Structure, function, and evolution of plant *O*-methyltransferases

Kevin C. Lam, Ragai K. Ibrahim, Bahareh Behdad, and Selvadurai Dayanandan

Abstract: Plant *O*-methyltransferases (OMTs) constitute a large family of enzymes that methylate the oxygen atom of a variety of secondary metabolites including phenylpropanoids, flavonoids, and alkaloids. *O*-Methylation plays a key role in lignin biosynthesis, stress tolerance, and disease resistance in plants. To gain insights into the evolution of the extraordinary diversity of plant *O*-methyltransferases, and to develop a framework phylogenetic tree for improved prediction of the putative function of newly identified OMT-like gene sequences, we performed a comparative and phylogenetic analysis of 61 biochemically characterized plant OMT protein sequences. The resulting phylogenetic tree revealed two major groups. One of the groups included two sister clades, one comprising the caffeoyl CoA OMTs (CCoA OMTs) that methylate phenolic hydroxyl groups of hydroxycinnamoyl CoA esters, and the other containing the carboxylic acid OMTs that methylate aliphatic carboxyl groups. The other group comprised the remaining OMTs, which act on a diverse group of metabolites including hydroxycinnamic acids, flavonoids, and alkaloids. The results suggest that some OMTs may have undergone convergent evolution, while others show divergent evolution. The high number of unique conserved regions within the CCoA OMTs and carboxylic acid OMTs provide an opportunity to design oligonucleotide primers to selectively amplify and characterize similar OMT genes from many plant species.

Key words: methylation, flavonoids, enzyme evolution, methyltransferase, caffeoyl CoA.

Résumé : Les *O*-méthyltransférases (OMT) végétales constituent une grande famille d'enzymes qui méthylent l'atome d'oxygène d'une panoplie de métabolites secondaires dont les phénylpropanoïdes, les flavonoïdes et les alcaloïdes. Chez les plantes, l'*O*-méthylation joue un rôle clé dans la synthèse de la lignine, la tolérance aux stress et la résistance aux maladies. Afin de mieux comprendre l'évolution de la diversité extraordinaire présente au sein des O-méthyltransférases végétales et pour développer un arbre phylogénétique cadre permettant une meilleure prédiction de la fonction chez des séquences nouvelles ressemblant à des OMT, les auteurs ont réalisé une étude comparative et phylogénétique de 61 séquences protéiques d'OMT végétales caractérisées sur le plan biochimique. L'arbre phylogénétique qui en résulte présente deux groupes principaux. Un des groupes inclut deux clades apparentés, l'un comprenant les caféoyl CoA OMT (CCoA OMT), lesquelles méthylent les groupes hydroxyles phénoliques chez les esters hydroxycinnamoyl CoA, et l'autre regroupe les carboxyl OMT, ces derniers méthylant les groupes carboxyles aliphatiques. Le second groupe comprend les autres OMT qui agissent sur une gamme variée de métabolites incluant les acides hydroxycinnamiques, les flavonoïdes et les alcaloïdes. Les évidences suggèrent que certaines OMT seraient le produit d'une évolution convergente tandis que d'autres résulteraient d'une évolution divergente. Les niveaux élevés de régions uniques conservées au sein des CCoAPMT et des OMT agissant sur les acides carboxyliques rendent possible la synthèse d'amorces permettant d'amplifier et de caractériser sélectivement des gènes codant pour des OMT semblables chez plusieurs espèces végétales.

Mots-clés : méthylation, flavonoïdes, évolution enzymatique, méthyltransférase, caféoyl CoA.

[Traduit par la Rédaction]

Introduction

One of the unique features of higher plants is their ability to synthesize a vast array of low molecular weight organic compounds, such as alkaloids, flavonoids, and terpenoids, collectively known as secondary plant metabolites. The origin and diversification of land plants may have been significantly influenced by the evolution of the extraordinary diversity of plant secondary metabolites and the biosynthesis of lignin, which provided higher plants with unprecedented opportunities to attract pollinators and seed dispersal agents, fight pathogens and herbivores, and adapt to terrestrial environmental conditions. The breadth of structural diversity of these metabolites is the result of a number of substitution reactions catalyzed by substrate- and position-specific enzymes. Among these reactions, enzymatic methylation, which is catalyzed by a large family of *S*-adenosyl-L-methionine–dependent methyltransferases, plays a major role. *O*-Methyltransferases (OMTs), one of the major groups of methyltransferases in plants, constitute a large family of enzymes that methylate the oxygen atom of a variety of secondary metabolites, mostly phenylpropanoids, flavonoids, and some alkaloids (Ibrahim et al. 1998; Ibrahim and Muzac 2000). Their methylated products play important roles in lignin biosynthesis, as pharmacologically active sub-

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stances, as antimicrobial compounds (phytoalexins), and in interactions of plants with the surrounding environment (Bohm 1998; Wink 2003 and references therein). Furthermore, *O*-methylation of secondary metabolites reduces the reactivity and (or) mutagenicity of their phenolic hydroxyl groups and alters their solubility, affecting the degree of carcinogenicity and antimicrobial activity (Zhu et al. 1994).

Most phenolic compounds, such as the simple phenols, phenylpropanoids, coumarins, and the flavonoid B-ring with its 3-C side chain (Fig. 1), are formed from L-phenylalanine, whereas the benzylisoquinoline (BIQ) alkaloids are formed from L-tyrosine. On the other hand, most *meta*-substituted phenolics, as well as the flavonoid A-ring, are derived from acetate moieties via the polyketide pathway. Flavonoid compounds, which include the flavanones, flavones, isoflavones, flavonols, and anthocyanidins, undergo a variety of enzymatic substitution reactions that bring about an enormous diversity of flavonoid structures (Ibrahim and Anzellotti 2003). Pterocarpans are structurally related to isoflavones, where position 3 is equivalent to position 7 of isoflavones (Fig. 1). They serve as direct precursors of phytoalexins, a class of widespread antimicrobial compounds characteristic of most species of the Leguminosae.

In contrast with mammalian enzymes, plant OMTs methylate a wide range of compounds with a high degree of selectivity, as exemplified by the stereospecific enzymes in *Chrysosplenium americanum* (Ibrahim et al. 1987) and *Mentha* ×*piperita* (Willits et al. 2004), both of which synthesize a variety of partially methylated flavonoids. However, a few OMTs have been shown to be multifunctional (promiscuous) enzymes that catalyze the methylation of structurally related compounds such as phenylpropanoids and flavonoids (Gauthier et al. 1998); BIQ alkaloids and phenylpropanoids (Frick and Kutchan 1999); stilbenes, phenylpropanoids, and flavonoids (Chiron et al. 2000); caffeic acid, caffeoyl CoA, catechol, and dimethylfuranone (Wein et al. 2002); and caffeoyl esters and flavonoids (Ibdah et al. 2003). In fact, most multifunctional enzymes utilize a variety of methyl acceptor molecules that possess 2 vicinal phenolic (catecholic) hydroxyl groups as substrates.

Several hypotheses have been proposed to explain the evolution of novel enzymes and biochemical pathways. Following the classical hypothesis, that mutations in genes leading to the utilization of other available substances when the primary substance is depleted are the key driving force in the evolution of biochemical synthesis (Horowitz 1945), several theories have been proposed to explain the evolution of novel genes in biochemical pathways (Pichersky and Gang 2000). Evolution of substrate-specific enzymes from catalytically promiscuous ones (Copley 2003) and evolution of promiscuous enzymes from substrate-specific ones (Aharoni et al. 2005) may have played equally significant roles in the evolution of various OMTs. In fact, Yoshikuni et al. (2006) recently constructed several specific terpene synthases from the promiscuous γ-humulene synthase gene by modifying a few amino acid residues. Although gene duplication and subsequent divergence are considered the main forces behind the evolution of proteins with novel functions (Ohno 1970), adaptive selection of specific alleles may also contribute to the evolution of novel genes without prior gene duplication (Pichersky and Gang 2000).

The recent increase in large-scale genome sequencing projects and the continued improvement of sequencing technology has led to the proliferation of a large number of gene sequences, including many OMT sequences, in public databases. In spite of the availability of protein sequence information for a large number of well-characterized and putative plant OMTs, the evolutionary relationships of these genes remain poorly understood. Comparative and phylogenetic analyses of these sequences are crucial for understanding the evolutionary history of the gene family, as well as prediction of the putative function of newly identified OMT sequences.

The objectives of the present study were to perform a comparative analysis of biochemically characterized plant OMT protein sequences and to reconstruct a framework phylogenetic tree. The results will facilitate (*a*) inference of evolutionary relationships among various OMTs, (*b*) identification of evolutionary trends among plant OMTs, and (*c*) improved prediction of the putative function of newly identified OMT-like gene sequences. Understanding the evolutionary relationships among plant OMTs will aid our understanding of the evolution of the extraordinary diversity of secondary metabolites in plants.

