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cDNA cloning and functional characterization of flavonol 3-*O*-glucoside-6″-*O*-malonyltransferases from flowers of *Verbena hybrida* and *Lamium purpureum*☆

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Abstract

Complementary DNAs coding for two flavonol 3-*O*-glucoside malonyltransferases, *Vh3MaT1* and *Lp3MaT1*, were cloned from flowers of *Verbena hybrida* and *Lamium purpureum*, respectively, expressed in *Escherichia coli* cells, and functionally characterized. The cloning strategy took full advantage of the specific conservation of a sequence (–Tyr–Phe–Gly–Asn–Cys–, termed motif 2) in the anthocyanin-specific members of the versatile acyltransferase (VAT) family. Both of the expressed proteins, Vh3MaT1 and Lp3MaT1, effectively catalyzed the regiospecific transfer of the malonyl group from malonyl-CoA to the 6″-hydroxyl group of quercetin 3-*O*-glucoside and were highly specific for these acyl donor and acceptor. Therefore, these enzymes are malonyl-CoA:flavonol 3-*O*-glucoside-6″-*O*-malonyltransferases. Kinetic parameters were determined at pH 7.0 and 30 °C as follows: for Vh3MaT1, k_{cat}, 2.9 s^{-1}; k_{cat}/K_m for quercetin 3-*O*-glucoside, 17 s^{-1} mM^{-1}; and k_{cat}/K_m for malonyl-CoA, 930 s^{-1} mM^{-1}; for Lp3MaT1, k_{cat}, 9.0 s^{-1}; k_{cat}/K_m for quercetin 3-*O*-glucoside, 28 s^{-1} mM^{-1}; and k_{cat}/K_m for malonyl-CoA, 360 s^{-1} mM^{-1}. These results suggested that Vh3MaT1 and Lp3MaT1 should serve as useful biocatalysts for the malonylation of quercetin 3-*O*-glucoside to control the bioactivities and pharmacokinetics of the flavonoid. The results also show that VAT members having motif 2 are not restricted to "anthocyanin" acyltransferases but should include an extended class of enzymes, i.e. "flavonoid glucoside" acyltransferases.

Keywords: cDNA cloning; Flavonol 3-*O*-glucoside-6″-*O*-malonyltransferase; Quercetin; *Verbena hybrida*; *Lamium purpureum*

1. Introduction

The flavonol quercetin is a dietary flavonoid found in abundance in plant foods such as onion, apple, kale, and broccoli [1]. A variety of bioactivities that may be beneficial to human health have been reported for this flavonoid [2,3]. Although flavonoids, including quercetin, generally exist as their conjugated forms (e.g. glycosides and/or their acylated forms) in plant tissues, most of these activities have been examined with their aglycon forms, which is also the case for quercetin [1,3]. However, evidence shows that quercetin conjugates have different bioactivities compared with those of the parent aglycon—the conjugation may reduce or even abolish the bioactivity of the aglycon and may also affect the pharmacokinetics of the flavonoid [4]. This, in turn, suggests that the functional properties of quercetin may vary with the conjugation types. Thus, altering the conjugation types by enzymatic modification potentially serves as a strategy for controlling the bioactivities of this flavonoid and should be of pharmaceutical and nutritional importance. We consider the malonylation

☆ The nucleotide sequences reported in this paper have been submitted to the Genbank database under the accession numbers AY500350 (for Vh3MaT1) and AY500352 (for Lp3MaT1).

