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Evolution of novel *O*-methyltransferases from the *Vanilla planifolia* caffeic acid *O*-methyltransferase

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Abstract

The biosynthesis of many plant secondary compounds involves the methylation of one or more hydroxyl groups, catalyzed by *O*-methyltransferases (OMTs). Here, we report the characterization of two OMTs, Van OMT-2 and Van OMT-3, from the orchid *Vanilla planifolia* Andrews. These enzymes catalyze the methylation of a single outer hydroxyl group in substrates possessing a 1,2,3-trihydroxybenzene moiety, such as methyl gallate and myricetin. This is a substrate requirement not previously reported for any OMTs. Based on sequence analysis these enzymes are most similar to caffeic acid *O*-methyltransferases (COMTs), but they have negligible activity with typical COMT substrates. Seven of 12 conserved substrate-binding residues in COMTs are altered in Van OMT-2 and Van OMT-3. Phylogenetic analysis of the sequences suggests that Van OMT-2 and Van OMT-3 evolved from the *V. planifolia* COMT. These *V. planifolia* OMTs are new instances of COMT-like enzymes with novel substrate preferences.

Abbreviations: COMT, Caffeic acid *O*-methyltransferase; FOMT, flavonoid *O*-methyltransferase; IEMT, (iso)eugenol *O*-methyltransferase; IPTG, isopropyl-β-D-thiogalactopyranoside; OMT, *O*-methyltransferase; PCR, polymerase chain reaction; SAM, S-adenosyl-L-methionine

Introduction

The biosynthesis of many plant secondary compounds involves the methylation of one or more hydroxyl groups (Schroder *et al.*, 2002). Identification of the *O*-methyltransferases (OMTs) involved has been achieved for some secondary metabolites. These enzymes catalyze the transfer of the methyl group from S-adenosylmethionine (SAM) to a hydroxyl group of the secondary metabolite. Plant OMTS have been categorized into two classes, based primarily on protein sequence (Joshi and Chiang, 1998). The most widely studied members of the Class I OMTs are the caffeoyl coenzyme A OMTs, which methylate the lignin precursor caffeoyl-CoA (Humphreys and Chapple, 2002). The most widely studied Class II OMTs are caffeic acid *O*-methyltransferases (COMTs), which are involved in the synthesis of S-lignin. Although still referred to as COMTs, the preferred substrates are actually caffeoyl aldehyde and 5-hydroxyconiferaldehyde (Osakabe *et al.*, 1999; Li *et al.*, 2000; Dixon *et al.*, 2001; Parvathi *et al.*, 2001). Other Class II OMTs catalyze the methylation of flavonoids, flavonols, phenylpropenes, and phenolics.

In general, the Class II OMTs with similar substrate preferences group together in phylogenetic sequence comparisons (Ibrahim *et al*., 1998; Schroder *et al*., 2002; Gang, 2005). There are, however, some interesting cases where the experimentally determined substrate preferences of a Class II OMT are quite different from what would be predicted based on protein sequence comparisons. For example, a COMT and an (iso)eugenol *O*-methyltransferase (IEMT) from *Clarkia breweri* were characterized that have 83% amino acid identity yet have distinct substrate specificities (Wang and Pichersky, 1998). Site-directed mutagenesis (Wang and Pichersky, 1999) and molecular modeling (Zubieta *et al*., 2002) revealed that the distinct substrate specificities between the two *C. breweri* OMTs could be explained by differences in a few substrate binding residues. Similarly, two OMTs from *Chrysosplenium americanum* are 83% identical, yet one methylates both COMT substrates as well as some flavonoids and the other is inactive with COMT substrates but is active with a different group of flavonoids (Gauthier *et al*., 1996, 1998). The *C. americanum* flavonoid OMT (FOMT) differs from the COMT at seven of the COMT substrate binding sites (Zubieta *et al*., 2002), suggesting that these residues are important for the observed substrate discrimination between the two enzymes. These results from the *C. breweri* and *C. americanum* enzymes support the hypothesis that gene duplication followed by sequence changes in COMTs that affect substrate binding are the evolutionary origins of related OMTs that are involved in the synthesis of some of the numerous secondary metabolites synthesized by plants (Pichersky and Gang, 2000).

The cured pods (called beans) of the orchid *Vanilla planifolia* are the major natural source of the secondary metabolite vanillin (4-hydroxy-3-methoxybenzaldehyde), which is the most widely used flavor compound in the world (Havkin-Frenkel *et al*., 2005). The biosynthetic pathway for vanillin has not been demonstrated, although several possible pathways have been proposed (Havkin-Frenkel *et al*., 1999; Dignum *et al*., 2001; Walton *et al*., 2003). As the final step in one proposed pathway 3,4-dihydroxybenzaldehyde is methylated at the 3-position producing vanillin (Havkin-Frenkel *et al*., 1999). We have obtained evidence for such an activity in bean crude extracts (Pak *et al*., 2004). Through our search for such an enzyme we have fortuitously identified two *V. planifolia* OMTs that methylate 1,2,3-trihydroxy phenolic compounds, such as methyl gallate. One of the enzymes also methylates the flavonol myricetin. Both of the new *V. planifolia* enzymes require a 1,2,3-trihydroxybenzene moiety for significant activity, a substrate requirement that has not been reported for any other OMTs. The deduced protein sequences of these enzymes are most similar to reported COMTs, yet they have no activity against typical COMT substrates. These two *V. planifolia* OMTs are new instances of COMT-like enzymes with novel substrate preferences and are new examples of the evolution of novel biosynthetic enzymes from enzymes of primary metabolism.

Materials and methods

Materials

Mature *V. planifolia*, *V. pompona*, *V. phaeantha*, *Cattleya* sp., *Dendrobium* sp., *Epidendrum* sp., and *Iwanagara* sp. plants, maintained in the greenhouse, were the source of stem, leaf, root, flower, and bean tissues. Compounds used as possible substrates were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Indofine Chemical company Inc. (Hillsborough, NJ, USA).

Isolation of cDNA clones for Van OMT-2 and Van OMT-3

The *V. planifolia* COMT cDNA clone was isolated by using the polymerase chain reaction (PCR) with degenerate oligonucleotides that amplified a 350 bp fragment from a cDNA library prepared from *V. planifolia* tissue cultures (Pak *et al*., 2004). Fourteen of sixteen PCR clones that were sequenced were the COMT. Two of sixteen clones had a different DNA and deduced amino acid sequence, yet were also similar to COMTs. One of these PCR clones was used to screen a cDNA library constructed by Stratagene (LaJolla, CA, USA) in the λ ZAP-Express vector from poly (A^+) RNA from *V. planifolia* bean tissue. The PCR fragment was labeled with $[\alpha^{32}P]$ dCTP using a commercial kit (Prime-It II Random Primer Labeling kit, Stratagene) and was used to screen 500,000 plaque-forming units from the bean cDNA library.