Methods

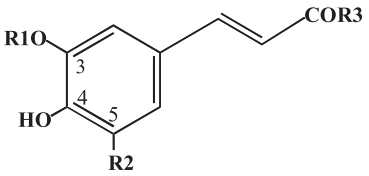
Multiple sequence alignment

We chose amino acid sequences of 61 biochemically characterized plant OMT genes (Table 1) from EMBL, GenBank, and SwissProt databases. Three non-plant OMT sequences (*Streptomyces*, *Bos*, and human) were used as outgroups. These sequences were aligned using CLUSTAL W software (Thompson et al. 1994), saved as a text file in PHYLIP format, and imported into the software program MacClade 4.03 (Maddison and Maddison 2000) for visual inspection and manual editing. The aligned sequence matrix was imported into GeneDoc version 2.7 software (Nicholas and Nicholas 1997) to annotate conserved residues within different groups of OMT sequences. The groupings were based on (*a*) clusters in the phylogenetic tree, (*b*) substrate type, (*c*) methylation of the A or B rings of flavonoids, and (*d*) methylation of the *meta* or *para* positions of flavonoid B rings. The PCR contrast option of GeneDoc was used for highlighting conserved residues unique to OMTs that methylate specific substrate types. Amino acid residues with known functional importance, such as active sites and substrate binding sites based on published data, were also annotated on the aligned sequence matrix.

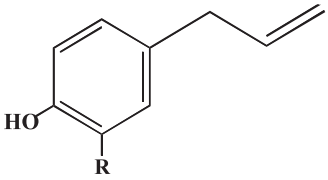
Phylogenetic analysis

The phylogenetic analyses were conducted using PAUP version 4.0, beta 10 (Swofford 2001). Maximum-parsimony phylogenetic trees were reconstructed through heuristic search with equal character weights, gaps treated as missing, and multi-state taxa interpreted as uncertainty. The starting tree was obtained via stepwise addition, and tree bisection–reconnection (TBR) was used as the branch-

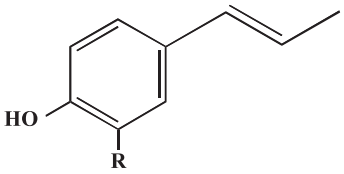
Fig. 1. Chemical structures of secondary metabolites utilized as methyl acceptor (substrate) molecules by various *O*-methyltransferases.



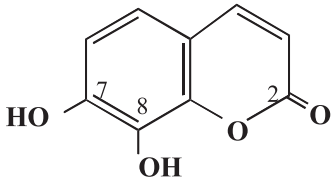
Hydroxycinnamic acids (HCAs)
Caffeic acid: R1, R2 = H; R3 = OH
5-Hydroxyferulic acid : R1 = Me;
R2 and R3 = OH
CoA derivatives: R3 = coenzyme A



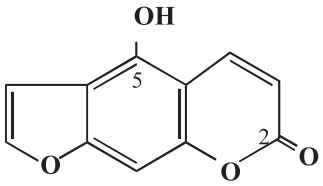
Phenylpropenes
Chavicol: R = H
Eugenol: R = OMe



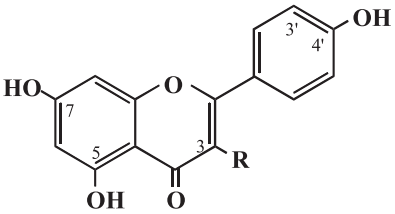
Isoeugenol: R = OMe



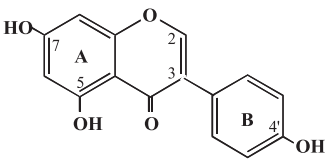
7,8-Dihydroxycoumarin
(Daphnetin)



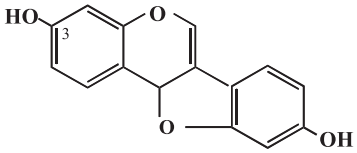
5-Hydroxyfuranocoumarin
(Bergaptol)



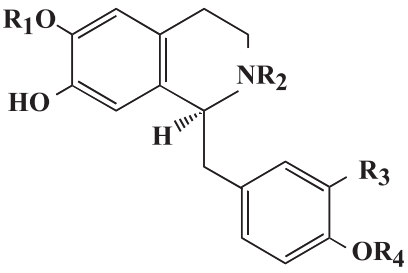
Flavonoids
Flavones: R = H
Flavonols: R = OH



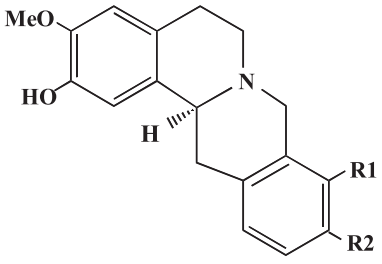
Isoflavone



Pteroarpan



Benzylisoquinoline (BIQ) alkaloids (all R, *S*)
Norcoclaurine: R1, R2, R3, R4 = H
Coclaurine: R1 = Me; R2, R3, R4 = H
Reticuline R1, R2, R4 = Me; R3 = OH



Tetrahydro-BIQ alkaloids
Scoulerine: R1 = OH; R2 = OMe
Tetrahydrocolumbamine: R1, R2 = OMe

swapping algorithm. Phylogenetic trees were rooted using 3 non-plant OMT sequences as outgroups. Bootstrap analysis with a fast heuristic search based on 1000 replicates was performed to assess the robustness of branches.

Results

The sequences used in the present analysis include OMTs that utilize caffeoyl-CoA esters (CCoA OMTs), hydroxycin-

namic acids, simple phenols, carboxylic acids (carboxyl OMTs), and (iso)flavonoids and alkaloids as well as a few multifunctional OMTs that utilize more than one substrate (Fig. 1, Table 1). Of the 61 selected sequences, 9 belong to group I OMTs that utilize acetyl-CoA derivatives, and the remaining sequences are group II OMTs (Joshi and Chiang 1998). The maximum-parsimony analyses yielded 2 equally parsimonious trees (total number of characters = 485; tree length = 6404 steps; consistency index = 0.542; retention index = 0.653) that differed at the positions of *Vitis*, *Populus*, and *Medicago* CCoA OMTs. Thus, these 3 branches collapsed in the strict consensus tree, and the remaining branches remained fully resolved. One of the 2 equally parsimonious trees is shown in Fig. 2. The resulting phylogenetic tree revealed 2 distinct monophyletic lineages. One of these, lineage A, included 2 sister clades: lineage A1 comprised the CCoA OMTs that methylate phenolic hydroxyl groups of hydroxycinnamoyl-CoA esters and the other, lineage A2, contained the carboxyl OMTs that methylate the aliphatic carboxyl groups to their methyl ester derivatives. Clustering of OMT sequences into these sister clades was strongly supported by bootstrap values of 100%. The grouping of these 2 clades together was supported by a bootstrap value of 71%. Moreover, these 2 groups of OMTs are structurally distinct, as evidenced by the relatively long branch lengths, reflecting a large number of amino acid changes from their most recent common ancestral sequence (Fig. 2). In the CCoA OMTs (group A1), the *Stellaria longipes* (chickweed) and *Mesembryanthemum crystallinum* (ice plant) OMTs clustered together, forming a basal branch among the CCoA OMTs (Fig. 2). These 2 sequences, which utilize both caffeoyl-CoA/glucose esters and flavonoid substrates, show close sequence similarities to each other but are highly divergent from the rest of the CCoA OMTs, although they share a number of conserved regions with this group (Fig. S2[2]).

Lineage B comprised the remaining OMTs, namely hydroxycinnamic acid (HCA), (iso)flavonoid, and BIQ alkaloid OMTs. The alkaloid columbamine *N*-methyltransferase of *Coptis japonica* was basal to this group, followed by a group comprising *M.* ×*piperita* flavonoid 4′- and 7-OMTs. The remaining OMTs of this group formed 2 major groups, B1 and B2. Group B1 included 17 OMTs comprising a mixture of alkaloid and flavonoid OMTs (Fig. 2). The flavonoid A-ring and B-ring 4′-methylating enzymes were also confined to this group. Group B2 comprised 29 sequences that included most of the HCA OMTs, several multifunctional OMTs, and flavonoid and alkaloid OMTs, as well as OMTs utilizing di- and tri-hydroxyphenolics, a carbohydrate (*myo*-inositol), a furanocoumarin, and isoeugenol. With the exception of *Catharanthus roseus* 3′/5′-OMT (group B1), all known OMTs that methylate the flavonoid 3′ or 3′/5′ positions were confined to group B2. A clade combining the bifunctional (CCoA/HCA) *Pinus taeda* OMT and the *Papaver somniferum* reticuline 7-OMT occupied a basal position in group B2, suggesting that this group may have derived from an ancestral sequence closely related to the *P. taeda* OMT. The *Hordeum vulgare* OMT (U54767), which was previously reported as an HCA OMT (Lee et al. 1997) but recently characterized as an alkaloid *N*-methyltransferase involved in gramine biosynthesis (Larsson et al. 2006), occupied the next basal position, followed by the chalcone OMT, a dihydric phenol OMT, and a group of multifunctional (dihydric phenol, BIQ alkaloids, and HCA) OMTs. The HCA OMTs occupied terminal branches of this clade. A few OMTs that methylate structurally similar compounds were located in different clades (Fig. 2). For example, the *Clarkia breweri* isoeugenol OMT clustered in clade B2, whereas the isomeric *Ocimum basilicum* chavicol and eugenol OMTs grouped within clade B1, suggesting that these OMTs may have undergone convergent or repeated evolution. Similarly, the *Triticum aestivum* flavone OMT (clade B2) and the *C. roseus* flavonol 3′/5′-OMT (clade B1) showed a polyphyletic origin.