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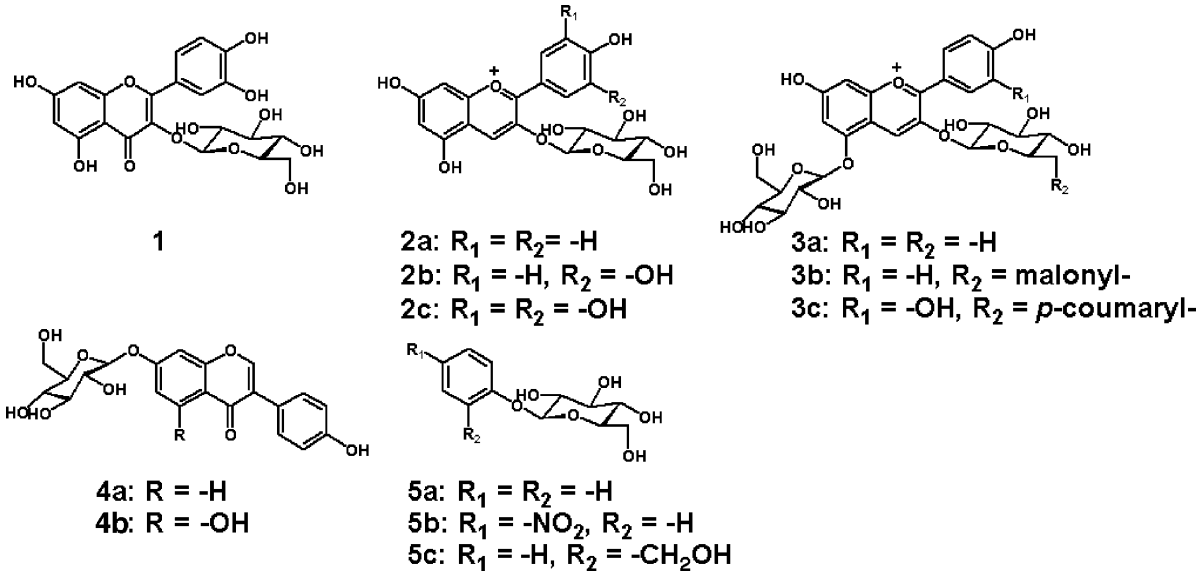

Fig. 1. Structures of some phenolics that are examined for acyl acceptors of Vh3MaT1 and Lp3MaT1 in this study. (**1**) Quercetin 3-*O*-glucoside; (**2**) anthocyanidin 3-*O*-glucosides (aglycon names: (**2a**) pelargonidin; (**2b**) cyanidin; and (**2c**) delphinidin); (**3a**) pelargonidin 3,5-*O*-diglucoside; (**3b**) pelargonidin 3-*O*-(6″-*O*-malonylglucoside)-5-*O*-glucoside; (**3c**) cyanidin 3-*O*-(6″-*O*-*p*-coumarylglucoside)-5-*O*-glucoside; (**4**) isoflavone 7-*O*-glucosides (aglycon names: (**4a**) daidzein; (**4b**) genistein); (**5a**) phenyl β-D-glucopyranoside; (**5b**) *p*-nitrophenyl β-D-glucopyranoside; and (**5c**) *o*-(hydroxymethyl)phenyl β-D-glucopyranoside (salicin).

of quercetin 3-*O*-glucoside (Fig. 1, compound **1**) as one such modification—the malonylation is one of the most common aliphatic acylations of flavonoids in nature and allows the enhancement of the solubility in water [5], the protection of glycosyl moiety from enzymatic degradation [6], and the stabilization of flavonoid structures [6]. Although malonyl-CoA:flavonol 3-*O*-glucoside malonyltransferase activity has already been identified in some plant species [7], the molecular identity of this enzyme remained to be established. During the course of this study, we extensively examined many plant species for the occurrence of activity of malonyl-CoA-dependent malonyl transfer to **1** and found that the flowers of verbena (*Verbena hybrida*) and dead nettle (*Lamium purpureum*) contained appreciable levels of such activity. Thus, we attempted to obtain cDNAs coding for malonyl-CoA:flavonol 3-*O*-glucoside malonyltransferases from the cDNA libraries of these flowers. To achieve this, we assumed that these malonyltransferases could be congeneric to anthocyanin acyltransferases, the well-established, anthocyanin-specific members of the versatile acyltransferase (VAT) family [8], because flavonols are structurally and metabolically related to anthocyanins (e.g. Fig. 1, **2a**–**2c**) [5], and, indeed, several anthocyanin malonyltransferases have been shown to exhibit weak flavonol 3-*O*-glucoside malonyltransferase activities [6,9,10]. Thus, a homology-based strategy that has been established for the cDNA cloning of anthocyanin acyltransferases [6,9,10] was used, and as a result, two flavonol 3-*O*-glucoside-6″-*O*-malonyltransferase cDNAs could be successfully obtained. We describe here the cDNA cloning, sequencing, heterologous expression, and functional characterization of malonyl-CoA:flavonol 3-*O*-glucoside-6″-*O*-malonyltransferases from flowers of *V. hybrida* and *L. purpureum* (Fig. 2). To our knowledge, this is the first isolation of cDNAs encoding flavonol glucoside-specific acyltransferases.