The plaque lifts were prehybridized at 42 °C in 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution [1 × Denhardt's solution is 0.02% (w/v) Ficoll, 0.02% (w/v) PVP, 0.02% (w/v) BSA], 50 mM sodium phosphate, pH 6.8, 1% (w/v) SDS, 100 μg ml^{-1} calf thymus DNA, and 2.5 % (w/v) dextran sulfate. The hybridization solution was 5×10^5 cpm ml^{-1} of ^{32}P-labeled fragment, 50% (v/v) formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate, pH 6.8, 1% (w/v) SDS, 100 μg ml^{-1} calf thymus DNA, and 5% (w/v) dextran sulfate. Hybridized membranes were washed with 2 × SSPE (20×SSPE is 20 mM disodium EDTA, 160 mM sodium hydroxide, 200 mM monobasic sodium phosphate, and 3.6 M sodium chloride), 0.5% (w/v) SDS for 15 min at room temperature, 2 × SSPE, 0.5% (w/v) SDS for 15 min at 65 °C, and 0.2 × SSPE, 0.2% (w/v) SDS for 15 min at 65 °C. The washed filters were exposed to X-ray film (XOMAT-AR, Kodak, Rochester, NY, USA) with an intensifying screen. Positive plaques were subjected to two additional rounds of screening to isolate single positive plaques. The cDNA inserts from positive plaques were excised from the λ-vector as recombinant pBK-CMV phagemids (Short *et al*., 1988). Two full-length clones, designated Van OMT-2 and Van OMT-3, were completely sequenced by primer walking.

Phylogenetic analysis

The CLUSTAL-X (Thompson *et al*., 1997) program was used to align amino acid sequences with the Gonnet scoring matrices. Phylogenetic analysis was performed with the PAUP* program (Version 4.0b10 for Macintosh) (Swofford, 2002). A rooted phylogenetic tree was generated using the maximum parsimony method employing an heuristic search strategy. Gaps were treated as missing data. For rooting the tree, the *Picea abies* COMT sequence was designated as the outgroup. To determine relative level of support for the tree topology, bootstrap values were generated from 1000 replicates.

Expression of Van OMT-2 and Van OMT-3 in E. coli

The coding sequences of Van OMT-2 and Van OMT-3 were amplified by PCR with oligonucleotides that introduced *Nde*I sites at the 5′ and 3′ ends. The oligonucleotide sequences at the 5′ ends of the coding sequences were 5′-GGCAGCCATATGATGGGTTCTATTCCAAAGAAC-3′ and 5′-GGCAGCCATATGATGGGTTCTATTCCAAACAAC-3′ for Van OMT-2 and Van OMT-3, respectively. For both, the sequence of the oliogonucleotide at the 3′ end of the coding sequence was 5′-CGGATCCATATGCTAATTGGTGAATTCCAT-3′. The amplification reaction was carried out by using the Elongase Amplification System (Invitrogen, Carlsbad, CA, USA) in a GeneAmp 9600 thermocycler (Perkin-Elmer Life Sciences, Boston, MA, USA). The 100 μl reactions contained 60 mM Tris–SO_4, pH 9.1, 18 mM $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$, 200 μM each dNTP, 20 ng of each oligonucleotide, and 2 μl Elongase enzyme mix. Five nanogram plasmid DNA that had the full-length Van OMT-2 or Van OMT-3 cDNA was used as template. The initial denaturation was conducted at 94 °C for 2 min, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 46 °C, and 2 min extension at 68 °C. An additional 10 min extension at 68 °C was performed. Five microlitre of the PCR product was checked in a 1% agarose gel and the rest of the amplified fragment was purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA).

The purified PCR fragment was digested with *Nde*I, separated in a 1% agarose gel and purified with a QIAquick Gel Extraction Kit (Qiagen). The pET-15b expression vector (Novagen, Madison, WI, USA) was digested overnight with *Nde*I and dephosphorylated with 10 u μg^{-1} shrimp alkaline phosphatase (Promega, Madison, WI, USA) by incubating at 37 °C for 15 min. The phosphatase was inactivated by a 20 min incubation at 65 °C. The dephosphorylated vector was ethanol precipitated and redissolved in TE to 0.1 μg μl^{-1}. The Van OMT-2 and Van OMT-3 PCR fragments were ligated to the *Nde*I-digested dephosphorylated vector overnight at room temperature and then transformed into XL1-Blue MRF' Kan electroporation-competent cells (pCR-Script Amp Electroporation Competent Cell Cloning Kit, Stratagene). Plasmids from positive transformants were extracted with a QIAprep Miniprep kit (Qiagen) and fully sequenced using the T7 primer (5′-TAATACGACTCACTATAGGG-3′) and an internal primer (5′-TCCCAGTTGACCAAC-

CATAA-3′) (Davis Sequencing, Davis, CA, USA). Plasmids containing the correct sequences of Van OMT-2 and Van OMT-3, as well as a pET-15b empty vector control, were transformed to BL21-CodonPlus (DE3)-RIPL competent cells (Stratagene).

For purification of the recombinant protein, a BL21-CodonPlus (DE3)-RIPL transformant was grown at 37 °C at 200 rpm overnight in 4 ml LB medium supplemented with 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} chloramphenicol. The entire culture was then transferred to 100 ml fresh LB medium supplemented with 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} chloramphenicol and grown at 37 °C to an OD600 of 0.5 (about 2 h). The culture was transferred to a shaker at room temperature (22 °C) for 10 min to cool. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.05 mM. The cells were grown at room temperature for 4 h and collected by centrifugation at 6000 rpm for 20 min. The bacterial cell pellet was stored at −80 °C.

The recombinant protein was purified with a MagneHis Protein Purification System (Promega, Mannheim, Germany) according to the manufacturer's suggested protocol. The cell pellet was suspended in 20 ml Cell Lysis Reagent and shaken at room temperature for 30 min. The cell lysate was gently mixed with 1.5 ml Ni particles at room temperature for 2 min and placed on a magnetic stand (PolyATract System 1000, Promega) for 2 min. The supernatant was gently removed. The sample tube was removed from the magnetic stand and the Ni particles were washed with 10 ml Binding/Wash Buffer by pipetting for 2 min. The sample tube was placed on the magnetic stand for 2 min and the supernatant was removed. The wash step was repeated twice. The recombinant protein was then eluted from the Ni particles with 2 ml Elution Buffer. The elution step was repeated once. The eluted protein samples were pooled and stored on ice. The purified recombinant protein was concentrated and the buffer changed to 50 mM Tris–Cl, pH 7.5 and 2 mM DTT using Centricon-30 concentrators (Amicon, Beverly, MA, USA). Protein concentration was determined with the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard. Generally 200–400 μg of purified protein could be recovered from a 100 ml culture. The purified recombinant proteins were stored in small aliquots at −80 °C.

Enzyme assay and preparation of tissue extracts

O-Methyltransferase assays were as described by Wang *et al.* (1997). For screening potential substrates, the assays were carried out in 50 μl volumes consisting of 10 μl assay buffer (250 mM Tris–Cl, pH 7.5, 10 mM dithiothreitol), 1 μl 50 mM substrate dissolved in DMSO, 3 μg purified recombinant enzyme, and 1 μl S-[methyl−^{14}C]adenosyl-L-methionine (SAM) (56–58 mCi mmol^{-1}) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The final concentration of [^{14}C]SAM was 8.4 μM. The samples were incubated at 30 °C for 30 min, after which the reactions were stopped by the addition of 2.6 μl 6 M HCL. [^{14}C]SAM was separated from the radiolabeled methylated product by extraction with 100 μl ethyl acetate. Twenty microlitre of the organic phase containing the labeled product was used for liquid scintillation counting. The control consisted of all reaction components except the enzyme, and those counts were subtracted from the sample counts. The counts per minute were converted to picomoles of product produced per second (pkat), based on the specific activity of the substrate and the efficiency of the scintillation counter.

