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Article

O-Methyltransferases from *Arabidopsis thaliana*

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***O*-methylation mediated by *O*-methyltransferases (OMTs) is a common modification in natural product biosynthesis and contributes to diversity of secondary metabolites. OMTs use phenylpropanoids, flavonoids, other phenolics and alkaloids as substrates, and share common domains for *S*-adenosyl-L-methionine (AdoMet) and substrate binding. We searched *Arabiposis* genome and found 17 OMTs genes (*AtOMTs*). AdoMet- and substrate-binding sites were predicted. AdoMet binding domain of AtOMTs is highly conserved, while substrate-binding domain is diverse, indicating use of different substrates. In addition, expressions of six *AtOMT* genes in response to UV and in different tissues were investigated using real-time quantitative reverse transcriptase-polymerase chain reaction. All the *AtOMTs* investigated were expressed under normal growth condition and most, except *AtOMT10*, were induced after UV illumination. *AtOMT1* and *AtOMT8* were expressed in all the tissues, whereas *AtOMT10* showed flower-specific expression. Analysis of these *AtOMT* gene expressions could provide some clues on *AtOMT* involvement in the cellular processes.**

Key words: Arabidopsis thaliana, *O-methyltransferase, Real time PCR, Substrate binding sites*

Recognition of gene sets unique to an organism has become possible by completion of genome projects on a variety of organisms and the development of the comparative genomics. Compared to animals and bacteria, plants have unique sets of gene families such as cytochrome P450s and those involved in secondary metabolisms. Cytochrome P450s, which appeared more than 250 in *Arabidopsis*, mediate many biological reactions found only in plants.[1,2] The secondary metabolisms, particularly phenylpropanoid pathway and alkaloid biosynthesis pathways, are also mediated by special sets of genes such as *O*-methyltransferases (OMTs) and glycosyltransferases (GTs),[3,4] which attribute to the expansion of diversity within the groups.

OMTs catalyze the transfer of a methyl group from *S*-adenosine-L-methionine (AdoMet) to a hydroxyl group of an acceptor molecule to form methyl ether derivatives. Plant OMTs can be divided into two groups based on their molecular weight: caffeoyl coenzyme A OMTs (*CCoAOMT*s) and caffeic acid OMT (*COMT*), representing those from 26 to 28-kDa and from 38 to 43 kDa, respectively.[5]

Biological functions of *O*-methylation in plants are diverse. For an example, ferulic acid and sinapic acid, which are both methylated compounds, are precursors of the monolignols that serve as building blocks for lignin biosynthesis.[6] *O*-Methylation of flavonoids results in decreased the chemical activity and increased their lipophilicity and antimicrobial activity[3] as well as contributes to the formation of volatile compound in various plants.[7] Plant OMTs are high-specific in contrast to those from animals. Many OMTs, which were found in diverse plants, were assigned as OMTs only based on their amino acid sequence similarity with the functionally identified OMTs.[8]

A. thaliana was the first plant to have a completely identified genome sequence.[9] With the whole genome sequence, it is possible to immediately analyze the particular family genes. In the case of OMTs, *A. thaliana* is estimated to have 17 OMTs. Because most of OMTs in *A. thaliana* appear to have different expression patterns and different substrates, and, thus, different active site structures. 17 OMTs from *A. thaliana* were analyzed. We report here results of the analysis of the active sites and the AdoMet binding sites of 17 OMT genes and the expression profiles of the six *AtOMT* genes in response to UV-irradiation and in various organs of *A. thaliana*.

Materials and Methods

Sequence Pileups. The OMT sequences obtained from TAIR (http://www.arabidopsis.org) were aligned using CLUSTAL W (http://www.ebi.ac.uk/clustalw). The BoxShade program (http://www.isrec.isb-sib.ch:8080/software/BOX_form.html) was used to highlight conserved and similar amino acids.