The annotation of conserved residues in groups based on clustering of sequences on the phylogenetic tree showed a large number of conserved residues in groups A1 and A2 and a moderate number of conserved residues in groups B1 and B2 (Fig. S1[2]). Similarly, in the grouping based on substrate type, a large number of conserved residues were found in CCoA OMTs (group A1) and carboxyl OMTs (group A2), and a moderate number were found in groups of OMTs that methylate various substrates (Fig. S2). A moderate number of residues were conserved in the groups based on OMTs methylating A or B rings of flavonoids (Fig. S3[2]) and *meta* or *para* positions of flavonoids (Fig. S4[2]). The PCR-contrast shading revealed 37 residues unique to CCoA OMTs and 28 residues unique to carboxyl OMTs. The other substrate-methylating OMT groups did not show unique residues conserved among all members of the group.

Discussion

The resulting phylogenetic tree (Fig. 2) shows that OMTs segregate into 2 major clusters, one consisting of group I CCoA OMTs and carboxyl OMTs and the other consisting of group II OMTs. The group I OMTs are believed to be mostly involved in the biosynthesis of the guaiacyl-type lignin derived from the methylation of caffeoyl-CoA, which occurs in the primitive forms of land plants including pteridophytes and gymnosperms. Thus, it is reasonable to assume that CCoA OMTs may have evolved earlier during the evolution of vascular plants.

The earlier work that led to the cloning and characterization of the first OMT cDNAs involved in lignin biosynthesis (Bugos et al. 1991; Gowri et al. 1991), as well as those reported later from other plant species, indicated that the HCAs, caffeic acid (CA) and (or) 5-hydroxyferulic acid (5HFA), designated here as phenylpropanoids (Fig. 1), were the preferred substrates for the OMTs involved in lignin biosynthesis. Their respective methylated products, ferulic and sinapic acids, act as precursors of the guaiacyl and syringyl residues of lignin, respectively (Lewis and Yamamoto 1990). More recently, it was demonstrated that the aldehydes and (or) alcohols of both phenylpropanoids also act

[2] Supplementary data for this article are available on the journal Web site (http://genome.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5226. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

Table 1. Structural and functional features of some representative *O*-methyltransferase (OMT) cDNA clones derived from different organisms and involved in the methylation of the indicated substrates.

Accession number	Organism	No. of amino acids (kDa)	Preferred substrates	Biological significance	Reference
Cinnamoyl CoA (CCoA) OMTs					
U20736	*Medicago sativa* (alfalfa)	247 (n.d.)	Caffeoyl-CoA > 5HF-CoA > 5HFA	Lignin biosynthesis	Inoue et al. 1998
Z54233	*Vitis vinifera*	242 (27)	Caffeoyl-CoA > 5HF-CoA	Lignin biosynthesis, stress response	Busam et al. 1997
AJ224894	*Populus trichocarpa*	247 (29)	Caffeoyl-CoA	Lignin biosynthesis	Meyermans et al. 2000
M69184	*Petroselium crispum* (parsley)	241 (27)	Caffeoyl-CoA	Lignin biosynthesis, defence response to pathogens	Schmitt et al. 1991
AF036095	*Pinus taeda* (loblolly pine)	259 (29.1)	Caffeoyl-CoA > 5HF-CoA	Lignin biosynthesis	Li et al. 1999
U38612	*Nicotiana tabacum* (tobacco)	239 (27)	Caffeoyl-CoA, 5HF-CoA	Lignin biosynthesis	Martz et al. 1998
U13151	*Zinnia elegans*	245 (27.6)	Caffeoyl-CoA	Lignin biosynthesis	Ye et al. 1994
L22203	*Stellaria longipes* (chickweed)	241 (28)	Caffeoyl-CoA	Phenylpropanoid metabolism	Zhang and Chinnappa 1997
AY145521	*Mesembryanthemum crystallinum* (ice plant)	237 (26.6)	Caffeoyl esters, 6-OH-flavonols	Phenylpropanoid and flavonoid metabolism	Ibdah et al. 2003
Hydroxycinnamic acid (HCA) OMTs					
U16793	*Chrysosplenium americanum*	343 (37.9)	5HFA ≈ flavonoids > CA	Phenylpropanoid and flavonoid metabolism	Gauthier et al. 1998
U19911	*Zinnia elegans*	354 (38.6)	5HFA > CA	Lignin biosynthesis	Ye and Varner 1995
U54767	*Hordeum vulgare* (barley)	376 (42)	CA > catechol 3-aminomethyl indole*	Lignin biosynthesis (reannotated as gramine biosynthesis)*	Lee et al. 1997; Larsson et al. 2006
U39301	*Pinus taeda*	381 (42)	CA > 5HFA and their CoA esters	Lignin biosynthesis (bifunctional OMT)	Li et al. 1997
M63853	*Medicago sativa*	365 (39.9)	CA > 5HFA	Lignin biosynthesis, defence response	Gowri et al. 1991
AF064693	*Thalictrum tuberosum*	364 (74) (dimer)	*o*-Diphenols >> BIQ alkaloids	Antimicrobial alkaloid biosynthesis	Frick and Kutchan 1999
AF064694	*Thalictrum tuberosum*	362 (74)	CA > catechol >> BIQ alkaloids	Antimicrobial alkaloid biosynthesis	Frick and Kutchan 1999
AF064695	*Thalictrum tuberosum*	362	*o*-Dihydric phenols >> BIQ alkaloids	Antimicrobial alkaloid biosynthesis	Frick and Kutchan 1999
AF064696	*Thalictrum tuberosum*	364	*o*-Dihydric phenols, BIQ alkaloids	Antimicrobial alkaloid biosynthesis	Frick and Kutchan 1999
U86760	*Clarkia breweri*	368 (40)	Isoeugenol > eugenol	Floral scent biosynthesis	Wang et al. 1997
AF010291	*Lolium perenne* (ryegrass)	361 (38.7)	CA	Lignin biosynthesis	McAlister et al. 1998
M73235	*Zea mays*	364 (39.6)	CA	Lignin biosynthesis	Collazo et al. 1992
X62096	*Populus tremuloides* (aspen)	365 (39.8)	5HFA > CA	Lignin biosynthesis	Bugos et al. 1991
AY177404	*Secale cereale* (rye)	355 (38)	Daphnetin	Regulation of cold acclimation (8-OMT)	NDong et al. 2003
AF139533	*Liquidambar styraciflua* (sweet gum)	367 (n.d.)	Coniferyl aldehyde	Lignin biosynthesis	Osakabe et al. 1999
AF435007	*Ocimum basilicum* (basil)	356 (39.9)	Chavicol	Phenylpropene biosynthesis	Gang et al. 2002
AF435008	*Ocimum basilicum*	357 (40.2)	Eugenol	Phenylpropene biosynthesis	Gang et al. 2002
AY443007	*Ammi majus*	365 (n.d.)	Methyl caffeate > 5HFA > CA	Phenylpropanoid metabolism	Hehmann et al. 2004

Table 1 (*continued*).