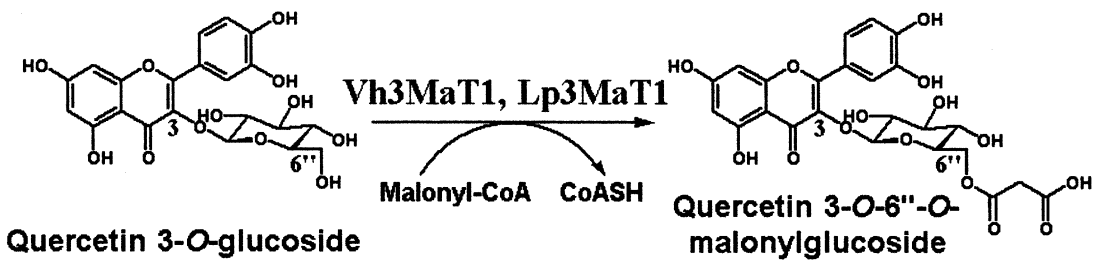

Fig. 2. Malonyl transfer from malonyl-CoA to quercetin 3-*O*-glucoside catalyzed by Vh3MaT1 and Lp3MaT1. The malonyl group is specifically transferred to the 6″-hydroxyl group of the flavonol glucoside.

2. Materials and methods

2.1. Plant materials, flavonoids, and other chemicals

Red verbena (*V. hybrida*) was purchased from a local market in Sendai. Dead nettle (*L. purpureum*) is a widespread plant throughout the temperate regions, including Japan, and the plant used in this study was that of a wild species grown in Sendai. Petals of recently opened flowers were collected and stored at –80 °C until use. Quercetin 3-*O*-glucoside (**1**) was kindly provided by Professor T. Iwasina (Tsukuba Botanical Garden, National Science Museum, Tsukuba, Japan). Anthocyanins (**2a**–**2c** and **3a**–**3c**) were isolated and purified as described previously [11]. Isoflavone 7-*O*-glucosides (i.e. daidzein 7-*O*-glucoside (**4a**), genistein 7-*O*-glucoside (**4b**)), phenyl β-D-glucopyranoside (**5a**), *p*-nitrophenyl β-D-glucopyranoside (**5b**), and *o*-(hydroxymethyl)phenyl β-D-glucopyranoside (salicin, **5c**) were purchased from Nacalai Tesque, Kyoto, Japan.

2.2. cDNA cloning of anthocyanin acyltransferase homologs

For the cDNA cloning of *Vh3MaT1* and *Lp3MaT1*, a pair of PCR primers was designed based on two amino-acid sequences conserved among anthocyanin acyltransferases [6,8,11]. The forward primer was 5′-(A/G)(A/C)(T/C)TA (T/C)TT(T/C)GG(T/G/C) AA(C/T)TG-3′, which was based on the sequence around motif 2 (–Tyr–Phe–Gly–Asn–Cys–, Fig. 3), and the reverse primer was 5′-CTT(T/C)CCCCA (C/T)CC(A/G) AAATC-3′, which was based on the sequence around motif 3 (–Asp–Phe–Gly–Trp–Gly–, Fig. 3). Cloning of *Vh3MaT1* cDNA was carried out as follows. Total RNA was prepared from petals of *V. hybrida* using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the QIAGEN OneStep RT-PCR Kit (Qiagen) with the primers mentioned above and the total RNA as a template. Thermal cycling conditions were as follows: the RT-PCR mixture was incubated at 50 °C for 30 min for reverse transcription and then at 95 °C for 15 min for the activation of DNA polymerase and inactivation of reverse transcriptase, followed by 35 cycles of PCR (one cycle consists of 94 °C for 30 s, 40 °C for 30 s, and 72 °C for 1 min), and finally incubated at 72 °C for 10 min. The amplified fragment, which was 250 bp in length, was cloned into TOPO-pCR2.1 (Invitrogen, Carlsbad, California, USA) and subjected to sequencing using the Dye-Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA) with the CEQ 2000 DNA analysis system (Beckman Coulter). The 250 bp fragment was DIG-labeled using the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Basel, Switzerland). The mRNA was isolated from the petals of *V. hybrida* using an mRNA isolation kit (Roche Molecular Biochemicals) and used for the construction of a cDNA library using the λZAPII-cDNA synthesis kit (Stratagene, La Jolla, CA, USA). The cDNA library (∼200,000 plaques) was screened by plaque hybridization with the DIG-labeled 250 bp fragment as a probe. The hybridization was performed at 37 °C for 16 h in 5 × SSC containing 0.02% (w/v) SDS, 0.1% (w/v) *N*-lauroylsarcosine, 2% (w/v) blocking reagent (Roche Molecular Biochemicals), and 30% (v/v) formamide. The filters were washed twice in 0.1 × SSC and 0.1% (w/v) SDS at 60 °C for 15 min. The DIG-DNA Labeling and Detection Kit (Roche Molecular Biochemicals) was used to detect DIG-labeled DNA. The cDNAs of positive clones were subcloned into the pBluescript SK-phagemid following the manufacturer's instructions and subjected to DNA sequencing. Cloning of Lp3MaT1 was completed essentially as described above except that total RNA and mRNA were prepared from petals of *L. purpureum*.

