTs was identified from Mesembryanthemum crystallinum (Ibdah et al., 2003). The enzyme, along with two highly homologous OMTs, exhibited substrate preferences for phenylpropanoids such as caffeic acid esters and flavonoids, and are clearly distinct from other CCoAOMTs, forming a new subgroup of CCoAOMT enzymes, designated PFOMT or CCoAOMT-like. Subsequently, several CCoAOMT-like proteins capable of methylating flavonoids were identified from different species, such as OsOMT15 and OsOMT17 from Oryza sativa subsp. japonica (Lee et al., 2008) together with VpOMT4 and VpOMT5 from Vanilla planifolia (Widiez et al., 2011), which are active against the meta-position of the flavonoid B-ring in vitro.

Recombinant ObPFOMT-1 from Ocimum basilicum was shown to be a promiscuous enzyme with a preference for flavones favoring the 8-OH more than the 3'-OH (Berim and Gang, 2013). AtCCoAOMT7 identified from Arabidopsis thaliana displayed unusual para-methylation activity, compared with other reported CCoAOMTs. The recombinant enzyme methylated flavones and flavonols in the meta-position, while it methylated dihydroflavonones and dihydroflavonols in the para-position, and the role of a single amino acid required for the conversion of para to meta was verified by incubating substrates with the CCoAOMT7 wild type and CCoAOMT7 single mutant, respectively (Wils et al., 2013). In addition, CCoAOMT-like enzymes involved in the methylation of anthocyanins have been functionally characterized in vivo and vitro, including VvAOMT from Vitis vinifera (Hugueney et al., 2009), SlAnthOMT from Solanum lycopersicum (Gomez Roldan et al., 2014), and PsAOMT from Paeonia suffruticosa cv. ‘Gunpohden’ (Du et al., 2015). The characterization of the above CCoAOMT-like enzymes indicated that this newly proposed subgroup involved in flavonoid methylation was likely to be present in many other species, making it of considerable importance to undertake further studies of this small group of plant enzymes, given their vital role in flavonoid methylation.

OMTs involved in flavonoid methylation have been functionally characterized from various plant species (Berim and Gang, 2016), including Arabidopsis, tomato, sweet basil, and peppermint. Recently, OMTs from citrus have been investigated and in silico analysis revealed 58 OMT genes in sweet orange (Liu et al., 2016). Nobiletin and tangeretin are examples of methylated flavonoids with significant bioactivity found exclusively in citrus species. Phylogenetic analysis with functionally characterized OMTs from other plants suggested that 27 of these genes could be involved in O-methylation of flavonoids in citrus, and their expression profiles were analyzed in different tissues and fruit developmental stages (Liu et al., 2016). Five OMT genes belonging to the COMT subfamily were isolated from Citrus depressa and one, termed CdOMT5, was successfully expressed in Escherichia coli (Itoh et al., 2016). Recombinant CdOMT5 exhibited broad substrate regioselectivity for the 3, 5, 6, and 7 positions of flavones, and bioconversion of quercetin into its 3,3',5,7 tetra-methyl ether was accomplished in E. coli overexpressing CdOMT5. This multifunctional property implied the possible role of the enzyme in the biosynthesis of PMFs. The expression patterns of this gene and the catalytic activity of the protein with different substrates need to be analyzed to confirm this hypothesis.

In this study, we describe the identification and biochemical characterization of CrOMT1, a CCoAOMT-like enzyme involved in the biosynthesis of PMFs in citrus, its expression in the peel of ‘Ougan’ fruit (Citrus reticulata cv. ‘Suavissima’), and the correlation between its expression profile and PMF accumulation. Recombinant CrOMT1 shows a remarkable preference for analogs of potential PMF-type substrates, and transient overexpression assays in the peel of citrus confirmed the role of the enzyme in enhancing accumulation of PMFs. This is the first identification of a citrus CCoAOMT-like enzyme responsible for PMF biosynthesis both in vitro and vivo, and opens the way for investigation of biosynthesis of PMFs in citrus.
Materials and methods

**Chemicals sources**

Acetonitrile and methanol of HPLC grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin 3-methyl ether, eriodictyol, apigenin, and formic acid for HPLC were obtained from Aladdin (Shanghai, China). Myricetin, apigenin, and 7,8-dihydroxyflavone hydrate were purchased from TCI (Shanghai, China). Caffeic acid, naringenin, hesperetin, neoerectin, and ferric acid were also purchased from Sigma-Aldrich.

**Plant materials and treatment**

Fruits of the ‘Ougan’ cultivar (Citrus reticulata cv. Suavissima) were sampled from Wenzhou City, China, at eight progressive maturation stages: S1, 30 days after flowering (DAF); S2, 60 DAF; S3, 80 DAF; S4, 100 DAF; S5, 120 DAF; S6, 140 DAF; S7, 170 DAF; and S8, 200 DAF. Samples of ‘Ougan’ fruit developmental stages and UV-B treatments were frozen immediately in liquid nitrogen and stored at –80°C for subsequent analysis.

UV-B treatments were irradiated by UV-B light and the unexposed side were set as the control group. The fruit samples were separated into four biological replicates, and the peel was divided into flavedo and albedo parts (albedo could be separated from the peel at S5, S6, S7, and S8). ‘Ougan’ fruits at S7 were also subjected to UV light irradiation. A total of five replicates were performed in UV-B treatment. For each fruit, the side irradiated by UV-B light and the unexposed side were set as the treatment group and control group, respectively. The procedure was carried out with UV-B light and the unexposed side were set as the treatment group and control group, respectively. The procedure was carried out with UV-B light and the unexposed side were set as the treatment group and control group, respectively.

