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Predicting the substrates of cloned plant *O*-methyltransferases

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Abstract

Plant *O*-methyltransferases (OMTs) have important roles in secondary metabolite biosynthesis. Sequencing projects and homology-based cloning strategies yield sequences for proteins with similarities to known OMTs, but the identification of the physiological substrates is not trivial. We investigated with a cDNA cloned from *Catharanthus roseus* the possibilities for predicting the substrates of OMTs, using the information from previous work and two newly identified motifs that were based on information from the crystal structures of two plant OMTs. The results, confirmed by functional analysis of the recombinant protein, indicated that a careful analysis of the deduced protein sequence can provide clues for predicting the substrates of cloned OMTs.

Keywords: *Catharanthus roseus*; Apocynaceae; Madagascar periwinkle; Homology based cDNA cloning; Recombinant protein expression; *O*-Methyltransferase; Phenylpropanoid; Flavonoids; Caffeic acid

1. Introduction

Sequencing projects provide information that can be extremely useful for many aspects of basic and applied research. One of the problems in using it, however, is that the annotation of the sequences often cites only similarities to other proteins, and sometimes the functions of those are described as putative. Understanding gene functions obviously requires more substantial information on the biochemical functions, but there is also a need for improved prediction possibilities because these could considerably facilitate the identification of possible substrates, if the general type of reaction can be predicted with some reliability. This is of particular importance in attempts to aim, by homology-based cloning strategies, for proteins with specific functions in interesting pathways.

We became interested in *O*-methyltransferases (OMTs) because the second step in the pathway from tabersonine to vindoline in the biosynthesis of the complex indole alkaloids is an *O*-methylation. Little is known about that enzyme (Fahn et al., 1985), but a homology-based cloning strategy appeared possible because all plant OMTs share common sequence elements. Three general OMT motifs had been defined previously (Kagan and Clarke, 1994), and the increasing number of sequences permitted a more detailed analysis of plant OMTs a few years later (Ibrahim, 1997; Joshi and Chiang, 1998; Ibrahim et al., 1998). Two additional motifs were proposed to be specific for caffeic acid 3-*O*-methyltransferases (COMTs) (Selman-Housein et al., 1999). The recently published crystal structures of two plant OMTs (Zubieta et al., 2001) added a new dimension because they revealed functionally important residues and subunit interactions that could not be deduced from the comparison of primary protein sequences.

We wished to test whether the sum of this information will at present permit reasonable predictions of the substrate specificity of putative OMTs cloned by PCR-based strategies. The example is a cDNA from *Catharanthus roseus* cell suspension cultures that was obtained by starting with a set of degenerate PCR primers originally used for cloning OMTs in the biosynthesis of isoquinoline alkaloids (Frick and Kutchan, 1999) which do not occur in *C. roseus*.

2. Results and discussion

A cDNA library was established with RNA from *C. roseus* cell suspension cultures induced by irradiation and simultaneous nutritional downshift. This treatment

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was chosen because it was known to induce the P450 reaction directly preceding the OMT activity of interest (Schröder et al., 1999). The first partial sequences for putative OMTs obtained by PCR revealed only one class of clones with at most a few base differences, suggesting that the cells contained a dominant OMT mRNA or that the primers possessed an unsuspected specificity for certain OMTs in *C. roseus*. Overlapping cDNAs representing the complete coding region were obtained from the cDNA library by PCR with primers specific for the putative OMT and phage-specific primers for sequences flanking the cDNA inserts in the phages.

Fig. 1 shows the protein sequence deduced from the *C. roseus* OMT1 and the position of conserved motifs described in previous work (Ibrahim, 1997; Joshi and Chiang, 1998; Ibrahim et al., 1998; Selman-Housein et al., 1999). All of the motifs conserved in plant OMTS were readily identified. A database search indicated that the polypeptide shared 77–83% identity with known COMTs (e.g. from *Nicotiana tabacum*, GenBank NTOMTIA and NTOMTIB; *Ocimum basilicum,* GenBank AF154917 and AF154918; various *Capsicum* species, GenBank CAU83789 and AF081214; various *Populus* species, GenBank PTLBCA, POPOME; *Medicago sativa*, Genbank ALFSALMCAT), and with several other polypeptides only identified by sequence similarity.