For determination of the kinetic parameters for Van OMT-3, the reaction conditions were modified to include unlabeled SAM. The methyl gallate K_M assays were carried out as above with 1 μl substrate (final concentrations of 2, 1, 0.5, 0.25, 0.1, and 0.05 mM), 2 μg purified Van OMT-3 enzyme, 10 μl 10 mM unlabeled SAM and 4 μl [^{14}C]SAM. All reactions were done in triplicate. The myricetin K_M assays were carried out with final concentrations of 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 mM, 2 μg purified Van OMT-3 enzyme, 10 μl of 10 mM unlabeled SAM, and 6 μl [^{14}C]SAM. The V_{max} and K_M were calculated from non-linear regressions of the Michaelis–Menten plots using the PRISM 4 program (GraphPad Software, San Diego, CA, USA).

Preparation of crude protein extracts of greenhouse grown *V. planifolia* bean (11-month-old), flower, stem, leaf, and root tissues was modified from that described by Pak *et al.* (2004). Three grams of fresh tissue was homogenized in 6 ml of

extraction buffer in an Ultra-Turrax T25 tissue homogenizer (IKA Works, Wilmington, NC, USA). The extraction buffer was 50 mM Bis-Tris–HCl, pH 6.9, 10 mM 2-mercaptoethanol, 5 mM $Na_2S_2O_5$, 1% (w/v) PVP-40, 1 mM phenylmethanesulfonyl fluoride, 10% (v/v) glycerol, and 100 μl protease inhibitor cocktail (Sigma-Aldrich) for each sample. The homogenate was filtered through cheesecloth and centrifuged 15 min at 10,000 rpm at 4 °C. The homogenate was passed through a PD10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the OMT assay buffer. The extract was eluted from the column with 3.5 ml assay buffer. Twenty micrograms of protein was assayed for activity. The assays were done in triplicate with 1 mM substrate. The background counts obtained from the crude extracts assayed with no added substrate were subtracted from the sample counts.

GC–MS analysis of Van OMT-3 reaction products

The methylated products from enzyme reactions using the purified recombinant Van OMT-3 with pyrogallol and myricetin as substrates were analyzed to determine the position of methylation. The reactions were carried out with 2 μg purified Van OMT-3, 1 mM substrate, and 10 μl of 10 mM unlabeled SAM. The ethyl acetate extracts from five 50 μl assays were pooled for the analysis.

For comparison with the reaction product from pyrogallol, 1,3-dihydroxy-2-methoxybenzene, 1,2-dihydroxy-3-methoxybenzene, 2,3-dimethoxyphenol, 2,6, dimethoxyphenol, and 1,2,3, trimethoxybenzene were used as standards. For comparison with the reaction product from myricetin, syringetin and 3,5,7-trihydroxy-3′,4′,5′-trimethoxyflavone (myricetin trimethyl ether) were used as standards.

Stock solutions of the reference standards were prepared at 1.0 mg ml^{-1} in ethyl acetate. Aliquots (10 μl) of the stock solutions representing approximately 10 μg of reference standard were transferred to 1.0 ml tapered glass reaction vials fitted with Teflon-lined, screw cap closures. Silylation reagent, 10 μl of *N*-methyl-*N* (trimethylsilyl)-trifluoroacetamide (Sigma-Aldrich), was then added to each vial. The vials were tightly capped and incubated at 80 °C for 60 min in a heating block immediately prior to GC–MS analysis. Samples were then directly injected and analyzed by GC–MS using the conditions described below. The reference standard 1,2,3-trimethoxybenzene was not derivatized prior to analysis.

Sample extracts of the enzymatic products from pyrogallol and myricetin as substrates were derivatized using the procedure described above except that an excess of silylation reagent was used (100 μl) to compensate for potential quenching of reagent by matrix-related interferences.

The reference standards and test samples were analyzed by GC–MS on a Varian 3400 gas chromatograph directly interfaced to a Finnigan MAT 8230 high resolution, double-focusing, magnetic sector mass spectrometer. The GC was equipped with a 30 m × 0.32 mm i.d. MDN-5 capillary column (Supleco, Bellefonte, PA, USA) containing a 0.25 μm film thickness. The injection port temperature was 280 °C and splitless injections were made with a 100:1 split time programmed at 0.5 min post-injection to serve as a septum purge. The GC was temperature programmed from 100 °C (3 min) to 320 °C at a rate of 10 °C min^{-1} with a 15 min hold at the upper limit. The GC–MS transfer line was maintained at 320 °C isothermal. The MS was operated in electron ionization mode (EI, 70 eV) scanning masses 35–650. The scan rate was 0.6 s per decade with a 0.8 s interscan time. For GC–MS analyses of the myricetin-TMS derivative and the derivatized enzymatic product of myricetin, the mass range of the scanning was 35–800. Data were recorded and processed using a Finnigan MAT SS300 data system and Micromass MassLynx data system and software.

Antibody production and immunoblot analysis

The purified recombinant Van OMT-3 protein was used for preparation of antiserum. It was mixed with an equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant and injected into the subscapular space of a rabbit. Three injections of 100 μg of protein each were given at 4-weeks intervals.

For immunoblot analysis, proteins from greenhouse grown bean, flower, root, leaf, and stem tissue were extracted by homogenizing tissue samples using a mortar and pestle in phosphate-

buffered saline (1.5 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, and 145.4 mM NaCl) in a ratio of 0.5 g tissue in 1 ml buffer. The extracts were centrifuged to remove debris and the protein concentrations of the supernatants determined using the Bio-Rad protein assay reagent. Protein samples were mixed with an equal volume of 2 × SDS sample buffer [2×: 125 mM Tris, pH 6.8, 4.6% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.002% bromophenol blue (w/v) (Laemmli, 1970)], then boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes (NitroPure, Osmonics, Westborough, MA, USA) in 10 mM 3-(cyclohexylamino)-1-propane–sulfonic acid (CAPS), pH 11, 10% methanol (v/v). Processing and detection by chemiluminescence (Western Lightening Chemiluminescence kit, Perkin-Elmer Life Science) was according to the manufacturer's instructions.

Results

Isolation of novel V. planifolia planifolia O-methyltransferase cDNA clones

We previously reported the characterization of a COMT from *V. planifolia* that exhibits substrate preferences typical of COMTs known to be involved in lignin synthesis (Pak *et al.*, 2004). The COMT cDNA clone was isolated using PCR with degenerate oligonucleotides based on conserved amino acid sequences in COMTs from other species. An additional PCR product was isolated from this approach and had distinct DNA and deduced amino acid sequences, but was also similar to other COMTs. This minor PCR product was used to screen a cDNA library prepared from *V. planifolia* bean tissue and two similar full-length clones were obtained. These two cDNAs were designated Van OMT-2 and Van OMT-3. The Van OMT-2 cDNA sequence contained a 5′-untranslated sequence of 346 bp, a 1077 bp open-reading frame, and a 168 bp 3′-untranslated sequence. The Van OMT-3 cDNA sequence contained a 5′-untranslated sequence of 50 bp, a 1077 bp open-reading frame, and a 198 bp 3′-untranslated sequence. Van OMT-2 encodes a 359 amino acid protein with a molecular weight of 38,920 and Van OMT-3 encodes a 359 amino acid protein with a molecular weight of 38,769. Van OMTs-2 and 3 are 95% identical to each other at the deduced amino acid level.