Accession number	Organism	No. of amino acids (kDa)	Preferred substrates	Biological significance	Reference
AY443006	*Ammi majus*	354 (38.7)	Bergaptol	Furanocoumarin biosynthesis (5/8-OMT)	Hehmann et al. 2004
AY028439	*Catharanthus roseus* (periwinkle)	363 (n.d.)	5HFA > CA > flavonoids	Phenylpropanoid and flavonoid biosynthesis	Schröder et al. 2004
Simple phenol OMTs					
X71430	*Nicotiana tabacum*	365 (40.5)	*o*-Dihydric phenols	Lignin biosynthesis (defence response)	Pellegrini et al. 1993
AB121046	*Rosa chinensis*	371 (44.0)	Phloroglucinol	Biosynthesis of rose floral scent compounds	Wu et al. 2004
AF502433	*Rosa hybrida*	367 (n.d.)	Orcinol	Biosynthesis of rose scent volatiles	Lavid et al. 2002
AF502434	*Rosa hybrida*	366 (n.d.)	Orcinol monomethylether		
Carboxylic acid OMTs					
At5g55250	*Arabidopsis thaliana*	386 (n.d.)	Indole-3-acetic acid	Growth-hormone regulation	Zubieta et al. 2003
AY008434	*Arabidopsis thaliana*	389 (45)	Jasmonic acid	Jasmonate-regulated responses	Seo et al. 2001
AF198492	*Antirrhinum majus*	364 (49)	Benzoic acid	Floral scent biosynthesis	Dudareva et al. 2000
AF133053	*Clarkia breweri*	359 (40.3)	Salicylic acid > benzoic acid	Floral scent biosynthesis	Ross et al. 1999
Other OMTs					
M87340	*Mesembryanthenum crystallinum*	365 (40.2)	*Myo*-inositol	Osmotic stress	Vernon and Bohnert 1992
Flavonoid OMTs					
U70424	*Arabidopsis thaliana*	363 (40.5)	Quercetin > myricetin	Flavonoid biosynthesis (3′-OMT)	Muzac et al. 2000
L10211	*Medicago sativa* (2′-OMT)	373 (37.6)	Isoliquiritigenin (chalcone)	Induction of *Nod* genes (2′-OMT)	Maxwell et al. 1993
U16794	*Chrysosplenium americanum*	345 (37.6)	Partially methylated flavonols	Flavonoid biosynthesis (3′/5′-OMT)	Gauthier et al. 1996
AY337457	*Mentha ×piperita* (peppermint)	344 (38)	Flavones > flavonols >> isoflavones	Flavonoid biosynthesis (7-OMT)	Willits et al. 2004
AY337458	*Mentha ×piperita*	344 (38)	Flavones > flavonols	Flavonoid biosynthesis (7-OMT)	Willits et al. 2004
AY337459	*Mentha ×piperita*	366 (40.8)	Various flavonols	Flavonoid biosynthesis (8-OMT)	Willits et al. 2004
AY337460	*Mentha ×piperita* (3′-OMT)	364 (40.3)	Various flavonoids	Flavonoid biosynthesis (3′-OMT)	Willits et al. 2004
AY337461	*Mentha ×piperita*	343 (37.8)	Various flavonoids	Flavonoid biosynthesis (4′-OMT)	Willits et al. 2004
U97125	*Medicago sativa*	352 (41)	6,7,4′-triOH-isoflavone > daidzein >> pterocarpan	Phytoalexin biosynthesis	He et al. 1998
AB091684	*Glycyrrhiza echinata*	367 (n.d.)	2,7,4′-triOH-isoflavanone	Phytoalexin biosynthesis	Akashi et al. 2003
AB091686	*Lotus japonicus*	365 (n.d.)	2,7,4′-triOH-isoflavanone	Phytoalexin biosynthesis	Akashi et al. 2003
AB091685	*Glycyrrhiza echinata*	357 (n.d.)	Daidzein	Phytoalexin biosynthesis	Akashi et al. 2003
U69554	*Pisum sativum*	360 (40.4)	6a-hydroxymaackiain > maackiain > medicarpin	Phytoalexin biosynthesis	Wu et al. 1997
X77467	*Hordeum vulgare*	390 (42.3)	Apigenin > naringenin	Phytoalexin biosynthesis (7-OMT)	Christensen et al. 1998
AY127568	*Catharanthus roseus*	348 (39.1)	Myricetin > dihydromyricetin	3′,5′-Methylation of flavonoids	Cacace et al. 2003
DQ223971	*Triticum aestivum*	356 (38.5)	Tricetin > chrysoeriol >> CA	Sequential 3′,4′,5′-methylation of tricetin	Zhou et al. 2006
Benzylisoquinoline (BIQ) alkaloid OMTs					
D29811	*Coptis japonica* (6-OMT)	347 (40)	Norcoclaurine	BIQ alkaloid biosynthesis	Morishige et al. 2000

Table 1 (*concluded*).

Accession number	Organism	No. of amino acids (kDa)	Preferred substrates	Biological significance	Reference
AB073908	*Coptis japonica*	351 (40)	Columbamine	Palmatine biosynthesis	Morishige et al. 2002
D29812	*Coptis japonica* (3′-/4′-OMT)	350 (41)	Various BIQ alkaloids	BIQ alkaloid biosynthesis	Morishige et al. 2000
D29809	*Coptis japonica* (9-OMT)	364 (41)	Scoulerine	Tetrahydro-BIQ alkaloid biosynthesis	Takeshita et al. 1995
AY268893	*Papaver somniferum*	356 (39.8)	Guaiacol > reticuline	BIQ alkaloid biosynthesis	Ounaroon et al. 2003
AY268894	*Papaver somniferum* (6-OMT)	347 (38.5)	Norcoclaurine	BIQ alkaloid biosynthesis	Ounaroon et al. 2003

Note: BIQ, benzylisoquinoline; CoA, coenzyme A ester; CA, caffeic acid; 5HFA, 5-hydroxyferulic acid; n.d., not determined.
*Reannotated gene.

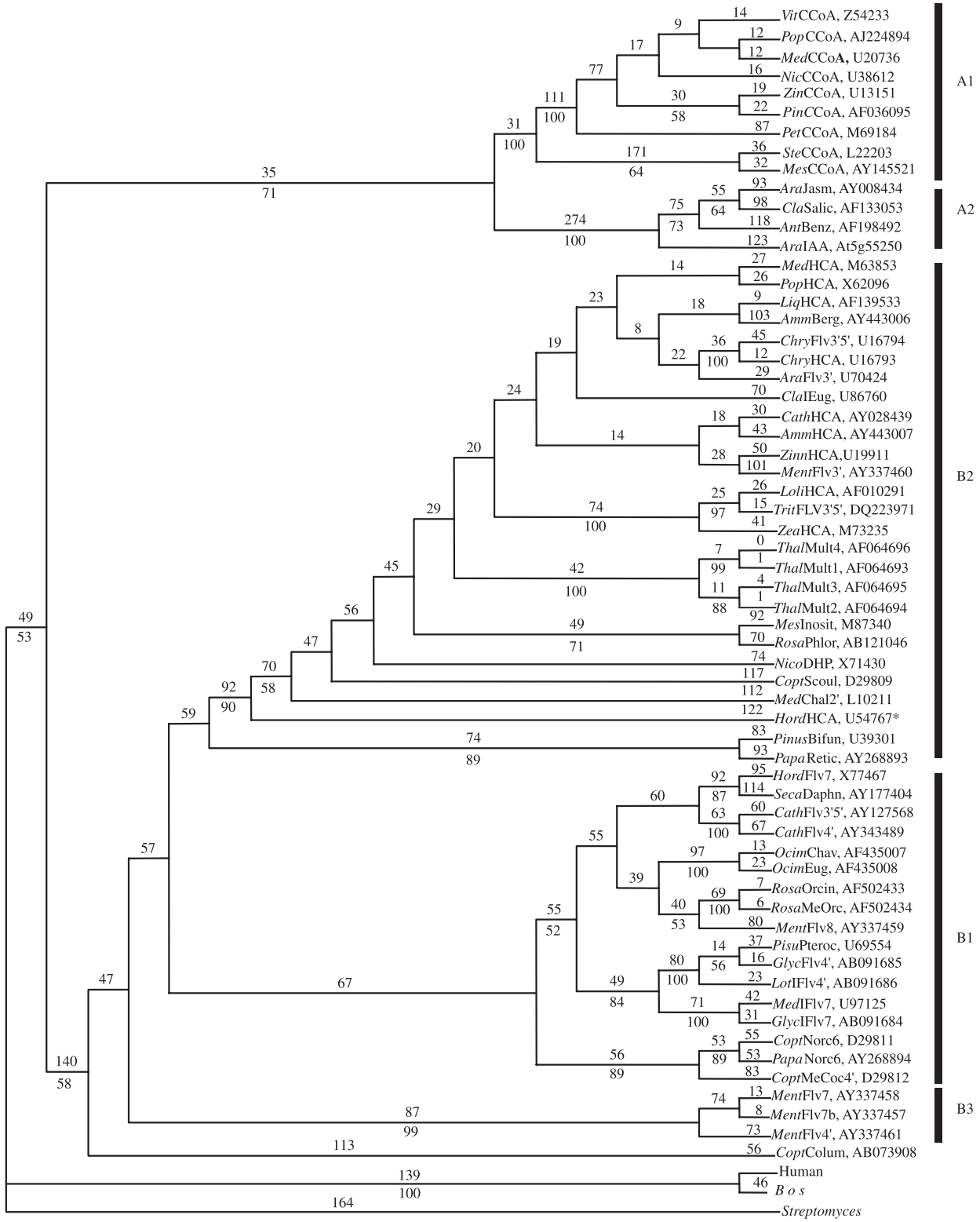
as good methyl acceptors (Parvathi et al. 2001). The phenylpropanoid OMTs are subdivided into 2 groups (Joshi and Chiang 1998). The OMTs that accept only the coenzyme A (CoA) esters of CA and 5HFA (i.e., CCoA OMTs) as the preferred substrates and that are involved in lignin biosynthesis as described above are categorized as group I. These OMTs are smaller (approx. 28–30 kDa) and lack the 5 consensus sequence motifs characteristic of group II OMTs (Ibrahim 1997). The group II OMTs, on the other hand, include all of the remaining plant OMTs and may have evolved later during the course of plant evolution. Other phenylpropanoid compounds recently reported as methyl acceptors include the phenylpropenes chavicol, eugenol, and isoeugenol (Fig. 1). These are characterized by having a terminal methyl or methylene group instead of a carboxyl group in the 3-carbon side chains, and that methylation takes place predominantly on a hydroxyl group *para* to the side chain. These phenylpropenes and their methylated products constitute an important component of the floral scent in *C. breweri* (Wang et al. 1997).