**RNA isolation, RNA sequencing, and qRT-PCR**

Total RNA of samples (‘Ougan’ developmental stages and UV-B treatment) was extracted according to the method described by Kiefer et al. (2000, and treated with gDNA Eraser to remove genomic DNA (gDNA) contamination via a PrimeScript™ RT reagent Kit (Takara, Dalian, China). RNA sequencing (RNA-Seq) was performed to analyze the expression pattern of OMT genes in flavedo of fruit peel during ‘Ougan’ developmental stages (S1, S3, S5, and S7) using an Illumina HiSeq sequencer (Shanghai Majorbio Bio-pharm Technology, Ltd, Shanghai, China). For RNA samples extracted from fruit flavedo after UV-B treatment, RNA-Seq (Quantification) was also conducted using a BGISEQ-500RS sequencer (Shenzhen Huada Gene Science and Technology Service Co., Ltd, Shenzhen, China). The gene expression level determined by RNA-Seq was evaluated by FPKM (fragments per kilobase of exon per million fragments mapped) values. All the reads were aligned to the Citrus clementina reference genome (http://www.citrusgenomedb.org/species/clementina/genome1.0) (Wu et al., 2014).

Real-time quantitative reverse transcription–PCR (qRT-PCR) was performed to verify the temporal and spatial expression pattern of CrOMT1 in ‘Ougan’ fruit. Briefly, total RNA was extracted and used to synthesize cDNA as described above. Gene-specific oligonucleotide primers were designed by NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with their gene specificity checked by melting curve and PCR re-sequencing. Citrus β-actin served as a housekeeping gene for normalization (Pilletteri et al., 2004) and ΔCt was used to calculate the relative abundance of gene transcripts. Real-time PCRs were performed as described before (Liu et al., 2017). Gene-specific primers mentioned above are listed in Supplementary Table S1 at JXB online.

**Phylogenetic analysis and sequence alignment**

The deduced amino acid sequences of 55 identified OMT genes expressed in the flavedo of ‘Ougan’ fruit were aligned with various currently known plant OMT members, including the COMT subfamily and CCoAOMT subfamily using the ClustalW bundled in MEGA X software (Mega Software, State College, PA, USA). Construction of an unrooted phylogenetic tree was based on the Neighbor-Joining statistical method (Saitou and Nei, 1987) and was tested with 1000 bootstrap replicates (Felsenstein, 1985). rTOL software (https://rtol.embl.de/) was used to display and annotate the phylogenetic tree. UniProt entries of proteins from other species used above are given in Supplementary Table S2.

**Isolation, cloning, and heterologous expression of CrOMT1**

The CrOMT1 gene was isolated based on the C. clementina reference genome. The full coding sequence (CDS) of CrOMT1 was amplified using the primers described in Supplementary Table S1 and then subcloned into the pET32a vector (without a stop codon) followed by transfer to E. coli strain BL21 (DE3) pLysS (Promega, Madison, WI, USA) for expression. Transformants carrying the expression plasmid pET-CrOMT1-PET were incubated to OD₆₀₀=0.6 in Luria–Bertani medium containing 0.1 g L⁻¹ ampicillin at 37 °C, followed by the addition of isopropyl-β-d-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and then the incubation was carried out for 24 h at 18 °C. After induction, the bacterial cells were collected (4000 rpm, 4 °C, 10 min) and resuspended in 1× PBS buffer. The clarified supernatant of cells disrupted by sonication was obtained by centrifugation at 4 °C and the corresponding recombinant proteins were purified using a HisTALON™ Gravity Columns Purification Kit (Takara, Dalian, China) according to the user manual. Collected fractions were transferred into storage buffer (50 mM Tris–HCl, pH 8.0, 10% glycerol, and 2 mM DTT) through a PD-10 desalting column (GE Healthcare, Uppsala, Sweden), and stored at –80 °C for further analysis. Protein concentrations were determined via the modified BCA Protein Assay Kit (Sangon Biotech, Shanghai, China).

**Enzyme assays and kinetics**

Enzyme reactions for recombinant CrOMT1-PET were performed with different phenolic substrates, including flavonoids and caffeic acid. Reaction mixtures consisted of Tris–HCl buffer (50 mM, pH 8.0), 1 mM SAM, 200 μM phenolic substrates, and 25 μl of purified protein in a final volume of 200 μl. Assays were incubated in 37 °C for 2 h and extracted twice with an equal volume of ethyl acetate. The upper organic phase was dried and resolved in methanol, and then filtered through a 0.22 μm filter for HPLC or LC-MS.

The effect of pH on CrOMT1 was estimated in Tris–HCl buffer for pH 7.0–9.0 and potassium phosphate buffer for pH 5.5–8.0. Temperature ranging from 25 °C to 80 °C was set to determine the optimum temperature for CrOMT1. Both assays were performed with luteolin.

Relative activity of various phenolic substrates was measured using the MalA-Glu™ Methyltransferase Assay (Promega). The product of SAM in a methylation reaction was converted to ADP and then the ADP was converted to ATP, which could be detected by a plate-reading luminometer, and relative luminescence was correlated to SAH (S-adenosylhomocysteine) concentration by generating an SAH standard curve. In detail, assays were processed with phenolic substrate concentration at 12.5 μM, SAM at 250 μM, and appropriate dilutions of the purified enzyme in Tris–HCl buffer (pH 8.0). After 30 min of incubation.
at 37 °C, the reaction was stopped by adding 0.5% TFA (trifluoroacetic acid), followed by estimation of SAH by luminescence.