Plant materials and UV-irradiation. *A. thaliana* (ecotype Columbia) seeds were surface-sterilized in 5% sodium hypochlorite and 0.15% Tween 20 for 5 min, rinsed in distilled water, and placed onto 0.8% agar-containing Petri dishes. The plates contained 1x Murashige-Skoog salts (Life Technologies, Inc. Carlsbad, CA, USA), 0.5 mM MES (pH5.7), and 1% sucrose. The seeds were cold-treated at 4°C

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Table 1. Primer sets for real-time PCR

Gene	Forward Primer	Reverse Primer
AtOMT1	5'-ctcttcgccatgcaactagc-3'	5'-ggagacatgggagaaccatt-3'
AtOMT8	5'-taacgacatggacggatgaa-3'	5'-ggtttccttaggcaagaccg-3'
AtOMT10	5'-gctccttcttatcccggagt-3'	5'-tttgtcgttccagtcacg-3'
AtOMT11	5'-atgctcattcttatcccgg-3'	5'-ttcgtccgtccaatcatgta-3'
AtOMT12	5'-tagctgggacgatgaccatt-3'	5'-ctcctgggaactctgggatt-3'
AtOMT16	5'-tgcgaagaccatcagcatta-3'	5'-atctcttgcggcatatcgtc-3'
AT-ACT	5'-ccacccgagaggaagtacag-3'	5'-cctggacctgcttcatcata-3'

for 3 days and then incubated in the light (light grown; L+) for 12 days at 25°C. Flower buds were collected from mature *Arabidopsis* plants (5 weeks old). The plant was placed 20-25 cm below there UV-B lamps (Philips TL 2W/12 UV).

Real-time quantitative RT-PCR. Total RNA was isolated from the frozen materials using the Plant RNeasy extraction kit (Qiagen, Germay). Residual genomic DNA present in the preparation was eliminated by treating with RNAse-free DNAse I (Boehringer Mannheim/Roche, USA) treatment. cDNA was synthesized as described by Kim, *et al.*[10] and amplified using SYBR-Green® PCR Kit containing Hotstart DNA polymerase (Qiagen, Germany) on the Rotor-Gene RG-3000A thermocycler (Corbett Research, USA). GenBank access numbers and primers used are listed in Table 1. PCR was performed by incubating at 95°C for 15 min to activate the hot start Taq DNA polymerase, followed by 45 cycles of 10 sec at 95°C, 15 sec at 60°C, and 20 sec at 72°C. Specificity of the PCR amplification was checked through heat dissociation kinetics. PCR products were sequenced to check the specificity of each primer set. The results obtained from different samples were standardized to the constitutive actin gene[11] expression level. The expression levels of each gene were based on the take-off time of each gene.

Results and Discussion

Search of AtOMTs. Based on the Conserved Domain Database classification (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.html),[12] OMTs were classified into class 2 methyltransferase. We performed Basic Local Alignment Search Tool (BLAST) of the *Arabidopsis* database using the conserved OMT domain (SGLSSLVDVGGGTGALAAAI VRAYPHLKGIVFDLPHVVADAPSADRVEFVGGD). Fifty OMT homologues were initially identified from the database. Because the same DNA or protein sequences are frequently assigned as multiple identification numbers in the databases, duplicated sequences were removed, and 17 different genes were selected. Thus, *AtOMTs* were classified into 17 genes (Table 2). All of AtOMTs except *AtOMT4*, *AtOMT15*, and *AtOMT16* consisted of about 350-400 amino acid residues encoding proteins whose molecular weights are about 40-kDa.