Searches of the NCBI protein database (http://www.ncbi.nlm.nih.gov/) revealed that the *V. planifolia* COMT was the sequence most similar to both Van OMTs-2 and 3. Both sequences also had significant similarity to COMTs from other plant species. A comparison of the deduced amino acid sequences of Van OMTs-2 and 3 with the *V. planifolia* and *Medicago sativa* (alfalfa) COMTs is shown in Figure 1. The Van OMTs-2 and 3 amino acid sequences are 52% identical to the *V. planifolia* COMT. The *V. planifolia* COMT, Van OMTs-2, and 3 are 57, 47, and 49% identical to the alfalfa COMT, respectively. The structure of the alfalfa enzyme has been determined by X-ray crystallography and the catalytic residues and residues involved in substrate binding or positioning were identified (Zubieta *et al.*, 2002). The catalytic residues, H269, E297, and E329, are conserved in all of the sequences presented in Figure 1.

The relationship of Van OMTs-2 and 3 to each other, to the *V. planifolia* COMT, and to COMTs from other species is shown in Figure 2. Two other COMT-like sequences, the *C. breweri* IEMT and the *C. americanum* FOMT, were also included in the analysis. Maximum parsimony analysis of the amino acid dataset was based upon 384 total characters, of which 81 were constant, 64 variable characters were parsimony uninformative, and 239 characters were parsimony informative. An ancestral COMT sequence, that from the gymnosperm *P. abies*, was designated the outgroup for rooting the tree. The sequences from the dicots and monocots grouped into two sister clades. Interestingly, the three *V. planifolia* sequences grouped together as a sister clade to the Poales sequences, rather than with the other Asparagales sequences in the comparison, *Iris hollandica* and *Allium cepa*. Although Van OMTs-2 and 3 are clearly distinct from the *V. planifolia* COMT, that is the sequence most closely related to them. In BLASTp searches of the NCBI database, the same COMTs from other plant species were the best matches for all three of the *V. planifolia* OMT sequences. Based on sequence

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Van COMT     MATWVEHQQQQNGSKDVDEEACMYAMQLSSMVVLPMTLRVAVELGILEQIQAGGPDSYLT 60
Medicago     MGSTGETQITPTHIS--DEEANLFAMQLASASVLPMILKSALELDLLEIIAKAGPGAQIS 58
Van OMT-2    MGSIPKNQE--------EVDVCNYAQKLVSCVVLPMTLNAAIQLGLFEEIVAAGPGARLS 52
Van OMT-3    MGSIPNNHA--------EVDVCNYALKLVSSAVLPMTLNSAIQLGLLEEIVAAGPGARLS 52
             *.:  : :         : :.  :* :* *  **** *. *::*.::* *  .**.: ::

Van COMT     AEDLAARLGNSNPLAPVMIERILRLLTSYSILNFTDTVDGEGR-TVRSYGAAHVCKYLTP 119
Medicago     PIEIASQLPTTNPDAPVMLDRMLRLLACYIILTCSVRTQQDGK-VQRLYGLATVAKYLVK 117
Van OMT-2    AEELASRIGSTNPLAPALLHRILRLLASYSIVTSSEAADNDGRGTTIRYGAAPVCKYLTR 112
Van OMT-3    AEELASRIGSTNPLAPALLDRILRLLASYSIVTSYEAADTDGRGTTTRYGAAPVCKYLTR 112
             . ::*::: .:** **.::.*:****:.* *:.    .: :*: .    ** * *.***.

Van COMT     NQDGVSMAPLVLMNTDKVLMESWYHMKDAVTNGGIPFNLAYG-MTAFEYHGKDLRFNKVF 178
Medicago     NEDGVSISALNLMNQDKVLMESWYHLKDAVLDGGIPFNKAYG-MTAFEYHGTDPRFNKVF 176
Van OMT-2    NEDGVSLASISQLTNHKIAMESWYHVKDAVLHGGVPFNMAHGGLNEFEYHATDPSFSKVF 172
Van OMT-3    NEDGVSLASLSQLTNHKIAMESWYHVKDAVLHGGVPFNMAHGGLNEFEYHATDPSFSKVF 172
             *:****:::.:  :.. *: ******:**** .**:*** *:* :. ****..*  * ***

Van COMT     NEGMKNNSIIITKKILERYKRFEDVNVLIDVGGGIGGTISMITAKYPHIHGINFDLPHVV 238
Medicago     NKGMSDHSTITMKKILETYTGFEGLKSLVDVGGGTGAVINTIVSKYPTIKGINFDLPHVI 236
Van OMT-2    NEAMRGHSVFIMRNILKVYRGFDEAKVMVDVCGGTGGTLGMITAKHPHIKGINFDLPHVI 232
Van OMT-3    NEAMRGHSVVIMRNILKVYRGLDEAKVMVDVCGGTGGTLGMITAKHPHIKGINFDLPHVI 232
             *:.* .:* .  ::**: *  ::  : ::** ** *..:. *.:*:* *:*********:

Van COMT     SEAPPFQGVEHVGGNMFESVPIGDAIFIKWILHDWSDEHCLKLLRNCAKSLPDKGKVIVV 298
Medicago     EDAPSYPGVEHVGGDMFVSIPKADAVFMKWICHDWSDEHCLKFLKNCYEALPDNGKVIVA 296
Van OMT-2    AQAPPLPGVEHVGGDVFASVPSGDTVLLKWVLHDWNDEDCVRILKNCKKAIPETGKVVVV 292
Van OMT-3    AQAPPLPGVEHVGGDVFASVPTGDTVLLKWVLHDWNDEDCVRILKNCKKAIPETGKVVVV 292
              :**.  *******::* *:* .*::::**: ***.**.*:::*:** :::*:.***:*.

Van COMT     ECILPDAPLVTPEAEGVFHLDMIMLAHNPGGKERTKKEFKELAMLSGFSNFKALFSYANV 358
Medicago     ECILPVAPDSSLATKGVVHIDVIMLAHNPGGKERTQKEFEDLAKGAGFQGFKVHCNAFNT 356
Van OMT-2    ESVVPESLESSDLAHFILHSDLVMLLESPCGKERTKKDFRSLAQQSGFSGFAVLCSFSSS 352
Van OMT-3    ESVVPESLESSDLAHFILHSDLVMLLESPFGKERTKKDFRSLAQQSGFSGFAVLCSFSSA 352
             *.::* :   :  :. :.* *::** ..* *****:*:*.:*..**  :**..* .  .  .