2.3. Heterologous expression of cDNAs and purification of the expressed products

The full-length *Vh3MaT1* cDNA, thus, obtained was amplified by PCR using the primers 5′-GGTACCATGGCTA CAACCAC-3′ (underlining indicates *Kpn*I site) and 5′-CTGCAGTTCAATCTTCAATCC-3′ (underlining, *Pst*I site) to introduce *Kpn*I and *Pst*I restriction sites at the 5′- and 3′-sides of the start and stop codons of the cDNA, respectively. After the amplified fragment was cloned into a pCR4Blunt-TOPO vector and sequenced to confirm the absence of PCR errors, the full-length *Vh3MaT1* cDNA generated by *Kpn*I–*Pst*I digestion of the plasmid was ligated with a *Kpn*I–*Pst*I-digested pQE-30 vector (Qiagen). The expression vector for the *Lp3MaT1* cDNA was constructed essentially as described above, except that the following PCR primers were used for cDNA amplification: 5′-GCATGCATGAACAATTTCGC-3′ (underlining, *Sph*I site) and 5′-CTGCAGTTATGTTTGTATCCC-3′ (underlining, *Pst*I site). Each of the resultant plasmids was used to transform *Escherichia coli* JM109 cells. Transformant cells were cultivated at 30 °C for 24 h in 100 ml of an LB medium supplemented with 50 μg ml^{-1} ampicillin and 1% (w/v) lactose, which allowed consecutive expression of a recombinant protein. The expressed products, Vh3MaT1 and Lp3MaT1, were extracted from transformant cells and purified to homogeneity as described previously [11]. Briefly, crude extracts of transformant cells were prepared by sonication of lysozyme-treated cells followed by centrifugation. After removal of nucleic acids with polyethyleneimine, the recombinant proteins were purified from the extracts to homogeneity by means of successive column chromatographies on Ni-NTA-agarose (QIAGEN), High-Q (Bio-Rad), and Phenyl Superose HR 5/5 (Amersham Bioscience, Piscataway, NJ, USA) [11]. Proteins were quantified by the method of Bradford [12] using a kit (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA), with bovine serum albumin as the standard.