Among the 17 OMT genes, *AtOMT1* is the only one whose *in vivo* and *in vitro* function was characterized. The recombinant AtOMT1 protein uses the flavonoid quercetin as a substrate but not caffeic, and 5-hydroxyferulic acids, suggesting that it is involved in the flavonoid metabolism.[13] However, *in vivo* analysis of *AtOMT1* showed that it is involved in the lignin biosynthesis.[14]

Table 2. *Aarabidopsis* OMT genes used in this study

Gene Name	Locus	GenBank
AtOMT1	At5g54160	gi:30696415
AtOMT2	At4g35150	gi:18418585
AtOMT3	At1g51990	gi:42571826
AtOMT4	At1g63140	gi:62320780
AtOMT5	At1g77520	gi:42563288
AtOMT6	At1g77530	gi:18411586
AtOMT7	At1g76790	gi:30699199
AtOMT8	At3g53140	gi:42565882
AtOMT9	At1g21100	gi:30687177
AtOMT10	At5g37170	gi:18421617
AtOMT11	At5g53810	gi:30696377
AtOMT12	At1g33030	gi:22329913
AtOMT13	At1g21120	gi:62320459
AtOMT14	At1g21130	gi:30687191
AtOMT15	At1g62900	gi:30696774
AtOMT16	At3g62000	gi:22331900
AtOMT17	At4g35160	gi:30690231

Prediction of AdoMet and Substrate Binding Sites. The multiple sequence alignment of 17 AtOMTs showed from 20 to 96 % similarity. Phylogenetic tree of 17 showed that AtOMT9, 13, and 14 displayed the highest similarities, followed by AtOMT5 and AtOMT6. AtOMT16 turned out to be less similar to other AtOMTs (Fig. 1). Genes with high similarity are adjacent to each other in the same chromosome, probably due to gene duplication and some deletion or addition. In addition, analysis of nucleotide sequences showed that point mutations and frame shifts by the deletion of a few amino acids deletion commonly occurred, which results in the higher identity at the nucleotide level than at the amino acid level. For instances, *AtOMT6* and *AtOMT10* showed 55% identity at the amino acid level while 73 % at the nucleotide level.

Domain analysis of 17 AtOMTs was also performed, because methyltransferases generally have two domains, *S*-adenosyl methionine (AdoMet) binding domain and substrate binding domain. It is predictable that AdoMet binding domain

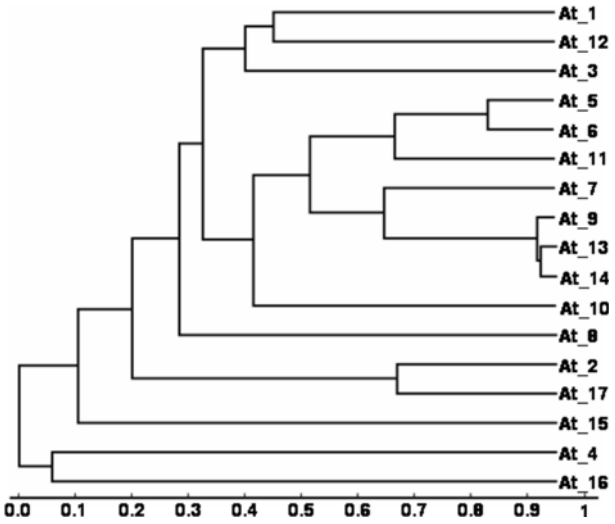

Fig. 1. Phyologenetic Tree of AtOMTs. Scale in bottom showed distance among genes.

from different OMTs would be conserved, while substrate-binding domain varies. AdoMet- and the substrate- binding sites were defined based on the crystal structure of chalcone *O*-methyltransferase (ChOMT) and isoflavone *O*-methyltransferase (IOMT).[15,16] The AdoMet-binding sites of 17 classes of AtOMTs genes were identical except for *AtOMT16* (Table 3) and also the same as COMT and ChOMT. Interestingly, the AdoMet binding sites from several OMTs including those able to modify flavonoids, phenolics and phenylpropanoids from different plants are conserved.