For kinetic analysis of the recombinant proteins, a serial dilution of phenolic substrates along with 2 mM SAM as methyl donor was used to conduct enzyme assays in the same conditions as mentioned above. The kinetic parameters \( K_m \) and \( V_{max} \) were estimated by non-linear regression curve fit using Michaelis–Menten bundled in GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA).

### Transient overexpression of CrOMT1 in ‘Ougan’ fruit peel

The full-length CDS of CrOMT1 was cloned into the pBII121 expression vector containing the CaMV35S promoter. The recombinant vector was transformed into Agrobacterium (EHA105). CrOMT1 transient overexpression in ‘Ougan’ fruit peel was conducted by Agrobacterium tumefaciens-mediated infiltration as described previously (Yin et al., 2016). Specifically, each fruit was set as a replicate, and seven fruits were used in transient analysis. Two points on the equator face on opposite sides of each fruit were selected, and one was injected with Agrobacterium suspension containing the target gene and the other with corresponding empty vector (pBII121) acting as a control. For each injection site, 1 ml of the corresponding Agrobacterium suspension (OD600=0.75) was used. Contents of PMFs and transcript levels of CrOMT1 were measured 5 d after infiltration by cutting out a region of tissue ~3 cm in diameter surrounding the injection site. Primers used in the construction of CrOMT1-pBII121 and for qRT–PCR, are listed in Supplementary Table S1.

### Metabolite analysis

Determination of flavonoid composition was carried out as described by Zhang et al. (2012) with minor modifications. Different tissues were ground under liquid nitrogen and up to 500 mg of frozen powder was extracted in 3 ml of 80% ethanol with sonication for 30 min; then the supernatants were obtained by centrifugation at 4000 rpm for 10 min. The samples were extracted three times and the supernatant fractions (~9 ml) were combined. The ethanol-based extract was filtered through a 0.22 μm membrane prior to HPLC analysis.

**HPLC, mass spectrometry, and NMR spectroscopy**

For HPLC, an Agilent (Agilent Technologies, Santa Clara, CA, USA) 1260 HPLC system equipped with a quaternary pump and a UV/Vis detector was used in all the chromatographic experiments. Samples were separated at room temperature by a Sunfire C18 ODS column (4.6×250 mm, 5 μm, Waters Corp., Milford, MA, USA), at a flow rate of 1 ml min−1. The mobile phases were acetonitrile (A) and 0.1% formic acid–water (B) with a linear gradient program as follows, 0/20, 5/20, 10/27, 15/27, 25/40, 35/60, 40/80, 42/100, 45/20, and 50/20 (min/A%). The injection volume of samples was 10 μl. Detection was by UV at 280 nm (dihydroflavones, dihydroflavonols, and caffeic acid), 330 nm (nobiletin, tangeretin, and 5-HPMF), and 350 nm (flavones and flavonols), 330 nm (nobiletin, tangeretin, and 5-HPMF). MS analysis was performed using an AB TripleTOF 5600plus System (AB SCIEX, Framingham, MA, USA). MS spectra were obtained in negative ion mode or positive ion mode (ESI). The exact mass calibration was measured automatically before each analysis employing the Automated Calibration Delivery System.

For NMR spectroscopy, a reaction mixture of 7.8-dihydroxyflavone methylated by CrOMT1 was extracted twice with an equal volume of ethyl acetate. The upper organic phase was dried using a rotary evaporator and then resolved in methanol for subsequent separation and collection by means of semi-preparative HPLC. Collected samples were completely dried and then dissolved in DMSO-d6 (bis-trideuteriomethyl sulfonite) (99.9 atoms % D) containing 0.03% TMS (tetramethylsilane). TMS standard was used for standardization of chemical shifts in NMR solvents. Proton (1H) NMR (500 MHz) and carbon (13C) NMR (125 MHz) spectra were recorded on a Bruker Avance III 500M spectrometer (Bruker, Switzerland).

### Statistical analyses

All data were obtained with at least three replicates and are presented as mean values and SEs. Graphics were drawn using OriginPro 2019 Learning Edition (Microcal Software Inc., Northampton, MA, USA) and GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Structural formulae were produced using ChemBioDraw Ultra 12.0 (PerkinElmer Informatics, Waltham, MA, USA).

### Results

**PMF accumulation in ‘Ougan’ peel during fruit maturation and in response to UV-B treatment**

PMFs were investigated in ‘Ougan’ fruit at eight different developmental stages. The peel of ‘Ougan’ fruit, which is known to accumulate abundant contents of PMFs (Wang et al., 2017), was used to determine detailed flavonoid metabolites. The three most abundant PMFs detected were nobiletin, tangeretin, and 5-HPMF according to the retention time of corresponding authentic standards and published literature (Wang et al., 2017). Nobiletin, which has six methoxy groups, was the most abundant PMF detected in citrus peel (Fig. 1). High contents of PMFs were observed in flavedo samples and peaked at S5; while they were barely detected in albedo tissue (Fig. 1A).

Significant increases were observed in the accumulation of nobiletin, tangeretin, and 5-HPMF in ‘Ougan’ fruit flavedo after irradiation for 24 h with UV-B, compared with the control group. This was maintained after irradiation for 48 h, although there was no significant further increase (Fig. 1B).