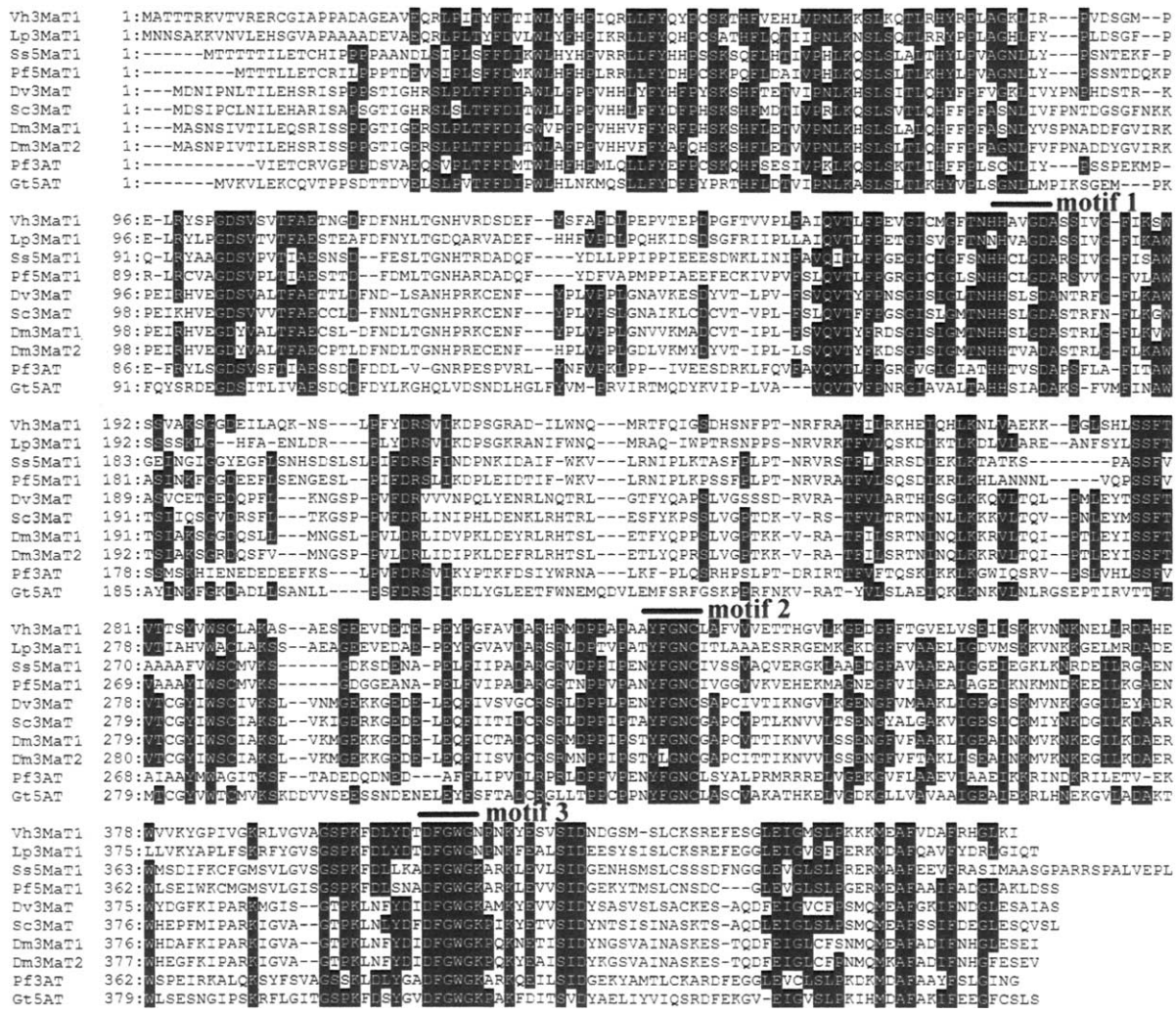

Fig. 3. Alignment of the amino acid sequences of flavonol 3-*O*-glucoside-6″-*O*-malonyltransferases [Vh3MaT1 and LP3MaT1 (this study)] and some anthocyanin malonyltransferases [Ss5MaT1, malonyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase of *Salvia splendens* (Genbank accession number, AAL50566); Pf5MaT, malonyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-malonyltransferase of *Perilla frutescens* (AF405204); Dv3MaT, malonyl-CoA:anthocyanidin 3-*O*-glucoside-6″-*O*-malonyltransferase of *Dahlia variabilis* (AF498108); Sc3MaT, malonyl-CoA:anthocyanidin 3-*O*-glucoside-6″-*O*-malonyltransferase of *Senecio cruentus* (AY190121); Dm3MaT1, malonyl-CoA:anthocyanidin 3-*O*-glucoside-6″-*O*-malonyltransferase of *Dendranthema* x *morifolium* (AY298809); Dm3MaT2, malonyl-CoA:anthocyanidin 3-*O*-glucoside-3″,6″-*O*-dimalonyltransferase of *D.* x *morifolium* (AY298810); Pf3AT, hydroxycinnamoyl-CoA:anthocyanin 3-*O*-glucoside-6″-*O*-acyltransferase of *P. frutescens* (BAA93475); and Gt5AT, hydroxycinnamoyl-CoA:anthocyanin 5-*O*-glucoside-6‴-*O*-acyltransferase of *Gentiana triflora* (BAA74428)]. The bars indicate the highly conserved sequences (motifs 1 and 3) of the VAT family and of motif 2 of anthocyanin (or flavonol glucoside) acyltransferases.

2.4. Acyltransferase assays

The standard reaction mixture (100 μl) consisted of 20 mM potassium phosphate, pH 7.0, 40 μM malonyl-CoA, 100 μM acyl-acceptor substrate, and enzyme. After incubation at 30 °C for 20 min, the reaction was stopped by adding 200 μl of ice-cold 0.5% (v/v) trifluoroacetic acid. Flavonols and anthocyanins in the resultant mixture were analyzed by reversed-phase HPLC using the Dynamax system (Rainin, Woburn, MA, USA) as described previously [11]. For the analyses of isoflavones (**4a** and **4b**) and other phenolic compounds (**5a**, **5b**, and **5c**), the following HPLC conditions were used: column, *J*'sphere ODS-M80 (4.6 mm × 150 mm, YMC, Kyoto, Japan); detection, 260 nm; flow rate, 0.7 ml min^{-1}. The column was previously equilibrated with 13.5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in water. After injection, the column was initially developed isocratically with 13.5% (v/v) acetonitrile for 3 min, followed by a linear gradient from 13.5 to 90% in 15 min. The column was then washed isocratically with 90% (v/v) acetonitrile for 1 min, followed by a linear gradient from 90 to 13.5% in 1 min.