Analysis of the substrate-binding sites of AtOMTs (Table 4), however, showed variations, even though those showing high amino acid homology had identical substrate-binding sites, possibly because the 17 OMTs methylate various substrates containing a hydroxyl group. Among the residues involved in the substrate-binding, two amino acid residues are known to be well-conserved; Met^{178} and Met^{318} in the case of AtOMT1.[15] The second methionine (Met^{318}) is conserved in most OMTs with a higher molecular weight while the first (Met^{178}) is conserved in 11 OMTs, and, in others such as AtOMT 7, 9, 13 and 14, this residue is replaced by glycine. These two conserved methionines are known to be involved in binding the phenyl group through thioester moieties,[15] which indicate that OMTs in these classes most likely use substrates that have the phenyl group such as flavonoids and phenyl propanoids. Others speculate to have different substrates. However, the Met^{178} does not bind to the phenyl group, but instead to the side chains such as propyl group found in phenylpropanoids, which suggests that variance of this group contributes to the expansion of substrate diversity.

Sequencing projects provide useful information on the biological substrate of OMTs based on the primary amino acid sequence homology. Basically, the prediction of biological function of OMTs is deduced from the conserved sequence information of OMTs. In particular, two motifs are critical to determining the substrate of OMTs.[17] The first motif, which showed variation depending on the substrates, is located at the beginning of the N-terminal, whereas the second motif is at the C-terminal. According to the crystal structure of chalcone OMT and isoflavond OMT,[15] the first motif is involved in dimerization of OMTs, and the second motif contains the part of substrate- binding sites. These two motifs, however, are not well-conserved in AtOMTs.

Other residues that are not the part of substrate-binding sites could be considered as critical residues because some of AtOMTs have the same substrate and AdoMet-binding sites. For example, *AtOMT6* and *7* not only have the same substrate and AdoMet-binding sites, but also have 87% overall identity at the amino acid level. Even though to know whether they

Table 3. A comparison of residues neighboring *S*-adenosine methionine binding sites

Genes	*S*-adenosine methionine binding sites							
AtOMT1	Asp204	Gly206	Asp229	Leu230	Asp249	Met250	Lys263	Trp269
AtOMT2	Asp159	Gly161	Asp184	Leu185	Asp204	Met205	Lys218	Trp224
AtOMT3	Asp204	Gly206	Asp229	Leu230	Asp249	Met250	Lys263	Trp269
AtOMT4	Asp223	Gly225	Asp248	Leu249	Asp268	Met269	Lys282	-
AtOMT5	Asp223	Gly225	Asp248	Leu249	Asp268	Met269	Lys282	Trp288
AtOMT6	Asp223	Gly225	Asp248	Leu249	Asp268	Met269	Lys282	Trp288
AtOMT7	Asp207	Gly209	Asp232	Leu233	Asp252	Met253	Lys266	Trp272
AtOMT8	Asp200	Gly202	Asp226	Leu227	Asp246	Met247	Lys260	Trp266
AtOMT9	Asp215	Gly217	Asp240	Leu241	Asp260	Met261	Lys274	Trp280
AtOMT10	Asp175	Gly177	Asp201	Leu202	Asp221	Met222	Lys235	Trp241
AtOMT11	Asp220	Gly222	Asp225	Leu226	Asp245	Met246	Lys279	Trp285
AtOMT12	Asp192	Gly194	Asp218	Leu219	Asp238	Met239	Lys252	Trp258
AtOMT13	Asp215	Gly217	Asp240	Leu241	Asp260	Met261	Lys274	Trp280
AtOMT14	Asp215	Gly217	Asp240	Leu241	Asp260	Met261	Lys274	Trp280
AtOMT15	Asp047	Gly049	Asp072	Leu073	Asp092	Met 93	Lys106	Trp112
AtOMT16	Tyr128	Gly130	Asn154	Ser155	Lys174	Gln175	Gln188	Ser194
AtOMT17	Asp216	Gly218	Asp241	Leu242	Asp260	Met261	Lys275	Trp281