**Isolation of a candidate OMT cDNA from ‘Ougan’ fruit peel**

To identify putative OMT genes involved in PMF biosynthesis in ‘Ougan’ fruit, expression profiles of OMT genes in the flavedo of ‘Ougan’ fruit during developmental stages (S1, S3, S5, and S7) were obtained from RNA-Seq data. On the basis of gene annotations in the C. dementina reference genome and subsequent conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), a total of 55 putative OMT genes were found to be expressed in the flavedo of ‘Ougan’ fruit during maturation (Supplementary Table S3).

To aid selection of potential OMT genes involved in PMF accumulation, correlation coefficients (Pearson \( r \)) between OMT gene transcript levels and PMF contents were calculated. Only nine of the 55 OMT genes were considered to have the potential to be involved in the synthesis of PMFs during fruit maturation using the following selection criteria: first, a correlation coefficient >0.7 for expression versus concentration of at least one PMF (nobiletin, tangeretin, or 5-HPMF); and, secondly, expression level of PFKM >50 for at least one developmental stage (Supplementary Table S3). Of the nine OMT genes that fulfilled these criteria, only one (Cider10026344m, termed CrOMT1) had the highest transcript levels (~12-fold and ~14-fold higher) in ‘Ougan’ flavedo irradiated by UV-B for 24 h and 48 h, compared with the control group (Fig. 2A). The expression pattern of CrOMT1 in the flavedo and albedo of ‘Ougan’ fruit at eight stages was verified by qRT–PCR.
CrOMT1 was specifically expressed in the flavedo, and the transcript abundance was highest at the S5 stage (relative expression level = 0.95). The transcript levels of CrOMT1 were much lower in the albedo, and the relative expression levels at the S5, S6, S7, and S8 stages were 0.006, 0.009, 0.016, and 0.007, respectively (Fig. 2B). The expression levels of CrOMT1 were highly correlated with the accumulation of PMFs in different developmental stages and tissues (Fig. 2C). These results suggested that CrOMT1 might potentially be involved in the synthesis of PMFs.

**In silico analysis of CrOMT1**

The cDNA encoding CrOMT1 was obtained from a cDNA library of ‘Ougan’ fruit peel using gene-specific primers. The ORF of CrOMT1 was 750 bp, coding for 249 amino acids. The calculated isoelectric point and molecular mass of the predicted amino acid sequence was 5.13 and 28.12 kDa, respectively (ExPASy Compute pI/Mw Tool).

CrOMT1 contained a conserved domain characteristic of the AdoMet_Mtases superfamily (accession: cl17173) and was clustered with members of the CCoAOMT subfamily on the phylogenetic tree (Fig. 3). Generally, members of this family have been demonstrated to be specifically active with phenylpropanoid-CoA esters, and function as key enzymes in the lignin biosynthesis pathway (Kim et al., 2010). However, a new subgroup of this subfamily, named PFOMT or CCoAOMT-like, was proposed recently (Ibdah et al., 2003). PFOMT methylates a wide range of metabolites and phenylpropanoids, with caffeic acid esters and flavonoids as preferred substrates. Consideration of the wide differences in substrate spectrum led to the CCoAOMT subfamily being divided into two subgroups: PFOMTs and true CCoAOMTs (Ibdah et al., 2003). Interestingly, CrOMT1 was clustered close to other PFOMTs (Fig. 3), with >50% amino acid sequence identity to PFOMTs from other plants, with the exception of VpOMT5 from *Vanilla planifolia*, which has a chloroplast transit peptide at the N-terminus (Widiez et al., 2011) (Supplementary Table S4). This close phylogenetic relationship with PFOMTs suggested that CrOMT1 was likely to accept flavonoids as favorable substrates (Fig. 3).

Amino acid sequences of enzymes in the PFOMT subgroup were aligned with several members of the true CCoAOMT subgroup and compared using secondary structural information.
The substrate specificity of the CCoAOMT subfamily is believed to be influenced by two regions: the N-terminus and an insertion loop near the C-terminus (Ibdah et al., 2003; Kopycki et al., 2008) (Supplementary Fig. S1). Two unique Tyr residues, boxed in red in Supplementary Fig. S1, were fully conserved within the true CCoAOMTs, and the two Tyr residues have
been implicated in caffeoyl-CoA binding (Walker et al., 2016). However, they were substituted by distinct residues within the PFOMTs, and in CrOMT1 they were also substituted by Leu and Trp, respectively. This indicated that CrOMT1 was likely to be active on substrates other than caffeoyl-CoA.

Regioselectivity of recombinant CrOMT1

The CrOMT1 enzyme fused with two histidine tags was expressed in E. coli, and the recombinant protein formed a band at ~46 kDa on SDS–PAGE, which is consistent with the predicted mass of 46.89 kDa (Supplementary Fig. S2). In order to explore the substrate specificity of CrOMT1, the recombinant enzyme was tested with numerous phenolic substrates representing three different flavonoid subclasses, flavonols, flavones, and dihydroflavones, and caffeic acid, assayed with SAM as the methyl donor. Identification of methylated products was conducted by retention time and MS/MS data compared with the authentic compound. For the product without commercial standards, methylation sites were inferred from the obtained results by similar substrates, published literature, or NMR spectroscopy. (Table 1; Supplementary Figs S3, S4). For better understanding of the methylation sites for each substrate, schematic diagrams with chemical structures are shown in Supplementary Fig. S5.