3. Results

3.1. cDNA cloning of anthocyanin acyltransferase homologs

In an attempt to isolate cDNAs encoding flavonol 3-*O*-glucoside malonyltransferases, we have extensively searched for cDNAs encoding anthocyanin acyltransferases, which are anthocyanin-specific members of VATs. The VATs are involved in the plant and fungal secondary metabolisms and play very important roles in the biosyntheses of a wide variety of natural products of pharmaceutical and agricultural importance, such as anthocyanins, phytoalexins [13], vindoline [14], benzylacetate (flower scent [15]), alkyl acetate (fruit flavor [16]), taxol (an anticancer drug [17–19]), and morphine (an anodyne [20]). Despite the observed diversity of the substrate specificities and regiospecificities of acyl transfer and the primary structures of enzymes, these acyltransferases were categorized into a single enzyme family, the VAT family (also called BAHD family [21]), on the basis of the strict conservation of the sequence motifs (motifs 1 and 3; Fig. 3) in their primary structures as well as the suggested conservation of their acyl-transfer mechanism [14,22]. Moreover, the anthocyanin-specific members of the VAT family have been shown to share an additional well-conserved sequence, –Tyr–Phe–Gly–Asn–Cys– (motif 2), in their primary structures. Thus, PCR primers were designed on the basis of the amino acid sequences around motifs 2 and 3, and, using these primers, a partial VAT cDNA, 0.25 kbp in size, was amplified from a total RNA prepared from red petals of *V. hybrida*. Using this 0.25 kbp fragment as a probe, the *V. hybrida* cDNA library of ∼200,000 clones was screened under high-stringency conditions, giving rise to 31 positive clones, from which a full-length cDNA (termed *Vh3MaT1*) that coded for a protein of 461 amino acids was obtained. Similarly, a 0.25 kbp cDNA fragment could be amplified from a total RNA extracted from red petals of *L. purpureum*, and, using this fragment as a probe, the *L. purpureum* cDNA library of ∼200,000 clones was screened to obtain two positive clones; one of these clones coded for a full-length VAT cDNA (termed *Lp3MaT1*), which coded for a protein of 461 amino acids. The deduced amino acid sequences of these cDNAs (Fig. 3) contained motifs 1, 2, and 3 and also showed significant sequence similarities to those of anthocyanin acyltransferases (identity: Vh3MaT1, 40–45%; Lp3MaT1, 38–44%). These results showed that Vh3MaT1 and Lp3MaT1 were anthocyanin acyltransferase homologs of the VAT family. It would be worth mentioning here that, in the amino acid sequences of known anthocyanin acyltransferases, motif 3 (–Asp–Phe–Gly–Trp–Gly–) is immediately followed by a lysine residue, which is replaced by an asparagine residue in the Vh3MaT1 and Lp3MaT1 sequences (Fig. 3). The Vh3MaT1 and Lp3MaT1 sequences were 61% identical to each other.

3.2. Heterologous expression and acyltranferase activities of Vh3MaT1 and Lp3MaT1

The *Vh3MaT1* and *Lp3MaT1* cDNAs were heterologously expressed in *E. coli* JM109 cells, giving rise to soluble, catalytically active proteins. These recombinant proteins were purified to homogeneity and then examined for their acyl donor and acyl-acceptor specificities (Fig. 1 and Table 1).