Table 4. A comparison of residues neighboring substrate binding sites

Genes	Residues neighboring substrate binding sites							
AtOMT1	Met128	Asn129	Phe174	Met178	Val314	Ile317	Met318	Asn322
AtOMT2	-	-	Ile128	Met132	Leu277	Val280	Met281	Thr285
AtOMT3	Ala127	Gly128	Phe174	Met178	Ala315	Thr318	Met319	Thr323
AtOMT4	Val148	Leu149	Phe193	Met197	-	-	-	-
AtOMT5	Leu147	Leu148	Phe193	Met197	Met334	Leu337	Met338	Cyc342
AtOMT6	Leu147	Leu148	Phe193	Met197	Met334	Leu337	Met338	Cyc342
AtOMT7	Val132	Thr133	Phe179	Gly181	Met318	Leu321	Met322	Leu326
AtOMT8	Gln123	His124	Met166	Met170	Asp312	Val315	Met316	Arg322
AtOMT9	Val140	Asn141	Phe187	Gly191	Met326	Leu329	Met330	Cyc334
AtOMT10			Phe145	Met149	Thr287	Leu290	Met291	Cyc295
AtOMT11	Leu144	Asp145	Phe191	Met195	Met331	Thr334	Met335	Cyc339
AtOMT12	Leu116	Phe117	Phe162	Met166	Phe302	Phe306	Met307	Asn311
AtOMT13	Val140	Asn141	Phe187	Gly191	Met326	Leu329	Met330	Cyc334
AtOMT14	Val140	Asn141	Phe187	Gly191	Met326	Leu329	Met330	Cyc334
AtOMT15	-	-	Phe017	Met021	Met158	Leu161	Met162	Ser166
AtOMT16	-	-	Ser098	Val102	His232	Val235	Ser236	Val240
AtOMT17	Phe134	Glu135	Ile185	Met189	Leu334	Val337	Met338	Thr342

prefer the same substrate would be the next questions to be asked, accumulated data on OMTs suggested that residues outside of the binding pocket have sometimes more influences in the substrate specificity and binding affinity rather than the overall homology among OMTs. *OMTII-1*, and *OMTII-4* of meadow rue *Thalictrum tuberosum* showed a high sequence identity with only one amino acid among the 364 amino acid residues being different. The binding affinity of the two OMTs for caffeic acid showed about fourfold difference,[18] even though the different amino acid was located at the 21st residue that is not a part of substrate-binding site. The differences in the binding activity were explained by the difference in the morphology in the dimmer formation.[19] In addition, the residues located in the middle of the protein, but are not a part of the binding site, are considered to be important for the affinity against different substrate. FOMT3' from *Chrysosplenium americanum* differs from FOMtx by only one amino acid (Ser239Arg) that is not a part of substrate- or AdoMet-binding site while only FOMT3' could catalyze flavonol.[20,21] Some OMTs use the same backbone substrates with different substitution such as different methylation or hydroxylation. FOMT3′ and OMT1 from *C. americanum* showed 84% identity, while FOMT3′ accepted the partial methylated flavonoids and *OMT1* used unmethylated ones as substrates.[20] These finding illustrated a possible scenario to increase the metabolic capacities by changing a few amino acids while maintaining the substrate-binding sites.

Prediction of substrate-binding sites prediction provides some clues as to why plant contains so many OMT genes. First, because plant OMTs are highly specific, multiple methylations to multiple substrates seems to require many genes. Second, *OMTs* that differ in a few amino acids but have the same substrate might be expressed differently in various tissues, which could result in the formation of more diverse OMTs. Another possible scenario for the diversity of metabolites with limited number of genes would be the heterodimerization process between different OMTs. Some alkaloid substrates such as (*S*)-coclaurine, (*S*)-norreticuline and (*R,S*)-6-O-methylnorlaudanosoline were methylated not by homodimer but by heterodimers in *Baculovirus*, while formation of heterodimers or homodimers in plants is uncertain.[18,22]

Expression profiling of *AtOMTs*. The biological function of OMTs is the modification of secondary metabolites such as monolignols, flavonoids, alkaloids, and other phenolics. Secondary metabolites consisting of these groups are known to be induced upon external stimuli such as UV-irradiation and accumulate in specific tissues. For example, lignin biosynthesis occurs mainly in stems and antocyanin in flowers. Genes involved in phenylpropanoid biosynthesis including the biosynthesis of lignins, flavonoids, and anthocyanins are known to be induced by stress.[23] UV-irradiation is one of the stresses, which cause the increase in flavonoid content in *Arabidopsis*.[24] Thus, expression patterns in different tissues and stimuli could provide clues about the biological function of the gene of interest.