Van COMT     WVMEFNK-- 365
Medicago     YIMEFLKKV 365
Van OMT-2    WVMEFTN-- 359
Van OMT-3    WVMEFTN-- 359
             ::*** :
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Figure 1. Comparison of deduced amino acid sequences of Van OMTs-2 and 3 with the COMT sequences from *Vanilla planifolia* and *Medicago sativa*. The substrate-binding residues, as determined in *M. sativa* (Zubieta *et al.*, 2002), are highlighted by bold larger font sizes and the catalytic residues H269, E297, and E329 (positions given are those in the *M. sativa* sequence) are highlighted by larger font sizes. An asterisk indicates identical residues in all sequences, a ":" indicates strongly conserved residues (score > 0.5), and a "." indicates weaker conserved residues (score < 0.5) (Thompson *et al.*, 1997).

comparisons, the Van OMTs-2 and 3 are clearly members of the COMT family and have a similar level of identity to the other COMTs in the comparison as does the *V. planifolia* COMT. Although there is up to 40% sequence variability among the COMTs, the substrate-binding residues are generally well-conserved in the COMTs reported from other species, as well as in the *V. planifolia* COMT. However, 7 of 12 conserved substrate binding residues are altered

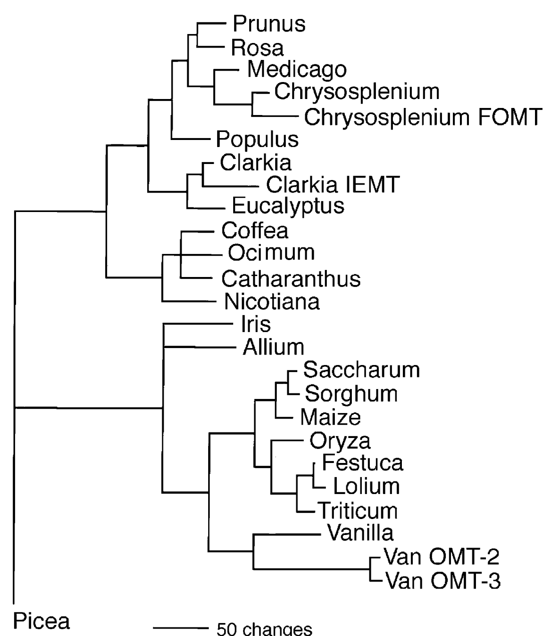


Figure 2. Rooted maximum parsimony phylogenetic tree comparing the Van OMTs-2 and 3 sequences with the similar COMT sequences from other species. Also included in the comparison were the *Chrysosplenium americanum* FOMT and the *Clarkia breweri* IEMT. The *Picea abies* COMT was designated as the outgroup for rooting. All branches were supported by greater than 50% bootstrap percentages based on 1000 replications. GenBank accession numbers for the corresponding DNA sequences are: *Allium cepa*, CF442066 (Kuhl *et al*., 2004); *Catharanthus roseus*, AY028439 (Schroder *et al*., 2002); *Chrysosplenium americanum*, U16793 (Gauthier *et al*., 1998); *Chrysosplenium americanum* FOMT, U16794, (Gauthier *et al*., 1996); *Clarkia breweri*, AF006009 (Wang and Pichersky, 1997); *Clarkia breweri* IEMT, U86760 (Wang *et al*., 1997); *Coffea canephora*, AF454631 (unpublished data); *Eucalyptus gunnii*, X74814 (Poeydomenge *et al*., 1994); *Festuca arundinacea*, AF153825 (unpublished data); *Iris hollandica*, AB183825 (unpublished data); *Lolium perenne*, AF010291 (McAlister *et al*., 1998); *Medicago sativa*, M63853 (Gowri *et al*., 1991); *Nicotiana tabacum*, X74452 (Jaeck *et al*., 1996); *Ocimum basilicum*, AF154918 (Wang *et al*., 1999); *Oryza sativa*, XM480185 (unpublished data); *Picea abies*, AJ868575, unpublished; *Populus tremuloides*, X62096 (Bugos *et al*., 1991); *Prunus amygdalus*, X83217 (Garcia-Mas *et al*., 1995); *Rosa chinensis*, AJ439740 (Scalliet *et al*., 2002); *Saccharum officinarum*, AJ231133 (Selman-Housein *et al*., 1999); *Sorghum bicolor*, AY217766 (Bout and Vermerris, 2003); *Triticum aestivum*, AY226581 (Jang *et al*., 2003); *Vanilla planifolia* COMT, AY555144 (Pak *et al*., 2004); *V. planifolia* OMT-2, DQ400399; *V. planifolia* OMT-3, DQ400400; *Zea mays*, M73235 (Collazo *et al*., 1992).

in Van OMTs-2 and 3 (Figure 1), which suggested that these enzymes may have different substrate preferences from COMTs.

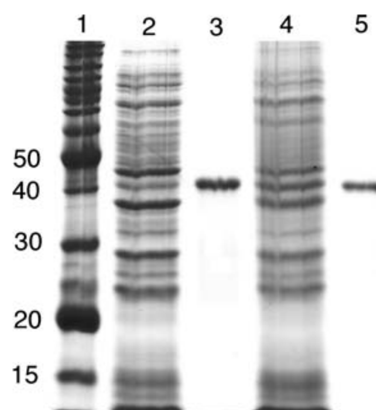


Figure 3. Purification of Van OMTs-2 and 3 recombinant His-tagged fusion proteins from *E. coli*. Lane 1, Protein mass standards. Masses in kiloDaltons indicated on the left. Lanes 2 and 4, 40 μg of crude extract of *E. coli* transformant cells containing the expression vector for Van OMTs-2 and 3, respectively. Lanes 3 and 5, 3 μg of purified Van OMTs-2 and 3 purified recombinant protein, respectively.

Expression of Van OMT-2 and Van OMT-3 in E. coli

The proteins encoded by Van OMTs-2 and 3 were expressed as N-terminal polyhistidine-tagged fusions in *E. coli* based on the expression vector pET-15b. The recombinant proteins were purified using nickel particle affinity chromatography (Figure 3). The recombinant proteins tended to rapidly accumulate in insoluble inclusion bodies, so conditions were developed using a low concentration of IPTG and a short room temperature incubation to allow accumulation of soluble Van OMTs-2 and 3 proteins. With these mild induction conditions, no clear difference in protein pattern between uninduced and induced cells could be seen in protein gels of the soluble extracts (data not shown), but recombinant protein could be purified from the extracts.

The OMT activity of the purified recombinant proteins was assayed against numerous potential substrates (Tables 1 and 2, Figure 4). Neither Van OMTs-2 or 3 had significant activity with the typical COMT substrates, caffeoyl aldehyde, 5-OH-coniferaldehyde, 5-OH-ferulic acid or caffeic acid. Neither enzyme had activity with the proposed vanillin precursor 3,4-dihydroxybenzaldehyde, and so cannot be components of the vanillin biosynthetic pathway. The preferred substrates for both enzymes were 1,2,3-trihydr-

Table 1. Relative activity (%) of Van OMT-2, Van OMT-3 and the *Vanilla planifolia* COMT purified recombinant proteins with selected substrates. Three microgram of each enzyme was used and all substrates were assayed at 1 mM. ND is not determined.