The flavonols quercetin, quercetin 3-methyl ether, myricetin, and kaempferol were tested (Fig. 4A–D). Quercetin was converted to its 3’-methyl ether (isorhamnetin), 4’-methyl ether (tamarixetin), and eventually formed the di-methyl ether (quercetin 3’,4’-dimethyl ether), whose structure was deduced from the time-dependent methylation reaction and MS (Fig. 4A; Supplementary Figs S3, S6). The reaction kinetics showed that...
the two mono-methyl ethers could be produced by CitOMT1 in vitro, but the amount of di-methyl ether increased while the two mono-methyl products decreased during extended reaction times (Supplementary Fig. S6). This indicated that quercetin could be converted into the final product quercetin 3',4'-dimethyl ether by CitOMT1 in vitro. To our knowledge, this is the first report that CCoAOMT-like enzyme could sequentially catalyze vicinal hydroxy groups on the B-ring of quercetin. The methylation site was restricted to the 3' position with quercetin 3-methyl ether as substrate, yielding a single product, its 3'-O-methylation product (quercetin 3,3'-dimethyl ether) (Fig. 4B). Three products were obtained with myricetin as substrate. Two were identified as myricetin 3'-methyl ether (laricitrin) and myricetin 3',5'-dimethyl ether (syringetin), and the third peak was myricetin di-methyl ether according to MS (Fig. 4C; Supplementary Fig. S3). Quercetin and myricetin have similar structures (the latter has an additional hydroxy group on the B-ring); therefore, methylation might be expected at the 3' and 4' positions, but so far this has not been confirmed. With kaempferol, which carries a single hydroxy group on the B-ring, only a tiny peak, representing 3-methylated kaempferol (isokaempferide), was detected (Fig. 4D).

Luteolin, tricetin, apigenin, baicalein, and 7,8-dihydroxyflavone, which are classified as flavones, were incubated with recombinant CitOMT1 separately (Fig. 4E–H). Luteolin, which lacks 3-OH compared with quercetin, was converted into a single product, chrysoeriol (luteolin 3'-methyl ether) (Fig. 4E). With tricetin as substrate, four products were produced by CitOMT1. One was identified as tricin (tricetin 3',5'-dimethyl ether) and the other three were two tricetin mono-methyl ethers and one di-methyl ether identified by MS (Fig. 4F; Supplementary Fig. S3). Production of four methylation products is similar to the results obtained with ObPFOMT-1 from Ocimum basilicum, which also yielded four products, two mono-methylated and two di-methylated, when incubated with tricetin (Berim and Gang, 2013). Therefore, the other three products were probably 3'-methyl ether, 4'-methyl ether, and 3',4'-dimethyl ether by comparison with tricetin derivatives produced by ObPFOMT-1. Apigenin, lacking vicinal dihydroxy groups on the B-ring, could not be methylated by the enzyme (Table 1; Supplementary Fig. S5). Baicalein, which has three hydroxy groups in positions 5, 6, and 7 on the A-ring, was converted into its 6-methyl ether (oroxylin A) (Fig. 4G). With 7,8-dihydroxyflavone as substrate, a single product was

Table 1. Compounds identified by LC-MS analysis of reaction products produced by recombinant CitOMT1

| Substrate                  | Retention time (min) | Theoretical [M+H]+ | Product                          | Retention time (min) | Theoretical [M+H]+ | Observed [M+H]+ | Methylated position |
|----------------------------|----------------------|--------------------|----------------------------------|----------------------|--------------------|-----------------|---------------------|
| Flavonoids                 |                      |                    |                                  |                      |                    |                 |                     |
| Quercetin                  | 23.251               | 303.0505           | Isorhamnetin                     | 28.985               | 317.0661           | 317.0644        | 3'                  |
|                            |                      |                    | Tamarixetin                      | 29.208               | 317.0661           | 317.0651        | 4'                  |
|                            |                      |                    | Quercetin 3',4'-dimethyl ether   | 30.303               | 331.0818           | 331.0819        | 3',4'               |
| Quercetin 3-methyl ether   | 25.056               | 315.0505           | Quercetin 3,3'-dimethyl ether    | 30.420               | 329.0661           | 329.0673         | 3'                  |
| Myricetin                  | 15.453               | 319.0454           | Larcitrin                        | 22.765               | 333.0610           | 333.0597        | 3'                  |
|                            |                      |                    | Syringetin                       | 28.189               | 347.0767           | 347.0754        | 3',5'-4'            |
|                            |                      |                    | Myricetin dimethyl ether         | 29.960               | 347.0767           | 347.0749        | 3',4'               |
| Kaempferol                 | 28.497               | 287.0556           | Isokaempferide                   | 29.856               | 301.0712           | 301.0718        | 3'                  |
| Flavones                   |                      |                    |                                  |                      |                    |                 |                     |
| Luteolin                   | 22.967               | 285.0399           | Chrysoeriol                      | 28.319               | 299.0566           | 299.0564        | 3'                  |
| Tricetin                   | 16.739               | 303.0505           | Tricetin mono methyl ether       | 22.735               | 317.0661           | 317.0663        | 3'                  |
|                            |                      |                    | Tricetin mono methyl ether       | 23.193               | 317.0661           | 317.0659        | 4'                  |
|                            |                      |                    | Tricin                           | 27.212               | 331.0818           | 331.0821        | 3',5'-4'            |
|                            |                      |                    | Tricetin dimethyl ether          | 29.279               | 331.0818           | 331.0825        | 3',4'               |
| Apigenin                   | 27.514               | ND                 |                                  |                      | ND                 |                 |                     |
| Baicalein                  | 28.778               | 271.0606           | Oxyrin A                         | 35.379               | 285.0763           | 285.0774        | 6'                  |
| 7,8-Dihydroxyflavone       | 21.403               | 255.0657           | 7-Hydroxy-8-methoxyflavone       | 28.918               | 269.0814           | 269.0806        | 8'                  |
| Dihydroflavones            |                      |                    |                                  |                      |                    |                 |                     |
| Eriodictyol                | 22.044               | 287.0556           | Homoeordictyol                   | 28.072               | 301.0712           | 301.0706        | 3'                  |
|                            |                      |                    | Hesperidin                       | 28.765               | 301.0712           | 301.0722        | 4'                  |
|                            |                      |                    | Homoeordictyol 7-O-rutinoside    | 12.336               | 609.1819           | 609.1848        | 3'                  |
| Eriocitrin                 | 8.004                | 595.1663           | Hesperidin                       | 13.144               | 609.1819           | 609.1847        | 4'                  |
|                            |                      |                    | Hesperidin 7-O-neohesperidioside | 13.240               | 609.1819           | 609.1838        | 3'                  |
| Neoeriocitrin              | 9.517                | 595.1663           | Neoeriocitrin                    | 13.941               | 609.1819           | 609.1845        | 4'                  |
| Naringenin                 | 27.975               | ND                 |                                  |                      | ND                 |                 |                     |
| Hesperetin                 | 29.059               | ND                 |                                  |                      | ND                 |                 |                     |