A. thaliana contains at least 17 OMT genes.[26] Comparison of nucleotide sequences of these genes indicated that some of them are too identical to define gene- specific primers (unpublished data). We chose six AtOMTs (*AtOMT1*, *AtOMT8*, *AtOMt10*, *AtOMT11*, *AtOMT12*, and *AtOMT16*) to investigate their expression patterns. Gene-specific primers could be relatively designed from these genes. Each amplied fragment was cloned and sequenced to determine the specificity of the primers; none of these *AtOMT*s except *AtOMT1*, which has been shown to be involved in the lignin biosynthesis *in vivo*, have been functionally characterized.[26]

Relative expression of six *AtOMT* genes was investigated

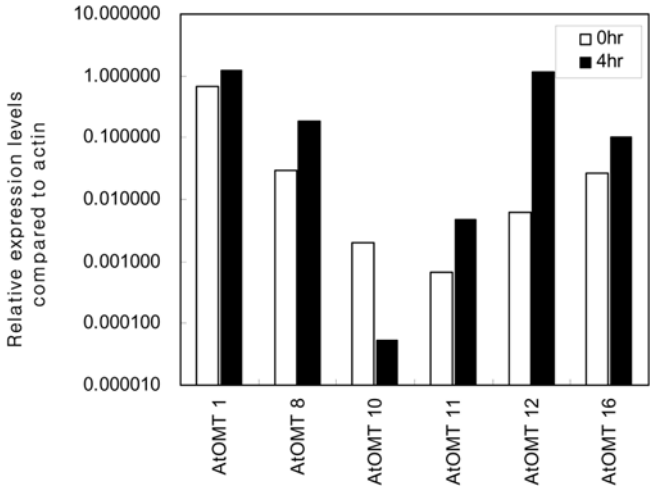

Fig. 2. Relative expression profile of the six *AtOMT* genes in *Arabidopsis* in response to UV irradiation. Relative transcripts levels were measured by the real time RT-PCR and standardized to the constitutive actin gene. All values are the means of three independent experiments.

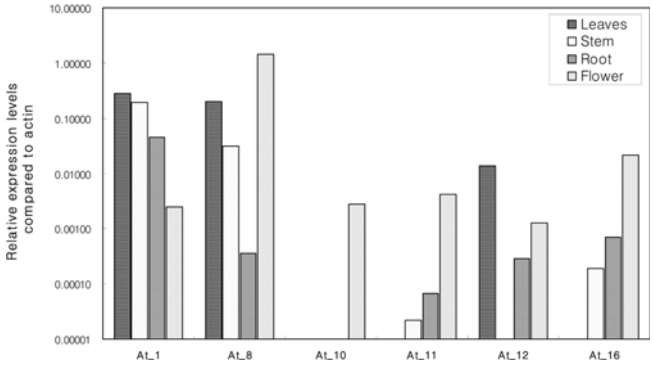

Fig. 3. Relative expression profile of *AtOMT* genes in *Arabidopsis* organs. The RNA was extracted from leaves, stems, roots, and flowers.

by the real-time RT-PCR. Total RNA was isolated from the 5-week-old whole *Arabidopsis* plants. Figure 2 illustrates the relative transcript levels of the six *AtOMT*s standardized to the constitutive actin gene.[11] A logarithmic scale was used to present the results due to high variations among the genes. Under normal growth condition, each transcript was detected, demonstrating that all the *AtOMT* genes tested were transcriptionally active. *AtOMT1* showed the highest expression, exhibiting about 67% of the actin expression level, whereas expression of *AtOMT11* was 0.7%. Other OMTs exhibited from 23- (*AtOMT8*) to about 997-fold (*AtOMT11*) lower expression than *AtOMT1* (Fig. 2). The influence of UV on the expression of OMTs was also analyzed. Five-week-old *Arabidopsis* plants were illuminated with UV light for 4 hrs and total RNA was isolated for the expression analysis. Under normal growth condition, *AtOMT*s exhibited transcript levels ranging from 68 (*AtOMT1*) to 0.2% (*AtOMT11*) of *actin*. However, UV illumination stimulated the expression of most *ATtOMT*s except *AtOMT10*. Induction rate ranged from 1.5-fold of *AtOMT1* to 180-fold of *AOMT11*. UV serves as a stimulus for accumulation of secondary metabolites including flavonoids, anthocyanins and alkaloids;[23] thus, the enhancement of *AtOMT* gene expression is correlated with the accumulation of these compounds.