A relationship tree of the plant OMTs available in the databases (status May 2001) is shown in Fig. 2. The *C. roseus* protein grouped in a large cluster that included most of the functionally characterized dicotyledonous COMTs. It included several proteins named after their activity with catechol (cat OMTs), but this activity appears to be a property of all COMTs, and the available evidence indicates that the common denominator is the acceptance of substrates possessing vicinal phenolic hydroxyl groups (Pellegrini et al., 1993; Maury et al., 1999). The position of the *C. roseus* protein in the COMT cluster suggested that the substrate should contain vicinal hydroxyl groups and might be a phenylpropanoid compound, but a closer inspection showed that a reliable prediction was not possible. The cluster contained several proteins that have no or very low activities with caffeic acid, e.g. the (*iso*)eugenol OMT that uses mono-hydroxylated substrates (Wang and Pichersky, 1999), and the 3′- and 3′/5′-flavonol OMTs that belong to flavonoid metabolism (Seguin et al., 1998; Muzac et al., 2000). Other polypeptides in the cluster possess very high activities with other than the typical COMT substrates, like the protein from *Chrysosplenium americanum* (AAA86982) that had comparable activities with some flavonoids (Gauthier et al., 1998), or proteins from *Thalictrum tuberosum* that were very active with alkaloids (Frick and Kutchan, 1999).

The recently published structures of two plant OMTs (chalcone and isoflavone OMT) (Zubieta et al., 2001) provided new possibilities to define motifs that may be characteristic for specific OMT groups. Interestingly, the models indicated that both subunits participated in

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a) CrOMT1    MGSANPDNKNSMTKEEEEACLSAMRLASASVLPMVLKSAIELDLLELIKK  50

a) SGPGAYVSPSELAAQLPTQNPDAPVMLDRILRLLASYSVLNCTLKDLPDGGIERLYSLAP 110

a) VCKFLTKNEDGVSMAALLLMNQDKVLMESWYHLKDAVLEGGIPFNKAYGMTAFEYHGKDP 170
d)           |----S1-----|              |-----S2-----|

a) RFNKVFNQGMSNHSTIIMKKILEIYQGFQGLKTVVDVGGGTGATLNMIVSKYPSIKGINF 230
b)                                    |---I--|              |---
c)                                   |---A---|            |-----

a) DLPHVIEDAPSYPGVDHVGGDMFVSVPKGDAIFMKWICHDWSDAHCLKFLKNCHEALPEN 290
b) II--|          |--III-|                                      |
c) J----|      |----K----|  |-----B----|                   |----

a) GKVILAECLLPEAPDSTLSTQNTVHVDVIMLAHNPGGKERTEKEFEALAKGAGFRGFIKV 350
b) IV-|                                |--V-|
c) -C---|                              |-----L----|

a) CCAYNSWIMELLK 363
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Fig. 1. Protein sequence of the *Catharanthus roseus* OMT1, and position of motifs described in previous work. Lines: (b) Ibrahim, 1997; Ibrahim et al., 1998, (c) Joshi and Chiang, 1998, (d) Selman-Housein et al., 1999. The boxed histidine (H) and the two glutamates (E) indicate residues essential for the catalytic activity, as predicted from the crystallized OMTs (Zubieta et al., 2001). The two boxed regions are thought to have major roles in the determination of the substrate preferences (Zubieta et al., 2001). The GenBank accession number for the *C. roseus* OMT1 is AY028439.