ND, not detectable; theoretical [M+H]+, exact mass of compound calculated by ChemBioDraw in positive mode; observed [M+H]+, exact mass of compound observed in MS/MS data. Values with a circle represent corresponding exact mass [M-H]– in negative mode. Values with a triangle represent methylation position identified by MS/MS in comparison with that of the authentic standard or by NMR.
Liu et al. identified 7-hydroxy-8-methoxyflavone by MS, ¹H NMR, and ¹³C NMR spectra. (Fig. 4H; Supplementary Figs S3, S4).

With the dihydroflavone eriodictyol as substrate, a 3'-methylation product (homoeriodictyol) and a 4'-methylation product (hesperetin) were both identified; furthermore, the enzyme was also capable of methylating its corresponding glycosides eriocitrin and neoeriocitrin (eriodictyol-7-O-β-rutinoside and eriodictyol-7-O-β-neohesperidoside) (Fig. 4I–K). Methylation of naringenin (which lacks a hydroxyl group in the 3' position compared with eriodictyol) and hesperetin (4'-methylated eriodictyol) by the recombinant enzyme was not observed (Table 1; Supplementary Fig. S5). Caffeic acid also acted as a substrate, and two peaks were detected, which were identified as ferulic acid (its 3-methyl ether) and isoferulic acid (its 4-methyl ether) (Fig. 4L).

**Substrate preference of recombinant CrOMT1**

Recombinant CrOMT1 was tested for its optimum pH and temperature using luteolin as a substrate (Supplementary Fig. S2). The highest reaction rate was at pH 8.0 (in Tris–HCl buffer) and 40 °C, and further tests were carried out at pH 8.0 and 37 °C. Overall, recombinant CrOMT1 exhibited a preference for flavones and dihydroflavones compared with flavonols and dihydroflavonols (Fig. 5A). In order to investigate the phenolic substrate preference of recombinant CrOMT1, kinetic properties were determined in Tris–HCl at pH 8.0 and 37 °C for 30 min and $K_m$ and $K_{cat}$ values were calculated based on non-linear regression curve fitting using Michaelis–Menten (Fig. 5B). $K_{cat}/K_m$ of these substrates tested was also calculated to better evaluate the preferred substrates of CrOMT1, and apparent differences in catalytic efficiency were observed (Fig. 5B). Specifically, CrOMT1 exhibited the best affinity as well as the highest catalytic efficiency for flavones compared with flavonols and dihydroflavonols. The $K_{cat}/K_m$ values of the four flavones, baicalein, tricetin, 7,8-dihydroxyflavone, and luteolin, were 1970.5, 1386.8, 669.4, and 447.7 M⁻¹ s⁻¹, respectively. The catalytic efficiencies of CrOMT1 for quercetin, myricetin, and kaempferol were obviously reduced compared with that of flavones, and the $K_{cat}/K_m$ values of the three flavonols were 212.7, 28.0, and 5.0 M⁻¹ s⁻¹, respectively. Kaempferol, which has no vicinal hydroxy groups, showed the worst affinity ($K_m=248.1$ μM) toward CrOMT1. The very similar structure of quercetin 3-methyl ether to that of luteolin might account for the similar catalytic efficiency of the two substrates. The catalytic efficiencies for the two glycosides of eriodictyol were more than three times higher than that for flavones and dihydroflavones compared with flavonols and dihydroflavonols (Fig. 5A).
of eriodictyol due to the much better affinity of the glycosides than the aglycone. Taken together, flavones with vicinal hydroxy moieties were the most favored substrates for CrOMT1, with the methylation sites towards baicalein, 7,8-dihydroxyflavone, and luteolin at the 6, 8, and 3’ position, respectively, and toward tricetin mostly at the 3’ and 5’ position (Figs 4E–H).
Transient overexpression of CrOMT1 enhanced PMF accumulation in ‘Ougan’ fruit peel

‘Ougan’ fruit peel is known to accumulate abundant PMFs (Wang et al., 2017), and transient overexpression experiments were conducted in order to explore the potential role of CrOMT1 in the methylation of flavonoids in vivo. Both the transcript levels of CrOMT1 and the content of PMFs in ‘Ougan’ peel infiltrated with CrOMT1-pBI121 increased significantly compared with that with empty vector (Fig. 6).