The genes encoding carboxyl OMTs may have evolved through duplication and subsequent divergence of the ancestral enzyme sequence that gave rise to CCoA OMTs. The carboxyl OMTs catalyze the methylation of a variety of acids including benzoic, salicylic, indoleacetic, and jasmonic acids. The methylated products of both benzoic and salicylic acids act as components of the floral scent in *Antirrhinum* (Dudareva et al. 2000) and *Clarkia* spp. (Wang et al. 1997), respectively. Carboxyl methylation of indoleacetic acid plays an important role in the regulation of plant growth and development, and methylated jasmonic acid acts as a signal molecule (Croteau et al. 2000). These OMTs are classified as group II enzymes and their cDNA sequences, although similar to each other, are distinct from those of other members of the group.

The fact that group II OMTs utilize substrates with a high degree of structural diversity suggests that many evolutionary forces may have played significant roles during the evolution of this group. Following initial duplication and evolutionary divergence, further fine-tuning of their assemblies, and convergent evolution, these genes may have given rise to the functional diversity of group II OMTs with their wide variety of substrates and regiospecificities. The segregation of the different group II OMTs into distinct subclasses follows, with few exceptions, the segregation by structural type of their preferred substrates, which comprise various phenylpropanoids including HCAs, phenylpropenes, (iso)flavonoids, and BIQ alkaloids. The biosynthetic origin of these molecules could also be considered an important factor in the evolution of these OMTs. Except for the BIQ alkaloids, which are derived from L-tyrosine, all the phenylpropanoid and flavonoid compounds are formed from L-phenylalanine.

The occurrence of structurally similar genes in different species that act on the same substrate, such as those for norcoclaurine 6-OMT in *C. japonica* and *P. somniferum*; isoflavone 7-OMT in *Glycyrrhiza echinata* and *Medicago sativa*; isoflavone 4′-OMT in *Lotus japonicus* and *G. echinata*; and HCA OMTs in *C. roseus*, *Ammi majus*, *Zea mays*, *Lolium perenne*, *Populus* spp., and *M. sativa*, supports the evolutionary conservation of these genes across several species.

Fig. 2. One of the two equally parsimonious trees (length = 6404 steps; consistency index = 0.542; retention index = 0.653) identified by equally weighted parsimony analysis using aligned amino acid sequences of biochemically characterized plant *O*-methyltransferases. The other equally parsimonious tree was identical to this tree with a single exception in which *Pop*CCoA clustered with *Vit*CCoA. A heuristic search was performed with stepwise-simple sequence addition, TBR branch swapping, steepest descent off, and MulTrees on using PAUP 4.0b10. Branch length (above the line) and bootstrap values based on 1000 replicates (below the line) are given at each branch. The name of each branch is an abbreviation based on genus name, substrate type, and GenBank accession number. See Table 1 for names of genera, substrate preference(s), and corresponding references. Human, *Bos*, and *Streptomyces O*-methyltransferase sequences were used as outgroups.



However, several examples supporting convergent as well as divergent evolution are also found among group II OMTs. Structurally similar genes that act on a variety of different substrates indicate that some OMT genes have undergone divergent evolution. Some of the examples that support divergent evolution are the flavonoid 4′- and 7-OMTs in *M.* ×*piperata*; orcinol and methyl orcinol OMTs in *Rosa* spp.; chavicol and eugenol OMTs in *O. basilicum*; and flavonol 4′-OMT and flavonol 3′/5′-OMT in *C. japonica* and flavanol 3′/5′-OMT and HCA OMT in *C. americanum*. Although some genes may have been lost during the course of evolution in certain species, some of the divergently evolved genes are retained in other species, as exemplified by the pterocarpan OMT in *Pisum sativum* and isoflavone 4′-OMT in *G. echinata*; flavonoid 7-OMT in *H. vulgare* and daphnetin 8-OMT in *Secale cereale*; CCoA OMT in *P. taeda* and reticuline OMT in *P. somniferum*; phloroglucinol OMT in *Rosa* spp.; *myo*-inositol OMT in *M. crystallinum*; furanocoumarin OMT in *A. majus*; and flavanol 3′-OMT in *M.* ×*piperita*. Several examples of convergent evolution, where structurally different enzymes act on similar substrates, include chavicol and eugenol OMTs in *O. basilicum* and isoeugenol OMT in *C. breweri*; flavonoid 4′-OMT in *M.* ×*piperita*, *G. echinata*, and *C. japonica*; flavonoid 7-OMT in *M.* ×*piperita*, *G. echinata*, and *H. vulgare*; flavonoid 3′/5′-OMT in *C. japonica* and flavonoid 3′-OMT in *M.* ×*piperita*, *Arabidopsis thaliana*, and *C. americanum*; and several other examples of HCA OMTs in a variety of species.

The alkaloid-specific OMTs show convergent or repeated evolution and occupy various clades on the phylogenetic tree. The 6-norcoclaurine and *N*-methylcoclaurine 4′-OMTs are located within a single clade. The C-terminal ends of these two OMTs are highly conserved for the *S*-adenosyl-L-methionine (AdoMet) binding site, whereas the N-terminal ends that constitute the putative alkaloid-binding pockets are more divergent. Therefore, the sequence differences between these two OMTs reflect the substrate specificity of this group (Morishige et al. 2002). However, the scoulerine and reticuline OMTs occupy different clades, and the columbamine-methylating enzyme, which catalyzes *N*- rather than *O*-methylation of BIQ alkaloids, occupies a separate clade. This pattern of distribution suggests that alkaloid-methylating enzymes may have evolved multiple times from different ancestral sequences. The 4 enzymes from *Thalictrum tuberosum* show broad substrate specificity for phenylpropanoids and BIQ alkaloids, among others (Frick and Kutchan 1999). The presence of several copies of the genes that encode multifunctional enzymes indicates that these genes may serve as a potential source for evolution of a diverse array of enzymes utilizing a variety of substrates. Similar multifunctional OMTs capable of methylating several compounds including caffeic acid and dimethyl methoxy furanone, one of the aroma compounds in strawberry fruits (Wein et al. 2002), have been reported in the literature. Such multifunctional enzymes may serve as precursors for evolution of a variety of OMTs.

In contrast with the phenylalanine-derived flavonoids, BIQ alkaloids are derived from L-tyrosine by the condensation of its decarboxylation/deamination products to form the first stable intermediate, (*S*)-norcoclaurine. Further hydroxylation and *N*- or *O*-methylation of the latter results in the formation of the analgesic morphinan and the antimicrobial berberine groups of BIQ alkaloids (Facchini 2006). These include norcoclaurine, coclaurine, *N*-methylcoclaurine, reticuline, scoulerine, and tetrahydrocolumbine (Fig. 1). Because of the structural similarity of the substrates involved, the AdoMet-catalyzed, position-specific methyl transfer reactions are essential for directing these intermediates to the specific biosynthetic pathways of this important group of alkaloids.

Based on the three-dimensional structure and evolutionary relationships, Martin and McMillan (2002) classified the protein structures found in various databases, including >100 structures of 50 distinct AdoMet-dependent methyltransferases from 31 different classes. The AdoMet-dependent methyltransferases exhibit 5 different structural protein folds (classes I–V), and the majority of known group II OMTs exhibit the class I fold (Schubert et al. 2003). Although highly conserved, the class I family contains several members with a variety of structural features such as monomers, homodimers, and tetramers; different topologies, including different numbers of α-helices and β-strands; and different structures of binding sites. This implies that the structural and catalytic requirements for methyl transfer from AdoMet are remarkably flexible. More recently, however, homology-based modeling of the *Arabidopsis* flavonol 3′-OMT, AtOMT1, using alfalfa COMT as a template (which shares high amino acid sequence similarity with AtOMT1) indicated that a single amino acid difference between the binding sites of AtOMT1 and COMT (Val^{314} versus Ile^{316}, respectively) may account for the strict substrate specificity of both enzymes (Yang et al. 2004).

Although the AdoMet-dependent OMTs share a common three-dimensional structure (Vidgren et al. 1994; Zubieta et al. 2001), they may have peripheral elements in their secondary structure and turn regions that differ in size and conformation depending on their evolutionary origin and biochemical function. Annotation of predicted secondary structure information and residues of known functional significance for the sequences analyzed in this study allowed us to determine the conserved regions common to each group, family, or clade of these OMTs (Fig. S1), as well as the residues conserved in groups based on substrate preference (Fig. S2), A-ring versus B-ring methylating OMTs (Fig. S3), and *para*- versus *meta*-methylating flavonoid OMTs (Fig. S4). Most of these characteristics are conserved within the same OMT group and, to some extent, between the different groups. This analysis is useful in prediction of the function of putative and new OMTs. Furthermore, these structural comparisons indicate that the functional characteristics of OMTs are in fact encrypted in their primary sequences and should allow the design of novel enzymes with desirable characteristics.