The reaction of the recombinant Vh3MaT1 with **1** and malonyl-CoA yielded a single malonylation product, as analyzed by reversed-phase HPLC (see Section 2.4). NMR analyses of the product showed downfield shifts (by 0.5–0.7 ppm) of resonance of the 6″-hydrogens of the 3-*O*-glucosyl moiety of **1**. ^{1}H-detected multiple-bond connectivity (HMBC) cross peaks were also observed between the *C*-1 carbonyl carbon of the malonyl group and 6″-hydrogens, indicating that the product was quercetin 3-*O*-6″-*O*-malonylglucoside. For the malonyl acceptors, the enzyme exhibited the highest reactivity to **1** with the following kinetic parameters: k_{cat}, 2.9 s^{-1}; k_{cat}/K_m for **1**, 17 s^{-1} mM^{-1}; and k_{cat}/K_m for malonyl-CoA, 930 s^{-1} mM^{-1}. The enzyme also showed appreciable levels of malonyl transfer activities to phenyl glucosides (**5a**–**5c**); however, the K_m values of these substrates were too large to be determined (Table 1). The enzyme showed low malonyl-transfer activities to isoflavone 7-*O*-glucosides (**4a** and **4b**; relative activity to **1**, 2–8%) and anthocyanins (**2a**–**2c** and **3a**–**3c**; relative activity, <0.6%). For acyl donors, Vh3MaT1 showed the highest activity with malonyl-CoA. Other aliphatic dicarboxylic acyl-CoAs (metylmalonyl-CoA and succinyl-CoA) could also serve as weak substrates, whereas aromatic acyl-CoAs (*p*-coumaroyl-CoA and caffeoyl-CoA) were inert as acyl donors.

The recombinant Lp3MaT1 could effectively catalyze the malonyl transfer from malonyl-CoA to **1** to produce quercetin 3-*O*-6″-*O*-malonylglucoside as well (Table 1). For malonyl acceptors, the enzyme exhibited the highest reactivity to **1**: k_{cat}, 9.0 s^{-1}; k_{cat}/K_m for **1**, 28 s^{-1} mM^{-1}; and k_{cat}/K_m for malonyl-CoA, 360 s^{-1} mM^{-1}. The enzyme showed appreciable levels of malonyl transfer activities to phenyl glucosides, although the K_m values of these substrates were too large to be determined, as was the case for Vh3MaT1. The enzyme showed only low levels of malonyl transfer activities to isoflavone 7-*O*-glucosides (<2%) and anthocyanins (<0.3%). For acyl donors, Vh3MaT1 showed the highest activity with malonyl-CoA. Methylmalonyl-CoA and succinyl-CoA could serve as weak substrates, whereas aromatic acyl-CoAs were inert as acyl donors.

4. Discussion

In this study, we isolated, by means of a homology-based strategy, two cDNAs coding for VATs having strong flavonol 3-*O*-glucoside malonyltransferase activity, one from flowers of *V. hybrida* and the other from those

Table 1
Acyl-acceptor and donor specificities of Vh3MaT1 and Lp3MaT1

Substrate[a]	Vh3MaT1				Lp3MaT1			
	Relative activity[b] (%)	k_{cat} (s^{-1})	k_{cat}/K_m for donor ($s^{-1}\,mM^{-1}$)	k_{cat}/K_m for acceptor ($s^{-1}\,mM^{-1}$)	Relative activity[b] (%)	k_{cat} (s^{-1})	k_{cat}/K_m for donor ($s^{-1}\,mM^{-1}$)	k_{cat}/K_m for acceptor ($s^{-1}\,mM^{-1}$)
Acyl acceptor[c]								
1a	100	2.9 ± 0.41	930 ± 42	17 ± 5.6	100.0	9.0 ± 1.0	360 ± 11	28 ± 2.0
2a	0.6	ND[d]	2.3 ± 0.1	0.087 ± 0.004	0.3	ND	7.0 ± 0.6	0.12 ± 0.07
2b	0.3	ND	3.5 ± 1.2	0.03 ± 0.005	0.2	ND	11 ± 2.1	0.14 ± 0.02
2c	0.2	ND	2.3 ± 0.5	0.02 ± 0.005	0.3	ND	11 ± 4.3	0.26 ± 0.08
3a	<0.1				<0.1			
3b	<0.1				<0.1			
3c	<0.1				<0.1			
4a	1.8	0.04 ± 0.01	1.2 ± 0.4	1.0 ± 0.3	0.6	0.01 ± 0.00	9.1 ± 0.5	0.70 ± 0.12
4b	7.6	0.07 ± 0.01	46 ± 4.9	20 ± 1.4	1.7	0.03 ± 0.00	11 ± 1.0	2.8 ± 0.52
5a	6.4	ND	2.0 ± 0.1	1.5 ± 0.1	11	ND	23 ± 1.6	2.9 ± 0.32
5b	48	ND	100 ± 17	5.0 ± 0.3	19	ND	28 ± 1.7	4.7 ± 0.38
5c	3.5	ND	1.3 ± 0.2	0.4 ± 0.0	7.7	ND	39 ± 13	1.3 ± 0.13
Acyl donor[e]								
Malonyl-CoA	100	2.9 ± 0.41	930 ± 42	17 ± 5.6	100	9.0 ± 1.0	360 ± 11	28 ± 2.0
Acetyl-CoA	0.4	0.01 ± 0.00	0.9 ± 0.2	0.2 ± 0.1	0.2	0.01 ± 0.00	0.8 ± 0.5	0.1 ± 0.0
Methylmalonyl-CoA	5.5	0.21 ± 0.02	3.1 ± 0.7	1.4 ± 0.3	2.5	0.15 ± 0.02	6.7 ± 2.2	0.7 ± 0.2
Succinyl-CoA	19	0.27 ± 0.01	170 ± 9.2	9.0 ± 0.1	27	1.2 ± 0.16	51 ± 19	37 ± 11
p-Coumaroyl-CoA	<0.1				<0.1			
Caffeoyl-CoA	<0.1				<0.1			