Substrate	Van OMT-2	Van OMT-3	Van COMT
n-Propyl gallate	100[a]	100[a]	3.1
Ethyl gallate	95	96	1.3
Methyl gallate	74	88	3.5
Myricetin	2	60	0.8
Pyrogallol	44	51	11.8
Delphinidin chloride	ND	17	ND
Myricitrin	ND	14	ND
Orcinol	4	13	0
2-Methoxyresorcinol	0.3	8	0
3,5-Dihydroxybenzaldehyde	4	8	0
Caffeoyl aldehyde	0.6	1.7	100[b]
5-OH-Coniferaldehyde	0	0	86
3,4-Dihydroxybenzaldehyde	0	0	40
Caffeic acid	0	0	16

[a] 57 pkat mg^{-1}.
[b] 70 pkat mg^{-1}.

oxybenzene derivatives such as n-propyl gallate, ethyl gallate, and methyl gallate. Van OMT-3 also had significant activity with the flavonol myricetin, which also has a 1,2,3-trihydroxybenzene moiety. Although the relative substrate activities of both enzymes were generally similar, Van OMT-2 did not have significant activity against myricetin.

The *V. planifolia* recombinant COMT (Pak *et al.*, 2004) was also purified and assayed with the Van OMTs-2 and 3 preferred substrates, as well as the typical COMT substrates. The *V. planifolia* COMT exhibited negligible activity with the Van OMTs-2 and 3 preferred substrates (Table 1). Although the deduced amino acid sequences of all three enzymes suggested they are all COMTs, in fact they have distinct substrate preferences. Based on the enzyme activity data, Van OMTs-2 and 3 are clearly not COMTs.

The best substrate for both Van OMTs-2 and 3, n-propyl gallate, is a synthetic compound with potent antioxidant activity that is often used as a food preservative (Shahidi, 2000). Since it is not a naturally occurring plant product it cannot be considered a possible *in vivo* substrate. Ethyl gallate and methyl gallate have been reported as secondary metabolites in some plants (Mendez and Mato, 1997; Abou-Zaid and Nozzolillo, 1999; Kandil *et al.*, 1999; Cui *et al.*, 2002; Riaz *et al.*, 2004). The flavonol myricetin is synthesized by a wide range of plant species and the B-ring monomethylated and dimethylated products, laricitrin and syringetin, respectively, are also naturally occurring flavonols (Harborne and Williams, 2001). Naturally occurring flavonols are generally glycosylated (Iwashina, 2000; Harborne and Williams, 2001). Van OMT-3 did have some activity against a glycosylated form of myricetin, myricitrin (myricetin-3-rhamnoside), but apparently the presence of the carbohydrate moiety was inhibitory to the methylation reaction. Van OMT-3 also had some activity with the anthocyanidin delphinidin. There was no activity with the similar flavonols, kaempferol or quercetin, which have only 1 or 2 hydroxyl groups on the B ring, respectively.

Both Van OMTs-2 and 3 required a 1,2,3-trihydroxybenzene moiety for significant activity but not all compounds with such a group were effective substrates (Table 2). The 1,2,3-trihydroxybenzene-containing compounds that were effective substrates all have in common that any other substituents on the molecule are *para* to the central hydroxyl group on the benzene ring and in the same plane as the benzene ring. The difference in activity between Van OMTs-2 and 3 with myricetin was surprising since their amino acid sequences are 95% identical. There are some amino acid differences between the two near the substrate binding residues, F/V182 and C/F322 that may contribute to the observed activity difference.

The kinetic parameters of purified recombinant Van OMT-3 were determined with the most likely *in vivo* substrates, methyl gallate and myricetin (Table 3).

Table 2. Compounds assayed for which there was no activity (<2% of the maximum) with Van OMT-3.

Compound
Compounds containing a 1,2,3-trihydroxybenzene moiety
(-)-Catechin gallate
(-)-Epigallocatechin gallate
(-)-Gallocatechin gallate
2,3,4-Trihydroxyacetophenone
2',3',4'-Trihydroxychalcone
Octyl gallate
Flavonols
Kaempferol
Quercetin
Rutin
COMT substrates and related compounds
Caffeic acid
Caffeic acid ethyl ester
Caffeoyl alcohol
Caffeoyl aldehyde
Ferulic acid
5-Hydroxyconiferyl alcohol
5-Hydroxyconiferyl aldehyde
5-Hydroxyferulic acid
5-Hydroxyferulic acid ethyl ester
Phenolics and related compounds
2-Allylphenol
1,2,4-Benzenetriol
Catechol
3,4-Dihydroxybenzaldehyde
2,5-Dihydroxybenzoic acid
2,3-Dimethoxyphenol
2,6-Dimethoxyphenol
3,4-Dimethoxyphenol
3,5-Dimethoxyphenol
4-Hydroxybenzaldehyde
4-Hydroxybenzoic acid
2-Hydroxybenzyl alcohol
3-Hydroxybenzyl alcohol
3-Hydroxy-5-methoxybenzaldehyde
4-Hydroxy-3-methoxybenzoic acid
Guaiacol
3-Methoxycatechol
4-Methoxyphenol
4-methylcatechol
3-Methylcatechol
3-Methoxyphenol
3-Methoxy-5-methylphenol
2-Methylresorcinol
Resorcinol
Syringaldehyde
Syringic acid
Vanillin
Phenylpropanoids and phenylpropenes
Coumaric acid
Eugenol
4-Hydroxy-3,5-dimethoxycinnamic acid
Trans-4-Hydroxy-3-methoxycinnamic acid
4-Hydroxy-3-methoxycinnamaldehyde

Determination of position of methyl group addition

All of the best substrates for Van OMTs-2 and 3 had multiple hydroxyl groups that were possible targets of the methylation reaction. To investigate the position of methylation, the activity of Van OMT-3 against pyrogallol (1,2,3-trihydroxybenzene) was compared with that with all of its potential methylated derivatives (Table 4). Although pyrogallol was not the best substrate for Van OMT-3, it is the only one for which all the possible methylated derivatives are commercially available. Activity was detected only with 1,3-dihydroxy-2-methoxybenzene, at 17% of the activity with pyrogallol. With this substrate the enzyme must have methylated one or both of the outer hydroxyl groups (positions 1 or 3). There was no detectable activity with 1,2-dihydroxy-3-methoxybenzene, indicating that once one of the outer hydroxyl groups were methylated, methylation of an additional group did not occur. Overall these data suggested that a single outer hydroxyl group in pyrogallol was methylated by Van OMT-3.

This interpretation was confirmed by GC–MS analysis of the methylated product produced from pyrogallol by Van OMT-3. After the reaction, the enzymatically methylated product was trimethylsilylated and compared with the trimethylsilylation products of the compounds in Table 4. A compound with the identical fragmentation pattern as that of 1,2-dihydroxy-3-methoxybenzene was detected in the Van OMT-3 reaction product (Table 5). None of the other potential methylated products of pyrogallol were detected, confirming that the enzyme methylated a single outer hydroxyl group in pyrogallol.

The GC–MS was also used to confirm the site of methylation of myricetin by Van OMT-3. The B ring of myricetin is similar to the structure of pyrogallol, suggesting that it is the site of methylation. If the methylation of myricetin was similar to that of pyrogallol, methylation of a single outer hydroxyl group on the B-ring would produce the flavonol laricitrin. Laricitrin was not commercially available for use as a standard, but its mass spectral data has been reported (Cacace *et al.*, 2003). A compound with the identical fragmentation pattern to that previously reported was detected in the reaction product of Van OMT-3 with myricetin (Table 5). The dimethylated product syringetin was not detected.