The large number of conserved residues in groups A1 and A2 (CCoA OMTs and carboxyl OMTs) indicates that members of these groups are structurally very similar to each other and less diverse compared with other OMTs. This may be an indication that either OMT sequences in these groups are evolutionarily more closely related or their divergence rate is limited because of structural or functional constraints. On the other hand, OMTs of the remaining clades,

Table 2. The locations of putative *O*-methyltransferase gene sequences similar to selected sequences representative of each major group of *O*-methyltransferases in complete genome sequences of *Arabidopsis*, *Oryza*, and *Populus*, identified through MegaBLAST analysis using the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview).

Group	Query	Genome	Location	Map element	Position
A1	U38612 (CCoA)	*Arabidopsis*	Chromosome 4	NC003075	16310925
		Oryza	Chromosome 6	NC008399	3313581
		Populus	Linkage group I	NC008467	26414046
		Populus	Linkage group IX	NC008475	4059506
A2	AY008434 (jasmonate)	*Arabidopsis*	Chromosome 1	NC003070	6791711
B1	AY337459 (flavonoid 8)	*Populus*	Linkage group XIX	NC008485	10083569
B2	U70424 (flavonoid 3′)	*Arabidopsis*	Chromosome 5	NC003076	21999223
		Oryza	Chromosome 8	NC008401	3334130
		Populus	Linkage group XII	NC008478	3089348
		Populus	Linkage group XV	NC008481	256147

or the ones that methylate other substrate types, show fewer conserved amino acid residues, indicating high heterogeneity of amino acid sequences within these groups. This is evidence that CCoA OMTs and carboxyl OMTs primarily undergo divergent evolution through gradual stepwise modification of preexisting sequences, whereas convergent evolution of OMTs in other groups may hinder the formation of group-specific amino acid residues common to all OMTs methylating a given substrate type. The existence of conserved residues unique to CCoA OMTs and carboxyl OMTs (Fig. S5[2]) provides an opportunity to design oligonucleotide primers specific for these groups and selectively amplify corresponding genes from a variety of plant groups to isolate and further characterize these OMTs in many plant species. Although OMTs of group A2 are important in many functional aspects in plants including signal transduction and stress tolerance, surprisingly only a few sequences of these genes are currently available. Isolation of genes of the group A2 family through direct amplification from genomic DNA using PCR technology as well as through screening of cDNA libraries with specific probes will be invaluable for expanding our knowledge of this family of genes and gaining insights into signal transduction and stress tolerance mechanisms in plants. This could serve as a foundation for plant improvement through breeding and biotechnological methods.

Investigation of the location and copy number of selected OMT sequences representative of each OMT group in currently available complete genome sequences of plants (*Arabidopsis*, *Oryza*, and *Populus*) using cross-species MegaBLAST analysis through the NCBI Map Viewer Web interface (http://www.ncbi.nlm.nih.gov/mapview/) revealed that the group A1 (CCoA) and group B2 (flavonoid 3′) related gene sequences were found in all 3 genomes examined, whereas the group A2 (jasmonate) and group B1 (flavonoid 8) related gene sequences were found only in *Arabidopsis* and *Populus* genomes, respectively (Table 2). Further studies involving detailed analysis of the distribution and diversity of OMT gene sequences in these 3 whole genomes will be valuable for furthering our understanding of the evolution and diversification of OMT genes in the plant kingdom.

In contrast with previous studies that were conducted on a limited number of OMTs, including many putative sequences (Joshi and Chiang 1998; Ibrahim et al. 1998; Ibrahim and Muzac 2000), the present study clearly demonstrates that the phylogenetic relationships among the biochemically characterized OMTs are corroborated, not only on the basis of amino acid sequence similarities and the number of amino acid changes from their common ancestors, but also on the basis of structural and regiospecific similarities of their methyl acceptor molecules. Oligonucleotide primers can be designed based upon amino acid motifs unique to branches leading to selected groups of OMTs and used as a direct tool for molecular cloning of target OMTs.

Addendum

Recently published gibberellin-methylating OMT sequences (Varbanova et al. 2007. Plant Cell, **19**: 32–45) cluster with AraIAA in the A2 clade.

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References

Aharoni, A.M., Gaidukov, L., Khersonsky, O., Gould, S.M., Roodveldt, C., and Tawfiq, D.S. 2005. The 'evolvability' of promiscuous protein functions. Nat. Genet. **37**: 73–76. PMID: 15568024.

Akashi, T., Sawada, Y., Shimada, N., Sakurai, N., Aoki, T., and Ayabe, S. 2003. cDNA cloning and biochemical characterization of *S*-adenosyl-L-methionine:2,7,4′-trihydroxyisoflavone 4′-*O*-methyltransferase, a critical enzyme of the legume isoflavone phytoalexin pathway. Plant Cell Physiol. **44**: 103–112. doi:10.1093/pcp/pcg034. PMID:12610212.

Bohm, B.A. 1998. Flavonoid functions in nature. *In* Introduction to flavonoids. Chemistry and biochemistry of organic natural products. Vol. 2. Harwood Academic Publishers, Amsterdam. pp. 339–364.

Bugos, R., Chiang, V., and Campbell, W. 1991. cDNA cloning, sequence analysis and seasonal expression of a lignin-specific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase of aspen. Plant Mol. Biol. **17**: 1203–1215. doi:10.1007/BF00028736. PMID:1932694.

Busam, G., Junghanns, K., Kneusel, R., Kassemeyer, H.-H., and

Matern, U. 1997. Characterization and expression of caffeoyl coenzyme A 3-*O*-methyltransferase proposed for the induced resistance response of *Vitis vinifera* L. Plant Physiol. **115**: 1039–1048. doi:10.1104/pp.115.3.1039. PMID:9390437.

Cacace, S., Schröder, G., Wehinger, E., Strack, D., Schmidt, J., and Schröder, J. 2003. A flavonol *O*-methyltransferase from *Catharanthus roseus* performing two sequential methylations. Phytochemistry, **62**: 127–137. doi:10.1016/S0031-9422(02)00483-1. PMID:12482447.

Chiron, H., Drouet, A., Claudot, A.-C., Eckerskorn, C., Trost, M., Heller, W., Ernst, D., and Sandermann, H., Jr. 2000. Molecular cloning and functional expression of a stress-induced multifunctional *O*-methyltransferase with pinosylvin methyltransferase activity from Scots pine (*Pinus sylvestris* L.). Plant Mol. Biol. **44**: 733–745. doi:10.1023/A:1026507707186.

Christensen, A., Gregersen, P., Olsen, C., and Collinge, D. 1998. A flavonoid 7-*O*-methyltransferase is expressed in barley leaves in response to pathogen attack. Plant Mol. Biol. **36**: 219–227. doi:10.1023/A:1005985609313. PMID:9484434.

Collazo, P., Montoliu, L., Puigdomenech, P., and Rigau, J. 1992. Structure and expression of the lignin *O*-methyltransferase gene from *Zea mays* L. Plant Mol. Biol. **20**: 857–867. doi:10.1007/BF00027157. PMID:1463825.

Copley, S.D. 2003. Enzymes with extra talents: moonlighting functions and catalytic promiscuity. Curr. Opin. Chem. Biol. **7**: 265–272. doi:10.1016/S1367-5931(03)00032-2. PMID: 12714060.

Croteau, R., Kutchan, T.M., and Lewis, N.G. 2000. Natural products (secondary metabolites). *In* Biochemistry and molecular biology of plants. *Edited by* B. Buchanan, W. Gruissem, and R. Jones. American Society of Plant Physiologists, Rockville, Md. pp. 1250–1318.

Dudareva, N., Murfitt, L., Mann, C., Gorenstein, N., Kolosova, N., Kish, C., Bonham, C., and Wood, K. 2000. Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. Plant Cell, **12**: 949–961. doi:10.1105/tpc.12.6.949. PMID:10852939.

Facchini, P.J. 2006. Regulation of alkaloid biosynthesis in plants. Alkaloids Chem. Biol. **63**: 1–44. PMID:17133713.

Frick, S., and Kutchan, T.M. 1999. Molecular cloning and functional expression of *O*-methyltransferases common to isoquinoline alkaloid and phenylpropanoid biosynthesis. Plant J. **17**: 329–339. doi:10.1046/j.1365-313X.1999.00379.x. PMID: 10205892.

Gang, D., Lavid, N., Zubieta, C., Chen, F., Beuerle, T., Lewinsohn, E., Noel, J., and Pichersky, E. 2002. Characterization of phenylpropene *O*-methyltransferases from sweet basil: facile change of substrate specificity and convergent evolution within a plant *O*-methyltransferase family. Plant Cell, **14**: 505–519. doi:10.1105/tpc.010327. PMID:11884690.