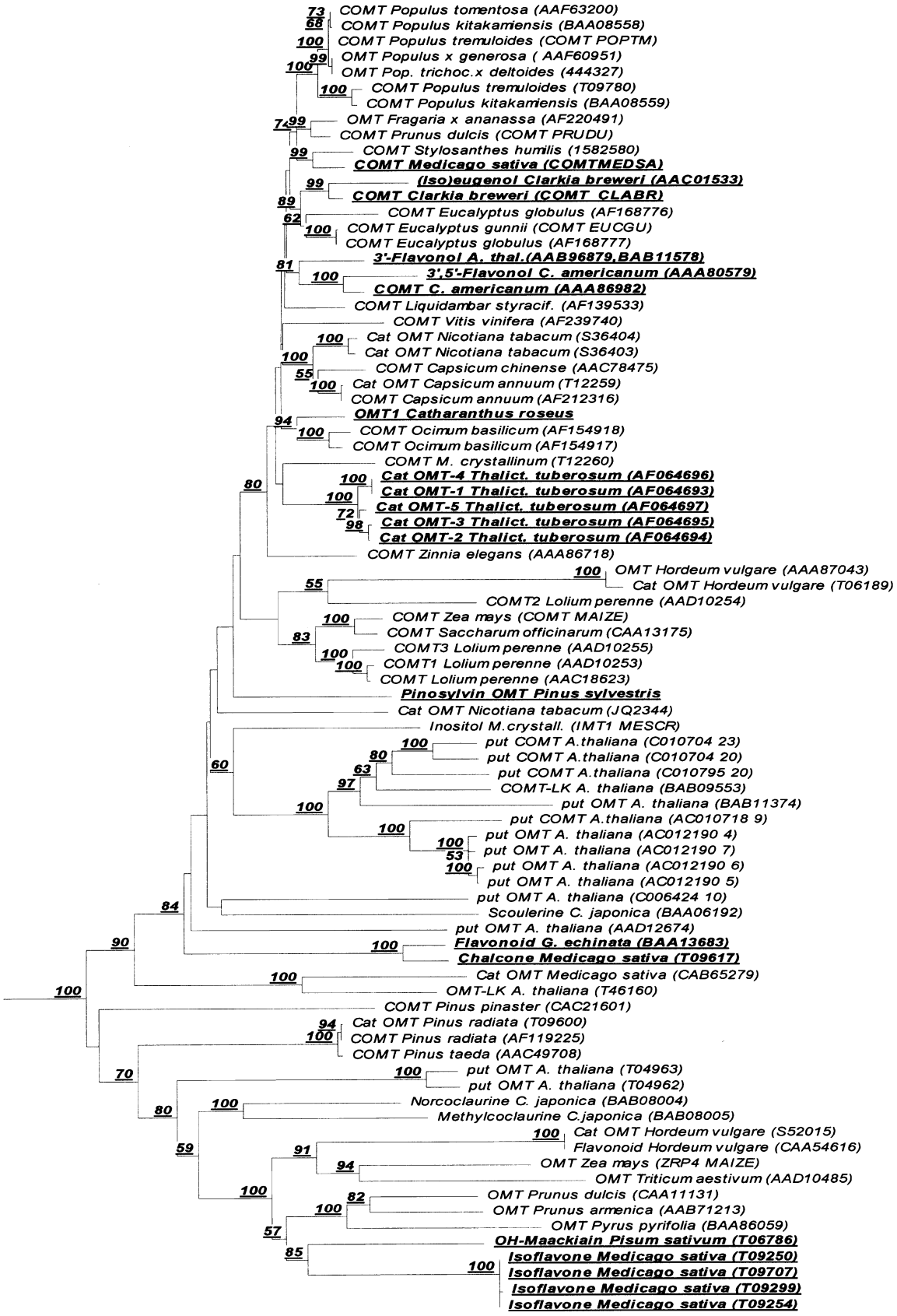


Fig. 2. Relationship tree of plant OMTs. The proteins discussed in more detail in the text are in bold print and underlined. The numbers at the branches are the bootstrap figures that indicate the percent values for obtaining this particular branching in 1000 repetitions of the analysis. The SwissProt accession numbers of the proteins, if available, are given in brackets. Put, putative; cat, catechol.

Table 1
Two regions with major influence on the substrate specificity in the two crystallized OMTs from *Medicago sativa* (Zubieta et al., 2001), and alignment with the corresponding regions in other OMTs[a]