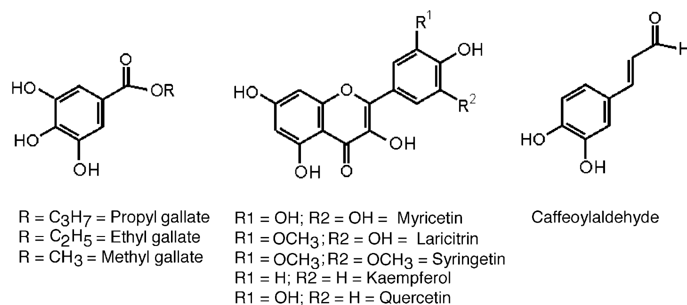
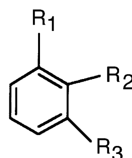


Figure 4. Structures of some of the compounds assayed as substrates for Van OMTs-2 and 3.

Table 3. Kinetic parameters of Van OMT-3.

Substrate	Apparent K_M μM	Apparent V_{max} nkat mg^{-1}	k_{cat} s^{-1}	k_{cat}/K_M mM^{-1} s^{-1}
Myricetin	114	1.7	6.8×10^{-2}	0.6
Methyl gallate	960	5.8	23×10^{-2}	0.2

Table 4. Relative activity of Van OMT-3 with pyrogallol (1,2,3 trihydroxybenzene) and its methylated derivatives.



Substrate	Relative Activity (%)
1,2,3-Trihydroxybenzene (R_1, R_2, R_3 = OH)	100
1,3-Dihydroxy-2-methoxybenzene (R_1, R_3 = OH; R_2 = OCH_3)	17
1,2-Dihydroxy-3-methoxybenzene (R_1, R_2 = OH; R_3 = OCH_3)	0
2,3-Dimethoxyphenol (R_1 = OH; R_2, R_3 = OCH_3)	0
2,6-Dimethoxyphenol (R_1, R_3 = OCH_3; R_2 = OH)	0

Table 5. GC-MS analysis of reaction products from Van OMT-3 with pyrogallol and myricetin as substrates. All samples were analyzed after trimethylsilylation.

Sample	EI mass spectra, m/z (relative abundance, %)		
	M^+	$[M–Me]^+$	Other ions
Enzymatic product from pyrogallol	284 (25)	269 (17)	254 (6.5), 73 (100)
1,2-Dihydroxy-3-methoxybenzene standard	284 (25)	269 (17)	254 (6.5), 73 (100)
Enzymatic product from myricetin	692 (3.7)	677 (100)	647 (31), 589 (12)
Monomethyl myricetin[a]	692 (–)	677 (100)	647 (17), 589 (12)

[a] Data from Cacace *et al*., 2003.

Van OMT-2 and Van OMT-3 expression in different tissues

To determine in which tissues Van OMTs-2 and 3 were expressed, activity assays were carried out on crude extracts of leaves, stems, aerial roots, flower, and bean tissue with methyl gallate and myricetin as substrates. The root extract had 4.9 and 1.6 pkat mg^{-1} activity with myricetin and methyl gallate as substrates, respectively. The flower

extract had 0.68 and 0.24 pkat mg^{-1} activity with myricetin and methyl gallate as substrates, respectively. The stem, leaf, and bean extracts had no activity with either substrate. That the root and flower crude extracts had activity with both substrates suggested the presence of Van OMT-3, and perhaps Van OMT-2. Both Van OMTs-2 and 3 were active with methyl gallate but only Van OMT-3 was active with myricetin. Purified Van OMT-3 was more active with methyl gallate than with myricetin. That the root and flower crude extracts were more active with myricetin than methyl gallate suggests there may be additional enzymes present that also methylate myricetin. Surprisingly there was no activity detectable with either substrate in the bean crude extract. Since the cDNA clones for both Van OMTs-2 and 3 originated from bean tissue, they clearly are expressed in that tissue. The lack of detectable activity in the bean extract suggests that the enzymes accumulate at low levels in the bean tissue. The root and flower tissues may have been generally more metabolically active than the other tissues, with consequently more enzymatic activity.

Immunoblot analysis of the tissues supported the interpretation of the activity assays (Figure 5). The antibody was prepared against Van OMT-3 but was also reactive against Van OMT-2, as would be expected considering the two proteins are 95% identical. The Van OMT-3 antibody did not cross-react with the purified recombinant *V. planifolia* COMT (data not shown). Strong immunoreactive bands were seen in both the aerial and underground root samples and in the flower sample, but no bands were detected in the leaf, stem, or bean samples. The results of the immunoblots therefore, revealed the presence of one or both Van OMTs-2 and 3 in the root and flower samples.

Since Van OMT-2 and/or Van OMT-3 appeared to be most highly expressed in the roots, aerial root samples from other orchid species were also examined by immunoblot analysis for the presence of these enzymes (Figure 6). A strong immunoreactive band was detected in another *Vanilla* sp. examined, *V. pompona*. Faint bands or no bands were detected in the other orchid samples.

Discussion

Through our search for an OMT in *V. planifolia* bean tissue that was active on the proposed vanillin precursor 3,4-dihydroxybenzaldehyde, we have fortuitously identified two new OMTs, Van OMTs-2

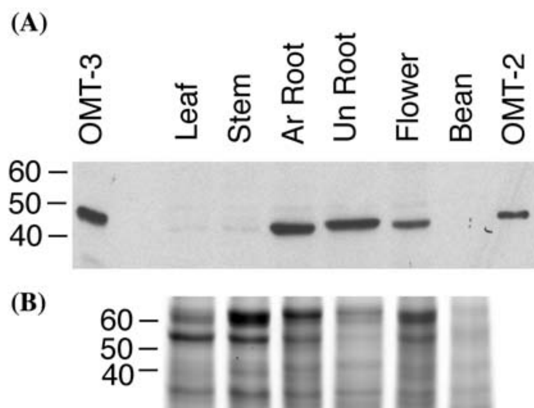


Figure 5. (A) Immunoblot analysis of Van OMT-3/Van OMT-2 in different tissues of *Vanilla planifolia*. Protein extracts (20 μg) were subjected to SDS-PAGE and immunoblot analysis using the Van OMT-3 antiserum. Ar Root is from aerial root tissue and Un Root is from underground root tissue. Also included on the blot were 20 ng each of purified recombinant Van OMTs-3 and 2 protein. (B) Coomassie Blue-stained gel of the same extracts used for the immunoblot in A. The positions of protein standards, in kiloDaltons, are indicated on the left.

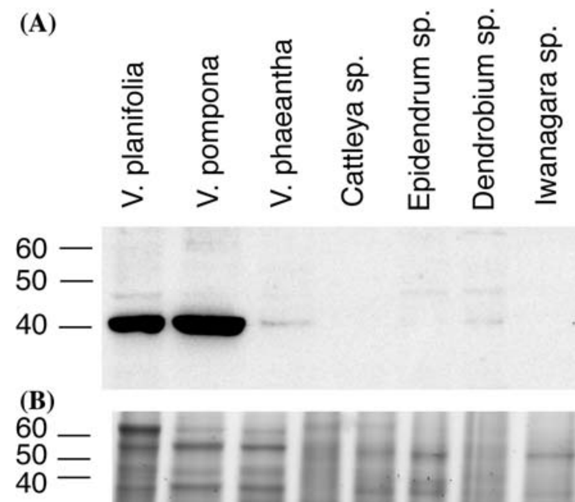


Figure 6. (A) Immunoblot analysis of aerial root extracts of different orchid species. Protein extracts (16 μg) were subjected to SDS-PAGE and immunoblot analysis using the Van OMT-3 antiserum. (B) Coomassie Blue-stained gel of the same extracts used for the immunoblot in A. The positions of protein standards, in kiloDaltons, are indicated on the left.

and 3, that are active on substrates containing a 1,2,3-trihydroxybenzene moiety. This is a substrate preference that has rarely been reported for other OMTs. The GC–MS analysis indicated that a single outer hydroxyl group on the 1,2,3-trihydroxybenzene moiety was methylated. The amino acid sequences of these two enzymes are most similar to COMTs that are involved in lignin biosynthesis, yet they have no activity with typical COMT substrates. The previously characterized *V. planifolia* COMT (Pak *et al.*, 2004) is the most similar sequence to Van OMTs-2 and 3 currently available. The *V. planifolia* COMT is not active with the preferred substrates of Van OMTs-2 and 3.