Gauthier, A., Gulick, P., and Ibrahim, R.K. 1996. cDNA cloning and characterization of a 3′/5′-*O*-methyltransferase for partially methylated flavonols from *Chrysosplenium americanum.* Plant Mol. Biol. **32**: 1163–1169. doi:10.1007/BF00041401. PMID: 9002616.

Gauthier, A., Gulick, P., and Ibrahim, R.K. 1998. Characterization of two cDNA clones which encode *O*-methyltransferases for the methylation of both flavonoid and phenylpropanoid compounds. Arch. Biochem. Biophys. **351**: 243–249. doi:10.1006/abbi.1997.0554. PMID:9514654.

Gowri, G., Bugos, R., Campbell, W., Maxwell, C., and Dixon, R.A. 1991. Stress responses in alfalfa (*Medicago sativa* L.). X. Molecular cloning and expression of *S*-adenosyl-L-methionine:caffeic acid 3-*O*-methyltransferase, a key enzyme of lignin biosynthesis. Plant Physiol. **97**: 7–14. PMID:16668418.

He, X.-Z., Reddy, J., and Dixon, R.A. 1998. Stress responses in alfalfa (*Medicago sativa* L.). XXII. cDNA cloning and characterization of an elicitor-inducible isoflavone 7-*O*-methyltransferase. Plant Mol. Biol. **36**: 43–54. doi:10.1023/A:1005938121453. PMID:9484461.

Hehmann, M., Lukacin, R., Ekiert, H., and Matern, U. 2004. Furanocoumarin biosynthesis in *Ammi majus* L.: cloning of bergaptol *O*-methyltransferase. Eur. J. Biochem. **271**: 932–940. doi:10.1111/j.1432-1033.2004.03995.x. PMID:15009205.

Horowitz, N.H. 1945. On the evolution of biochemical synthesis. Proc. Natl. Acad. Sci. U.S.A. **31**: 153–157. doi:10.1073/pnas.31.6.153. PMID:16578152.

Ibdah, M., Zhang, X.-H., Schmidt, J., and Vogt, T. 2003. A novel Mg^{2+}-dependent *O*-methyltransferase in the phenylpropanoid metabolism of *Mesembryanthemum crystallinum*. J. Biol. Chem. **278**: 43961–43972. doi:10.1074/jbc.M304932200. PMID: 12941960.

Ibrahim, R.K. 1997. Plant *O*-methyltransferase signatures. Trends Plant Sci. **2**: 249–250. doi:10.1016/S1360-1385(97)86345-5.

Ibrahim, R.K., and Anzellotti, D. 2003. The enzymatic basis of flavonoid biodiversity. *In* Integrative phytochemistry: from ethnobotany to molecular ecology. *Edited by* J.T. Romeo. Pergamon, New York. pp. 1–36.

Ibrahim, R.K., and Muzac, I. 2000. The methyltransferase gene superfamily: a tree with multiple branches. *In* Evolution of metabolic pathways. *Edited by* J.T. Romeo, R.K. Ibrahim, L. Varin, and V. De Luca. Pergamon, New York. pp. 349–384.

Ibrahim, R.K., De Luca, V., Khouri, H., Latchinian, L., Brisson, L., and Charest, J.-P. 1987. Enzymology and compartmentation of polymethylated flavonol glucosides in *Chrysosplenium americanum*. Phytochemistry, **26**: 1237–1245. doi:10.1016/S0031-9422(00)81789-6.

Ibrahim, R.K., Bruneau, A., and Bantignies, B. 1998. Plant *O*-methyltransferases: molecular analysis, common signature and classification. Plant Mol. Biol. **36**: 1–10. doi:10.1023/A:1005939803300. PMID:9484457.

Inoue, K., Sewalt, V., Balance, G., Ni, W., Stürzer, C., and Dixon, R.A. 1998. Developmental expression and substrate specificities of alfalfa caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase in relation to lignification. Plant Physiol. **117**: 761–770. doi:10.1104/pp.117.3.761. PMID: 9662519.

Joshi, C.P., and Chiang, V.L. 1998. Conserved sequence motifs in plant *S*-adenosyl-L-methionine-dependent methyltransferases. Plant Mol. Biol. **37**: 663–674. doi:10.1023/A:1006035210889. PMID:9687070.

Larsson, K.A.E., Zetterlund, I., Delp, G., and Jonsson, L.M.V. 2006. *N*-Methyltransferase involved in gramine biosynthesis in barley: cloning and characterization. Phytochemistry, **67**: 2002–2008. doi:10.1016/j.phytochem.2006.06.036.

Lavid, N., Wang, J., Shalit, M., Guterman, I., Bar, E., Bruerle, T., et al. 2002. *O*-Methyltransferases involved in the biosynthesis of volatile phenolic derivatives in rose petals. Plant Physiol. **129**: 1899–1907. doi:10.1104/pp.005330. PMID:12177504.

Lee, J., Vogt, T., Hause, B., and Löbler, M. 1997. Methyl jasmonate induces an *O*-methyltransferase in barley. Plant Cell Physiol. **38**: 851–862. PMID:9297848.

Lewis, N.G., and Yamamoto, E. 1990. Lignin: occurrence, biogenesis and biodegradation. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**: 455–496. doi:10.1146/annurev.pp.41.060190.002323. PMID:11543592.

Li, L., Popko, J., Zhang, X.-H., Osakabe, K., Tsai, C.-J., Joshi, C., and Chiang, V. 1997. A novel multifunctional *O*-methyltransferase implicated in a dual methylation pathway associated with

lignin biosynthesis in loblolly pine. Proc. Natl. Acad. Sci. U.S.A. **94**: 5461–5466. doi:10.1073/pnas.94.10.5461. PMID: 9144260.

Li, L., Osakabe, Y., Joshi, C., and Chiang, V.L. 1999. Secondary xylem-specific expression of caffeoyl coenzyme A 3-*O*-methyltransferase plays an important role in the methylation pathway associated with lignin biosynthesis in loblolly pine. Plant Mol. Biol. **40**: 555–565. doi:10.1023/A:1006244325250. PMID:10480380.

Maddison, D.R., and Maddison, W.P. 2000. MacClade. Version 4.02 [computer program]. Sinauer Associates, Sunderland, Mass.

Martin, J.L., and McMillan, F.M. 2002. *S*-Adenosylmethionine-dependent methyltransferase fold. Curr. Opin. Struct. Biol. **12**: 783–793. doi:10.1016/S0959-440X(02)00391-3. PMID: 12504684.

Martz, F., Maury, S., Pincon, G., and Legrand, M. 1998. cDNA cloning, substrate specificity and expression study of tobacco caffeoyl CoA 3-*O*-methyltransferase, a lignin biosynthetic enzyme. Plant Mol. Biol. **36**: 427–437. doi:10.1023/A:1005969825070. PMID:9484483.

Maxwell, C., Harrison, M., and Dixon, R.A. 1993. Molecular characterization and expression of alfalfa isoliquiritigenin 2′-*O*-methyltransferase, an enzyme specifically involved in the biosynthesis of an inducer of *Rhizobium meliloti* nodulation genes. Plant J. **4**: 971–981. doi:10.1046/j.1365-313X.1993.04060971.x. PMID:8281189.

McAlister, F., Jenkins, C., and Watson, J. 1998. Sequence and expression of a stem-abundant caffeic acid *O*-methyltransferase cDNA from perennial ryegrass (*Lolium perenne*). Aust. J. Plant Physiol. **25**: 225–235.

Meyermans, H., Morreel, K., Lapierre, C., Pollet, B., De Bruyn, A., Busson, R., et al. 2000. Modifications in lignin and accumulation of phenolic glucosides in poplar xylem upon down-regulation of caffeoyl coenzyme A *O*-methyltransferase, an enzyme involved in lignin biosynthesis. J. Biol. Chem. **275**: 36899–36909. doi:10.1074/jbc.M006915200. PMID:10934215.

Morishige, T., Tsujita, T., Yamada, Y., and Sato, F. 2000. Molecular characterization of *S*-adenosyl-L-methionine: 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase involved in isoquinoline alkaloid biosynthesis in *Coptis japonica.* J. Biol. Chem. **275**: 23398–23405. doi:10.1074/jbc.M002439200. PMID: 10811648.

Morishige, T., Dubouzet, E., Choi, K.-B., Yazaki, K., and Sato, F. 2002. Molecular cloning of columbamine *O*-methyltransferase from cultured *Coptis japonica* cells. Eur. J. Biochem. **269**: 5659–5667. doi:10.1046/j.1432-1033.2002.03275.x. PMID:12423366.

Muzac, I., Wang, J., Anzellotti, D., Zhang, H., and Ibrahim, R.K. 2000. Functional expression of an *Arabidopsis* cDNA clone encoding a flavonol 3′-*O*-methyltransferase and characterization of the gene product. Arch. Biochem. Biophys. **375**: 385–388. doi:10.1006/abbi.1999.1681. PMID:10700397.

NDong, C., Anzellotti, D., Ibrahim, R.K., Huner, N., and Sarhan, F. 2003. Daphnetin methylation by a novel *O*-methyltransferase is associated with cold acclimation and photosystem II excitation pressure in rye. J. Biol. Chem. **278**: 6854–6861. doi:10.1074/jbc.M209439200. PMID:12480941.