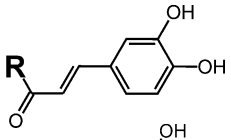
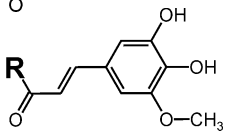
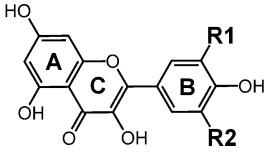
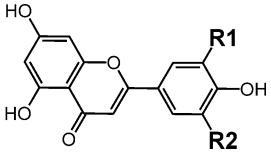
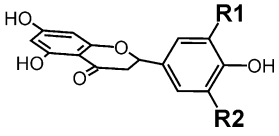
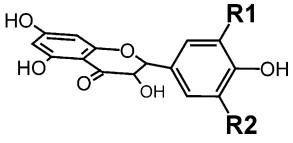
Protein	Motif 1	Motif 2
Crystallized OMTs and closely related		
Chalcone OMT *Medicago* sativa (T09617)	26 LSAMVLTTNLVYPA	324 TLDNLMFITV-G
Flavonoid OMT *Glycyrrhiza echinata* (BAA13683)	26 LSAMRLVTNLVYPA	319 TLDNIMFITV-G
Isoflavone OMT *Medicago sativa* (T09250)	18 ALLYKHIYAFIDSM	306 LMDVNMACLN-G
Hydroxy-maackiain OMT *Pisum sativum* (T06786)	18 IHLYKHVYNFVSSM	313 EYDVVMLTMFLG
COMTs		
COMT consensus	LFAMQLASASVLPM	H.D.IMLAHNPG
COMT *Medicago sativa* (COMT_MEDSA)	23 LFAMQLASASVLPM	315 HIDVIMLAHNPG
COMT *Clarkia breweri* (COMT_CLABR)	21 LFAMQLASASVLPM	320 HIDAIMLAHNPG
OMT1 *Catharanthus roseus*	21 LSAMRLASASVLPM	315 HVDVIMLAHNPG
COMT cluster: multifunctional OMTs		
COMT1 *Chrysosplenium americanum*	2 LFAMQLASASVLPM	291 HIDVITVAHNPG
COMT2 *Chrysosplenium americanum* (AAA86982)	2 LFAMQLACASVLPM	291 HIDVITVAHNPG
Cat OMTs *Thalictrum tuberosum*	22 LHAMQLASASVLPM	316 QLDNIMLAHNPG
Pinosylvin OMT *Pinus sylvestris*	21 LLGMELGNFSCVPM	315 HIDLLMLAYNPG
COMT cluster: OMTs without COMT activities		
3' Flavonol OMT *A. thaliana* (AAB96879)	21 LFAMQLASASVLPM	313 HVDCIMLAHNPG
3'/5'-Flavonol OMT *Chrysosplenium americanum* (AAA80579)	2 LFAMQLASASVLPM	291 TADVMIVTQNSG
(*Iso*)eugenol OMT *Clarkia breweri* (AAC01533)	23 LFAMQLASAAVLPM	318 HTDALMLAYNPG
Other OMTs		
Flavonoid OMT *Hordeum vulgare* (CAA54616)	42 LPCHSFGYLKSMAL	343 TMDLSMMMLFNG
Scoulerine OMT *Coptis japonica* (BAA06192)	40 LSGLGLSRLICLPM	330 TPDLLMMALNPG
Methylcoclaurine OMT *Coptis japonica* (BAB08005)	16 AHVWKIIYGFADSL	303 TLDIDMLV-NTG
Norcoclaurine OMT *Coptis japonica* (BAB08004)	12 AKLWNFIYGFAESL	299 TLDLDMML-NTG
Inositol OMT *Mesembryanthemum crystallinum* (IMT1_MESCR)	23 GLAVTLANAAAFPM	316 SLDCHTLVHNQG

[a] The residues found to be important in the chalcone and isoflavone OMTs are boxed in the first part of the Table. The COMT consensus was derived from the residues conserved in more than 80% of the functionally identified COMT. Boxed residues in the second part of the Table indicate differences to the COMT consensus. In parenthesis: protein identification in the SwissProt database, if available.

forming the active site pockets, and that major contributions to the substrate binding were made by residues in two specific small regions, one in each subunit of the dimer. In motif 1, the positions were not completely identical in the linear alignment of the two proteins, but nevertheless the two motifs defined small regions that could be identified in other OMTs because of the overall similarities in these and adjacent regions. The corresponding regions in the *C. roseus* OMT are marked in Fig. 1. They appear to represent novel motifs that did not correspond to any of the conserved motifs identified before.