[a] Chemical structures of acyl acceptors listed in this Table are presented in Fig. 1.

[b] The specific activities were determined under the conditions described in Section 2.4. The specific activities with quercetin 3-*O*-glucoside (17.2 nkatal mg^{-1} protein for Vh3MaT1 and 31.2 nkatal mg^{-1} protein for Lp3MaT1) are taken to be 100%.

[c] For evaluation, malonyl-CoA was used as an acyl donor.

[d] A linear relationship between initial velocity (v) and substrate concentration ([S]) was obtained in the range of [S] examined, suggesting that the K_m values for these substrates should be very large, because the enzyme-catalyzed reactions proceeds with first-order kinetics under the conditions of $[S] \ll K_m$. Therefore, only the k_{cat}/K_m value was determined from slope of v vs. [S] plots.

[e] For evaluation, quercetin 3-*O*-glucoside was used as an acyl acceptor.

of *L. purpureum*. Both VATs were highly specific for **1** and malonyl-CoA and exclusively produced quercetin 3-*O*-6-*O*″-malonylglucoside as a sole transfer product. Thus, these enzymes were malonyl-CoA:flavonol 3-*O*-glucoside-6″-*O*-malonyltransferases and should serve as useful biocatalysts for the modification of flavonol glucosides to control their bioactivities and pharmacokinetics.

The results obtained in this study clearly showed that VAT members having motif 2 are not restricted to "anthocyanin" acyltransferases but should include an extended class of enzymes, i.e. "flavonoid glucoside" acyltransferases. *Arabidopsis* is estimated to contain approximately 60 VAT genes in its genome [23], most of whose biochemical roles are unknown; thus, one plant species is generally expected to contain numerous VAT homologs, and flavonoid glucoside acyltransferases should represent only a small fraction of the total VATs encoded in the plant genome. Thus, our homology-based strategy is very useful for efficient and specific isolation of the flavonoid glucoside acyltransferase cDNAs (see also Sections 1 and 3.1). It must be mentioned that, during the course of the present studies, anthocyanin acyltransferase cDNAs could not eventually be isolated from cDNA libraries of both plant species. Although the reason for the result remains to be clarified, it might be possible that anthocyanin acyltransferases of these plant species lack the motif 2 in their amino acid sequences. Indeed, we have very recently identified a new VAT member, malonyl-CoA:anthocyanin 5-*O*-glucoside 4‴-*O*-malonyltransferase of *Salvia splendens* (Ss5MaT2), which did not contain motif 2 in its amino acid sequence—it was phylogenetically distant from known anthocyanin acyltransferases (H. Suzuki, T. Nakayama, T. Nishino, submitted). Thus, the sequences of all of the flavonoid glucoside acyltransferase do not necessarily have motif 2.

Finally, the high regioselectivity of Vh3MaT1- and Lp3MaT1-catalyzed malonylations is worth noting. With both enzymes, the malonylation of acyl-acceptor substrates, in all cases, yielded a single reaction product, as suggested from HPLC analyses (not shown). This is consistent with the general observation that VAT-catalyzed acyl transfer proceeds with high regioselectivity [8] and also illustrates the general usefulness of VAT for the specific acylation of industrially important compounds. Namely, because the compounds to be acylated, in many cases, carry multiple hydroxyl (or other nucleophilic) functions, there exists the problem of regiocontrol of acylation. Indeed, commercially feasible total chemical syntheses have not yet been achieved for some of such important compounds. Regioselective VATs with either strict or broad acyl-acceptor specificity should serve as useful molecular catalysts for specific acylation for the production of compounds of such importance.

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