Nicholas, K.B., and Nicholas, H.B. 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the authors. Available from http://www.nrbsc.org/downloads/.

Ohno, S. 1970. Evolution by gene duplication. Springer-Verlag, New York.

Osakabe, K., Tsao, C., Li, L., Popko, J., Umezawa, T., Carraway, D., et al. 1999. Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proc. Natl. Acad. Sci. U.S.A. **96**: 8955–8960. doi:10.1073/pnas.96.16.8955. PMID:10430877.

Ounaroon, A., Decker, G., Schmidt, J., Lottspiech, F., and Kutchan, T.M. 2003. (*R*,*S*)-Reticuline 7-*O*-methyltransferase and (*R*,*S*)-norcoclaurine 6-*O*-methyltransferase of *Papaver somniferum*: cDNA cloning and characterization of methyl transfer enzymes of alkaloid biosynthesis in opium poppy. Plant J. **36**: 808–819. doi:10.1046/j.1365-313X.2003.01928.x. PMID:14675446.

Parvathi, K., Chen, F., Guo, D., Blount, J.W., and Dixon, R.A. 2001. Substrate preferences of *O*-methyltransferases in alfalfa suggest new pathways for 3-*O*-methylation of monolignols. Plant J. **25**: 193–202. doi:10.1046/j.1365-313x.2001.00956.x. PMID:11169195.

Pellegrini, L., Geoffroy, P., Fritig, B., and Legrand, M. 1993. Molecular cloning and expression of a new class of *o*-diphenol *O*-methyltransferases induced in tobacco (*Nicotiana tabacum* L.) leaves by infection or elicitor treatment. Plant Physiol. **103**: 509–517. doi:10.1104/pp.103.2.509. PMID:7518088.

Pichersky, E., and Gang, D.R. 2000. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci. **5**: 439–445. doi:10.1016/S1360-1385(00)01741-6. PMID:11044721.

Ross, J., Nam, K., D'Auria, J., and Pichersky, E. 1999. *S*-Adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. Arch. Biochem. Biophys. **367**: 9–16. doi:10.1006/abbi.1999.1255. PMID: 10375393.

Schmitt, D., Pakusch, A.-E., and Matern, U. 1991. Molecular cloning, induction and taxonomic distribution of caffeoyl CoA 3-*O*-methyltransferase, an enzyme involved in disease resistance. J. Biol. Chem. **266**: 17416–17423. PMID:1894629.

Schröder, G., Wehinger, E., Lukačin, R., Wellmann, F., Seefelder, W., Schwab, W., and Schröder, J. 2004. Flavonoid methylation: a novel 4′-*O*-methyltransferase from *Catharanthus roseus*, and evidence that partially methylated flavanones are substrates of four different flavonoid dioxygenases. Phytochemistry, **65**: 1085–1094. doi:10.1016/j.phytochem.2004.02.010. PMID: 15110688.

Schubert, H.L., Blumethal, R.M., and Cheng, X. 2003. Many paths to methyltransfer: a chronicle of convergence. Trends Biochem. Sci. **28**: 329–335. doi:10.1016/S0968-0004(03)00090-2. PMID: 12826405.

Seo, H., Song, J., Cheong, J.-J., Lee, Y.-H., Lee, Y.-W., Hwang, I., Lee, J., and Choi, Y. 2001. Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. U.S.A. **98**: 4788–4793. doi:10.1073/pnas.081557298. PMID:11287667.

Swofford, D.L. 2001. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Version 4 [computer program]. Sinauer Associates, Sunderland, Mass.

Takeshita, N., Fujiwara, H., Mimura, H., Fitchen, J., Yamada, Y., and Sato, F. 1995. Molecular cloning and characterization of *S*-adenosyl-L-methionine:scoulerine 9-*O*-methyltransferase from cultured cells of *Coptis japonica*. Plant Cell Physiol. **36**: 29–36. PMID:7719631.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W — improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673–4680. doi:10.1093/nar/22.22.4673. PMID:7984417.

Vernon, D., and Bohnert, H. 1992. A novel methyltransferase induced by osmotic stress in the facultative halophyte *Mesem-*

bryanthemum crystallinum. EMBO J. **11**: 2077–2085. PMID: 1600940.

Vidgren, J., Svensson, L.A., and Liljas, A. 1994. Crystal structure of catechol *O*-methyltransferase. Nature (London), **368**: 354–358. doi:10.1038/368354a0. PMID:8127373.

Wang, J., Dudareva, N., Bhakta, S., Raguso, R., and Pichersky, E. 1997. Floral scent production in *Clarkia breweri* (Onagraceae). II. Localization and developmental modulation of the enzyme *S*-adenosyl-L-methionine:(iso)eugenol *O*-methyltransferase and phenylpropanoid emission. Plant Physiol. **114**: 213–221. doi:10.1104/pp.114.1.213. PMID:9159948.

Wein, M., Lavid, N., Lunkenbein, S., Lewinsohn, E., Schwab, W., and Kaldenhoff, R. 2002. Isolation, cloning and expression of a multifunctional *O*-methyltransferase capable of forming 2,5-dimethyl-4-methoxy-4(2H)-furanone, one of the key aroma compounds in strawberry fruits. Plant J. **31**: 755–765. doi:10.1046/j.1365-313X.2002.01396.x. PMID:12220266.

Willits, M.G., Giovanni, M., Prata, R., Kramer, C., De Luca, V., Steffens, J., and Graser, G. 2004. Biotransformation of modified flavonoids: an example of in vivo diversification of secondary metabolites. Phytochemistry, **65**: 31–41. doi:10.1016/j.phytochem.2003.10.005. PMID:14697269.

Wink, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry, **64**: 3–19. doi:10.1016/S0031-9422(03)00300-5. PMID:12946402.

Wu, Q., Preisig, C., and VanEtten, H. 1997. Isolation of the cDNA encoding (+)6a-hydroxymaackiain 3-*O*-methyltransferase, the terminal step for the synthesis of the phytoalexin pisatin in *Pisum sativum.* Plant Mol. Biol. **35**: 551–560. doi:10.1023/A:1005836508844. PMID:9349277.

Wu, S., Watanabe, N., Mita, S., Dohra, H., Ueda, Y., Shibuya, M., and Ebizuka, Y. 2004. The key role of phloroglucinol *O*-methyltransferase in the biosynthesis of *Rosa chinensis* volatile 1,3,5-trimethoxybenzene. Plant Physiol. **135**: 95–102. doi:10.1104/pp.103.037051. PMID:15122041.

Yang, H., Ahn, J.-H., Ibrahim, R.K., Lee, S., and Lim, Y. 2004. The three-dimensional structure of *Arabidopsis thaliana O*-methyltransferase by homology-based modelling. J. Mol. Graph. Model. **23**: 77–87. doi:10.1016/j.jmgm.2004.02.001. PMID: 15331056.

Ye, Z.-H., and Varner, J. 1995. Differential expression of two *O*-methyltransferases in lignin biosynthesis in *Zinnia elegans.* Plant Physiol. **108**: 459–467. doi:10.1104/pp.108.2.459. PMID: 7610157.

Ye, Z.-H., Kneusel, R., Matern, U., and Varner, J.E. 1994. An alternative methylation pathway in lignin biosynthesis in *Zinnia.* Plant Cell, **6**: 1427–1439. doi:10.1105/tpc.6.10.1427. PMID: 7994176.

Yoshikuni, Y., Ferrin, T.E., and Keasling, J.D. 2006. Designed divergent evolution of enzyme function. Nature (London), **440**: 1078–1082. doi:10.1038/nature04607. PMID:16495946.

Zhang, X.-H., and Chinnappa, C. 1997. Molecular characterization of a cDNA encoding caffeoyl coenzyme A 3-*O*-methyltransferase of *Stellaria longipes*. J. Biosci. **22**: 161–175.

Zhou, J.-M., Gold, N.D., Martin, V.J.J., Wollenweber, E., and Ibrahim, R.K. 2006. Sequential *O*-methylation of tricetin by a single gene product in wheat. Biochim. Biophys. Acta, **1760**: 1115–1124. PMID:16730127.

Zhu, B.T., Enzell, E.L., and Liehr, J.G. 1994. Catechol *O*-methyltransferase-catalyzed rapid *O*-methylation of mutagenic flavonoids. J. Biol. Chem. **269**: 292–299. PMID:8276810.

Zubieta, C., He, X.-Z., Dixon, R.A., and Noel, J.P. 2001. Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant *O*-methyltransferases. Nat. Struct. Biol. **8**: 271–279. doi:10.1038/85029. PMID:11224575.

Zubieta, C., Ross, J., Koscheski, P., Yang, Y., Pichersky, E., and Noel, J. 2003. Structural basis for substrate recognition in the salicylic acid carboxyl methyltransferase family. Plant Cell, **15**: 1704–1716. doi:10.1105/tpc.014548. PMID:12897246.