OMTs mainly participate in modification reactions of plant secondary metabolites. In *A. thaliana*, major secondary metabolites are flavonoids, hydroxycinnamic acid ester, glucosinolates, brassinosteroids, and indole phytoalexins,[13] some of which act as substrates for OMTs. The accumulation of these metabolites is tissue-specific. For example, flavonoids are accumulated mainly in leaves and hydroxylcinnamic acid esters in stems. Organ-specific expression of OMTs from *Arabidopsis* was also analyzed based on the EST.[25] However, EST database is not complete and, as such, will not represent the whole transcripts, which could only be detected when the real RNA sample is used. Because most OMTs have roles in the secondary metabolism, analysis of their tissue-specific expressions would reflect the tissue-specific deposition of such metabolites. We examined the expression pattern of six *AtOMT*s in different tissue such as flowers, roots, stems, and leaves by real-time PCR (Fig. 3). Expressions of *AtOMT1* and *AtOMT8* were detected at various levels in all tissues tested. Expression of *AtOMT1* was higher in leaves than in roots or flowers as described previously.[27] The expressions of *AtOMT1* in leaves and *AtOMT8* in flowers were about 28 and about 146 % of *actin*, respectively, whereas those of *AtOMT11* and *AtOMT16*, were not detected in leaves and *AtOMT12* was not expressed in stem (Fig. 3). *AtOMT10* was expressed only in flowers, suggesting its involvement in the antocyanin biosynthesis or volatile phenolic compounds such as 3, 5-dihydoxyanisole.[28] Analysis of the expression of these *AtOMT* genes might provide some clues regarding the cellular processes that *AtOMT* might be involved in, although the distinct enzymatic function of each gene cannot be directly elucidated. Assignment of the biochemical function of each *AtOMT* is an important next step. Two approaches could be applied to elucidate the biological functions of these genes. The first one is the *in vitro* analysis of individual genes by using the recombinant proteins. Drawback of this approach is that the substrate range must be narrowed due to the existence of many secondary metabolites. Therefore, the compounds represented by each group of flavonoids, alkaloids, and others would be the first choice. The second approach is to use the *Arabidopsis* mutant line. However, this approach also needs to be supported by *in vitro* analysis of individual genes, because *Arabidopsis* produces 170 secondary metabolites.[29] Without any information on the target metabolites, profiling of all the secondary metabolites and identification of the missing molecule would be a time-consuming task.[30]

Only a limited number of OMT genes have been functionally characterized in a particular organism. However, analysis of the plant genome shows several OMT genes, some of which are highly identical not only at the amino acid level but also at the nucleotide level such that they might result from gene

duplication. In *Arabidopsis*, at least 17 OMTs have been found. In the secondary metabolism, however, a high degree of sequence similarity with a known enzyme does not imply that the protein has the same substrate because highly divergent proteins catalyze the same reaction in different species.[31,32] Even though *AtOMT*s used in this study showed less than 40% homology at the amino acid level, *in vitro* substrate of some of *AtOMT*s might be same. But, their expression patterns in different tissuse might be different because their expression is likely to change depending on cell types or developmental stages. Thus, even among *AtOMT*s showing high sequence similarities and having same substrate, their expression patterns are tissue-specific, suggesting that their biological roles might be different depending upon tissues or stages of life.

Acknowledgments

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