Discussion

In this study, we identified CrOMT1 from citrus as a CCoAOMT-like enzyme that favored flavones with vicinal hydroxy groups in vitro. The accumulation profiles of CrOMT1 transcripts were highly correlated with PMF accumulation during ‘Ougan’ fruit maturation and in response to UV-B irradiation, and transient overexpression of CrOMT1 in the peel of ‘Ougan’ fruit enhanced the accumulation of PMFs.

CrOMT1 is a multifunctional enzyme with a preference for flavones

To test the substrate preferences of CrOMT1, a total of four representative classes of flavonoids and a simple phenolic acid (caffeic acid) were tested in in vitro assays. As found with other functionally characterized PFOMTs (Ibdah et al., 2003; Widiez et al., 2011; Berim and Gang, 2013; Wils et al., 2013), CrOMT1 could efficiently accept a wide range of phenolic acid with vicinal hydroxy groups. Retention of such a promiscuous catalytic function has been suggested to be an evolutionary advantage (Aharoni et al., 2005; Wils et al., 2013).

Relative activities of various substrates were determined and, among the tested substrates, flavone and dihydroflavones appeared to be favored by CrOMT1 (Fig. 5A). CrOMT1 showed the highest catalytic efficiency towards flavones based on subsequent kinetic analysis and preferred methylation at 6-0H and 8-0H on the A-ring to 3'-0H (Fig. 5B). Similarly, flavones were also favored by ObPFOMT-1 from O. basilicum, and methylation at 8-0H was preferred to 3'-0H (Berim and Gang, 2013). ObPFOMT-1, with 60% identity to CrOMT1, has been proposed to be a potential enzyme involved in the biosynthesis of nevadensin, a major methoxylated flavone in some basil cultivars (Berim and Gang, 2013). The dihydroflavone eriodictyol was converted into its 3'- and 4'-methyl ether at a constant ratio of ~20:80 (Fig. 4I), and this methylation pattern towards eriodictyol was identical to that of AtCCoAOMT7 from A. thaliana (Wils et al., 2013). When eriocitrin and neoeriocitrin, two glycosides of eriodictyol ubiquitous in citrus, were tested, CrOMT1 had significantly increased affinity for these substrates (Fig. 5B). Several CCoAOMT-like enzymes involved in methylated flavonoids also displayed much better activity with glycosides compared with the corresponding aglycone (Ibdah et al., 2003; Hugueney et al., 2009; Gomez Roldan et al., 2014; Du et al., 2015). CrOMT1 had lower catalytic activity towards flavonols. The methylation pattern for quercetin and myricetin was different from that of quercetin and myricetin.

Fig. 6. Transient overexpression of CrOMT1 increases PMF content in ‘Ougan’ fruit peel. (A) Expression profiles of CrOMT1 after infiltration with CrOMT1-pBI121. (B) Changes of PMFs after transient overexpression of CrOMT1 in ‘Ougan’ fruit peel. The effect of transient expression of CrOMT1 on the content of the three most abundant PMFs in the peel of ‘Ougan’ fruit was measured after infiltration with CrOMT1-pBI121, with empty vector as control: 5-HPMF, 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone. The content of flavonoids is expressed as mg g⁻¹ FW. Bars indicated in light blue and light green are empty vector and CrOMT1-pBI121, respectively. Values are means ± SE (n=7). *P<0.05 (right-tailed paired t-test, n=7).
most PFOMTs, which usually displayed methylation at the meta-position. Notably, the sequential methylation at the 3' and 4' position toward quercetin is the first report to our knowledge, and the unique capability could be used in enzyme engineering for generating quercetin 3',4'-dimethyl ether.

CrOMT1 is a novel CCoAOMT-like enzyme from citrus

The existence of OMTs with different methylation patterns towards flavonoids has been revealed in the cell-free extracts of citrus (Brunet et al., 1978; Brunet and Ibrahim, 1980), and in this report we identified CrOMT1 from ‘Ougan’ fruit as playing a role in flavonoid O-methylation. CrOMT1 has 96.8% identity to Cs1g22450 from sweet orange, a putative OMT (Supplementary Table S3); however, following a previous systematic analysis of OMTs in citrus, the latter enzyme was not suggested to be involved in flavonoid O-methylation because the selection criteria were restricted to members of the COMT subfamily (Liu et al., 2016). CdFOMT5 from Citrus depressa was identified as a COMT-type flavonoid-O-methyltransferase (Itoh et al., 2016), with 97.2% identity to Ciclev1003952m, a putative OMT in our study. Recombinant CdFOMT5 preferred flavonols to flavones and methylated flavones at the 3, 5, 6, and 7 position in vitro, although the 6-methylation was negligible. CrOMT1 has distinct catalytic properties compared with CdFOMT5, and the recombinant CrOMT1 favored flavones and exhibited the highest catalytic efficiency with the 6-, 8-, and 3'-OH groups of PMF-type flavones. Phylogenetic analysis (Fig. 3) and functional characterization in vitro showed that CrOMT1 was a new member of the PFOMTs or CCoAOMT-like subgroup (Ibdah et al., 2003) that could be involved in the methylation of flavonoids in citrus.