Table 1 summarizes the analysis of the two regions in other OMTs. The crystallized OMTs are both involved in flavonoid modifications, but the substrates are very different, and it was therefore not unexpected that they were not similar in the two motifs. However, as might be expected from the close overall relationship and the similarities in function, the crystallized chalcone OMT from *Medicago sativa* and the flavonoid OMT from *Glycyrrhiza echinata* (BAA13683) were very similar, and the same was found for the crystallized isoflavonoid OMT from *Medicago sativa* and the hydroxy-maackiain OMT from *Pisum sativum*. None of these proteins are known to have significant COMT activities. We next looked at the COMTs because the relationship tree suggested that the *C. roseus* cDNA encoded a polypeptide most closely related to the proteins in that group. The functionally identified COMTs in the cluster indicated a high conservation in these two regions. It was possible to establish a consensus, and the comparison showed that it was clearly different from the sequences in either one of the crystallized proteins (Table 1). Only two examples for typical COMTs are shown; they were chosen because they were from the plant with the crystallized OMTs (*Medicago sativa*) or from a plant with a

Table 2
Relative activities of the purified recombinant OMT1 from *Catharanthus roseus* with different substrates. The values were normalized to the activities obtained with the COMT standard substrate, caffeate (100% = 57.5 nmol/min/mg protein)

Structures	Compounds	(%)
	*Caffeate and CoA-ester*	
	Caffeate (R = OH)	100
	Caffeoyl-CoA (R = CoAS)	31
	*5-Hydroxyferulate and CoA-ester*	
	5-Hydroxyferulate (R = OH)	154
	5-Hydroxyferuloyl-CoA (R = CoAS)	41
	*Flavonols*	
	Kaempferol (R1 = R2 = H)	⩽0.1
	Quercetin (R1 = H, R2 = OH)	5.1
	Myricetin (R1 = R2 = OH)	13.2
	*Flavones*	
	Apigenin (R1 = R2 = H)	⩽0.1
	Luteolin (R1 = H, R2 = OH)	1.8
	Tricetin (R1 = R2 = OH)	7.1
	*Flavanones*	
	Naringenin (R1 = R2 = H)	⩽0.1
	Eriodictyol (R1 = H, R2 = OH)	3.4
	Pentahydroxyflavanone (R1 = R2 = OH)	8.0
	*Dihydroflavonols*	
	Dihydrokaempferol (R1 = R2 = H)	⩽0.1
	Dihydroquercetin (R1 = H, R2 = OH)	⩽0.1
	Dihydromyricetin (R1 = R2 = OH)	⩽0.1

closely related cloned OMT with another substrate specificity (the (*iso*)eugenol OMT from *Clarkia breweri*). The *C. roseus* protein followed the COMT consensus except for two deviations in motif 1. Interestingly, these two exchanges had the effect that the first six residues were identical to those in the flavonoid OMT from *Glycyrrhiza echinata* (Table 1). This analysis suggested a prediction that the *C. roseus* OMT1 could be a COMT that may also have activities with other substrates.

The prediction was tested with the purified recombinant protein expressed with a *N*-terminal His-tag in *E. coli*. The results are summarized in Table 2. The highest activities were indeed obtained with phenylpropanoids. The free acids were better substrates than the CoA-esters, and 5-hydroxyferulic acid was a better substrate than caffeic acid. Although not many cloned COMTs were comparatively tested with these substrates, the available data suggest that these properties may be typical for COMTs (Li et al., 1997; Inoue et al., 1998; Meng and Campbell, 1998; Maury et al., 1999). No activity was detected with 16-hydroxytabersonine, the substrate for the methylation reaction in the pathway to the complex indole alkaloids. Interestingly, the assays with various flavonoids showed that the *C. roseus* enzyme had significant activities with certain flavonoids, and pronounced preferences were notable (Table 2). The acceptance as substrate was strictly dependent on the presence of at least two hydroxyl groups in the B ring, and the presence of three hydroxyl groups increased the activities. The TLC chromatograms showed two radioactive products in these cases, suggesting that the enzyme could perform two methylations with these substrates. The activity was influenced by the structure of the ring C, i.e. the absence/presence of a double bond and/or a hydroxyl group. These variations have a significant effect on the 3D-structure of the molecules, as shown in Table 2. Good activities required planar substrates (flavonols, flavones) or at least no hydroxyl group at the C ring (flavanones), if the absence of the double bond in the C ring permitted a turn of the B ring by almost 90°