The Van OMTs-2 and 3 appear to be unique in requiring a 1,2,3-trihydroxybenzene group for activity. Enzymes that can catalyze the methylation of myricetin have rarely been reported, although the glycosylated B-ring methylated products, laricitrin and syringetin, have been reported from numerous species (Harborne and Williams, 2001), including orchids (Kuehnle *et al.*, 1997). A Class I caffeoyl CoA-like multifunctional OMT from *Mesembryanthemum crystallinum* could carry out the methylation of both the 3′-OH and 5′-OH of myricetin as well as the methylation of several quercitin and ferulic acid derivatives (Ibdah *et al.*, 2003). A Class II OMT from *Catharanthus roseus* also methylated both the 3′ and 5′ hydroxyls of the myricetin B-ring, but had little sequence identity with the *C. roseus* COMT, and grouped in a clade containing other flavonoid and alkaloid OMTs (Cacace *et al.*, 2003). Neither the *M. crystallinum* nor the *C. roseus* sequences were similar to that of Van OMT-3. Unlike the *M. crystallinum* and *C. roseus* enzymes, Van OMT-3 methylated only one outer hydroxyl group on the myricetin B-ring. Two nearly identical OMTs from *Chrysosplenium americanum* could methylate myricetin, but preferred the B-ring dihydroxy compounds luteolin and quercetin (Gauthier *et al.*, 1998). These enzymes were also effective with the typical COMT substrates 5-hydroxyferulic acid and caffeic acid. The *C. americanum* enzymes are unusual in being capable of acting on both phenylpropanoid and flavonoid substrates. Only one of these enzymes is included in the phylogenetic analysis in Figure 2 and is considered to be a COMT. The similar *C. americanum* FOMT is 83% identical to the *C. americanum* COMT but was inactive with COMT substrates. The *C. americanum* FOMT was active on several flavonoids, but not myricetin (Gauthier *et al.*, 1996).

The enzymes involved in the synthesis of the vast array of plant secondary metabolites are considered to have evolved from enzymes of primary metabolism via gene duplication followed by mutations that result in changes in the activity of the enzyme (Pichersky and Gang, 2000; Gang, 2005; Ober, 2005). Some interesting cases have been reported where a change in a few amino acids has resulted in a complete change in substrate specificity. The *C. americanum* FOMT discussed above is one such example. Another example is the *Clarkia breweri* IEMT, also discussed above (Wang and Pichersky, 1998). The *C. breweri* IEMT is 83% identical to the *C. breweri* COMT but methylates the 4-hydroxyl of isoeugenol and eugenol whereas COMTs methylate the 3-hydroxyl of caffeic acid and the 5-hydroxyl of 5-hydroxyferulic acid. From site-directed mutagenesis it was determined that seven amino acid differences were responsible for the discrimination between eugenol and isoeugenol and caffeic acid/5-hydroxyferulic acid (Wang and Pichersky, 1999). These seven amino acids are around the substrate-binding region of the enzymes. From molecular modeling of the IEMT, based on the crystal structure of alfalfa COMT, it was suggested that these changes resulted in a reorientation of the phenolic ring of (iso)eugenol positioning the 4-hydroxyl for methylation (Zubieta *et al.*, 2002). A similar situation was found in basil where a few amino acid differences were found to be responsible for substrate discrimination in a chavicol OMT and eugenol OMT (Gang *et al.*, 2002). The basil eugenol OMT is only distantly related to the *C. breweri* (iso)eugenol OMT, indicating independent evolution of the same enzyme activity (Gang *et al.*, 2002).

The *C. breweri* IEMT and the *C. americanum* FOMT are both COMT-like enzymes with novel substrate specificities. Based on the 83% identity between the *C. breweri* COMT and IEMT sequences and their phylogenetic relationship it was concluded that the IEMT had recently evolved from the COMT (Wang and Pichersky, 1998). The Van OMTs-2 and 3 are more divergent from the *V. planifolia* COMT (52% sequence identity) than the *C. breweri* IEMT and the *C. americanum* FOMT from their respective COMTs. Nonetheless, the *V. planifolia* COMT sequence is the sequence most closely related to

Van OMTs-2 and 3 (Figure 2), and phylogenetic analysis suggests that they have also evolved from the COMT. Presumably more recent duplication and divergence events resulted in the two, 95% identical, enzymes. The Van OMTs-2 and 3 are thus new instances of evolution of novel OMTs from COMTs, resulting in enzymes with distinct substrate specificities. The seven amino acid differences in the substrate binding residues from those identified from the alfalfa COMT crystal structure originally suggested the hypothesis that Van OMTs-2 and 3 had substrate preferences that differ from COMTs. The activity data presented here on the purified recombinant proteins confirmed the hypothesis. The basis of the differences in substrate preferences relative to COMT was not apparent from homology modeling of Van OMTs-2 and 3 based on the crystal structure determined for the alfalfa COMT (J-L. Ferrer, personal communication). In the future, X-ray crystallography will be required to understand the basis of the substrate preferences of Van OMTs-2 and 3.

There are now three documented cases from different plant species of apparent evolution from COMTs of new OMTs with new substrate specificities. As more sequence data becomes available, additional instances are likely to be found. As more such cases are described, and an understanding of the effects of specific amino acids on the active site develops, it may someday be possible to use a COMT as the starting point in designing an OMT that can act on a particular substrate of interest.

Extensive chemical analyses of the compounds in extracts of cured *Vanilla* beans have been reported due to the importance of the extracts as flavors (Hartman *et al*., 1992; Adedeji *et al*., 1993; Ehlers and Pfister, 1997; Werkhoff and Guntert, 1997). The phenolic and flavonol components of living *Vanilla* tissues, however, have not yet been examined. This information will be needed to determine the most likely *in vivo* substrates for Van OMTs-2 and 3. Based on the reported presence of methyl gallate and myricetin in numerous other plant species, these are potential *in vivo* substrates for Van OMTs-2 and 3.

Although the cDNA clones for Van OMTs-2 and 3 were isolated from a library prepared from bean tissue, from immunoblot analysis and enzyme assays of crude extracts they appeared to be most highly expressed in root and flower tissues. Immunoblot analysis indicated that similar proteins were expressed in roots of another *Vanilla* sp., *V. pompona*. Further molecular analyses will be required to determine if Van OMTs-2 and 3 homologues are present in the other orchid species examined.

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