CrOMT1 is responsible for PMF biosynthesis in ‘Ougan’ fruit peel

Three major PMFs (nobiletin, tangeretin, and 5-HPMF) exist in ‘Ougan’ fruit, and they all have three methoxy groups on the A-ring (6,7,8-OCH₃) and one methoxy group on B-ring (4'-OCH₃) (Fig. 1C). The similar structure of the three PMFs indicated that they might be derived from common precursors in vivo, with their structure as 5,6,7,8,3',4'-hexahydroxy flavones except for tangeretin which has no substitution at the 3' position. Although the natural PMF-type substrates were not available for in vitro studies, several substrate analogs, including baicalein (6,7,8-trihydroxyflavone), 7,8-dihydroxyflavone, and luteolin (5,7,3',4'-tetrahydroxyflavone), were demonstrated to be favored by recombinant CrOMT1. In addition to the three most abundant PMFs in citrus, several methylated flavonoids were also detected in a trace amount at the mass spectrometric level, such as tricin (Wang et al., 2016). Tricetin was converted into four products by CrOMT1 in vitro, and one of them was tricetin (Fig. 4F). CrOMT1 showed the best affinity (Kₘ=0.6 μM) and high catalytic efficiency (Kₚ/Kₘ=1386.8 M⁻¹ s⁻¹) toward tricetin (Fig. 5); therefore, CrOMT1 might also participate in the biosynthesis of the low-accumulated tricetin in vivo.

Analysis of PMF contents in ‘Ougan’ fruit peel indicated a dramatic tissue-specific accumulation pattern for three major PMFs, which were mainly detected in the flavedo of ‘Ougan’ fruit (Fig. 1A). These results indicated that CrOMT1 was highly correlated with PMF accumulation by both tissue and developmental stages of ‘Ougan’ fruit. Furthermore, transcript abundance of CrOMT1 sharply increased after UV-B irradiation, and the accumulation of three PMFs also increased (Figs 1B, 2A). Phenolic compounds, especially flavonoids, are thought to serve as a UV-absorbing sunscreen in epidermal tissues of plants (Li et al., 1993; Landry et al., 1995; Rozema et al., 1997; Agati and Tattini, 2010), and several upstream genes involved in the flavonoid pathway are also induced by UV-B irradiation (Falcone Ferreyra et al., 2010; Huang et al., 2016; Henry-Kirk et al., 2018). Similarly, it has been shown that methylation of flavonoids was induced by high light irradiation in the ice plant (M. crystallinum), and the transcript level of McPFOMT rapidly increased after exposure to elevated light conditions (Ibdah et al., 2002, 2003). Notably, McPFOMT along with CCoAOMT7 from A. thaliana and CCoAOMT from chickweed (Stellaria longipes) were proposed to form the new subclass (PFOMTs subgroup) within the CCoAOMT subfamily, with potential function not restricted to lignin biosynthesis (Ibdah et al., 2003).

Transient overexpression of CrOMT1 in the peel of ‘Ougan’ fruit enhanced the accumulation of the three most abundant PMFs (Fig. 6). Kinetic analyses (Fig. 5B) confirmed that recombinant CrOMT1 preferred to accept flavones with vicinal hydroxyl groups in vitro, which are similar to potential PMF-type substrates. This substrate preference is consistent with the accumulation of PMFs in response to overexpression of CrOMT1.

In summary, we characterized a CCoAOMT-like enzyme, CrOMT1, in citrus for the first time. After testing a wide range of substrates in vitro, we concluded that CrOMT1 showed a strong preference for flavones, especially those with methylation at the 6 and 8 positions, which were vital sites for PMFs synthesis. This together with the high correlation between accumulation of CrOMT1 transcripts and PMF accumulation both developmentally and in response to UV-B irradiation and the enhanced PMF accumulation in the peel of ‘Ougan’ overexpressing CrOMT1 support the suggestion that CrOMT1 is responsible for PMFs biosynthesis in ‘Ougan’ fruit peel. Identification of multifunctional enzymes responsible for production of methylated products is important for the synthesis of various methylated flavonoids by enzyme engineering and for bioactivity research.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Sequence alignment of CrOMT1 with other members of the CCoAOMT subfamily.

Fig. S2. Characterization of recombinant CrOMT1.

Fig. S3. MS/MS spectrometry of methylated products generated by CrOMT1 in vitro and corresponding authentic standards.
Fig. S4. 1H and 13C NMR data of 7,8-dihydroxyflavone and its methylated product by CrOMT1.

Fig. S5. Overview of the methylation of substrates by CrOMT1.

Fig. S6. HPLC chromatograms of time-dependent methylation of quercetin by recombinant CrOMT1 in vitro.

Table S1. Primers used for cloning CrOMT1 and qPCR.

Table S2. UniProt entries of proteins from other organisms used in phylogenetic analysis.

Table S3. Fifty-five OMT genes detected in the flavedo of ‘Ougan’ fruit.

Table S4. Percentage amino acid identity shared by PFOMTs described in the phylogenetic tree.

Table S5. Accumulation of the three most abundant PMFs in two different tissues during ‘Ougan’ fruit maturation.

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