with respect to the AC ring system. The dihydroflavonols were not accepted, even with three free hydroxyl groups, presumably because they combined angular structure and a C-ring hydroxyl group. Comparable studies of other COMTs are rare (see below for an exception), and therefore it is difficult to judge at present whether the activities observed in our experiments reflect a general property of all COMTs or whether they indeed are a consequence of the differences in the motif discussed here. It is therefore noteworthy that *C. roseus* contains anthocyanins that are methylated in R1 (petunidin) or in both R1 and R2 (malvidin and hirsutidin) (Milo et al., 1985). They were not only found in the plants, but also in calli (Carew and Krueger, 1976) and in light-induced suspension cultures (Knobloch et al., 1982). The enzyme activities were also present in extracts of the cells (not shown), and other experiments indicated that the entire pathway to anthocyanins is expressed in the induced suspension cultures (Kaltenbach et al., 1999). The anthocyanin pattern could be explained by methylations of a substrate with three hydroxyl groups that was performed by a COMT-type enzyme (single or two methylations). Although the precise position of the methyl groups was not determined in our experiments, the results are consistent with the possibility that OMT1 from *C. roseus* may be involved in the methylation of both phenylpropanoid and flavonoid substrates in vivo.

The results suggested that the two motifs might provide useful clues for identifying potential substrates, and therefore we looked at these regions in other OMTs with defined functions. We focussed on three groups with biochemical functions characterized with recombinant enzymes, and the results are summarized in Table 1.

(a) Multifunctional OMTs, defined for the purpose of this work as enzymes with high activities with typical COMT and also non-phenylpropanoid substrates. One example was a protein from *Chrysosplenium americanum* (AAA86982) that is still labelled as COMT in the database, but was identified as a multifunctional OMT with high activity against both phenylpropanoid and flavonoid substrates (Gauthier et al., 1998). The sequences in the motifs deviated from the COMT consensus in one residue in motif 1 and two residues in motif 2. Interestingly, the report also described a variant that lacked one of the three differences (a Cys to Ser exchange, Table 1), and this exchange was one of the few differences thought to be responsible for the notable differences in the substrate preferences of the two proteins (Gauthier et al., 1998). A second example were the five OMT cDNAs cloned from *Thalictrum tuberosum* which provide remarkable demonstrations that a single or a few differences can be responsible for drastic differences in activity and substrate preference (Frick and Kutchan, 1999). All five proteins were identical in the motifs considered here, with two differences to the COMT consensus, one in each motif. These may be taken as a hint that other than COMT substrates can be used (in this case alkaloids), but the large activity differences between the proteins require other explanations. It may be significant, however, that one of the crucial residues for different substrate preferences (a Cys-Tyr exchange) was located in position 21, just one residue before the begin of motif 1 as defined in this work. It may be possible that extensions of the motifs considered here may provide additional information. A third case was the OMT from *Pinus sylvestris* that had roughly comparable activities with typical COMT substrates, some flavonoids, and pinosylvin (Chiron et al., 2000). That enzyme revealed more differences to the COMT consensus (Table 2), and it also did not cluster in the large branch containing the majority of the COMTs in dicots (Fig. 1).

(b) Proteins in the large COMT cluster, but without significant activities against typical COMT substrates. Examples were the two closely related flavonol OMTs from *Chrysosplenium americanum* (AAA80579) and *A. thaliana* (AAB96879) (Fig. 1). The first was shown to be a 3′/5′-flavonol OMT with no activity against phenylpropanoid substrates (Gauthier et al., 1996; Seguin et al., 1998); this protein was clearly different from the COMT consensus, but only in the second motif (Table 1). The *A. thaliana* protein, originally described as COMT based on the high similarity with known COMTs (Zhang et al., 1997), was later identified as a 3′-flavonol OMT possessing little activity with caffeic acid, but high activities against flavonoids with vicinal hydroxyl groups in the B-ring (Muzac et al., 2000). The two motifs revealed only one difference to the COMT consensus, a Cys in a position that in most cases is a hydrophobic acid in COMTs. An interesting case is the (*iso*)eugenol OMT from *Clarkia breweri* that is closely related to the COMT from the same plant (83% identity, see also Fig. 1 for positions in the relationship tree). The COMT followed the consensus, while the (*iso*)eugenol OMT revealed three differences (Table 1). Other experiments with mutagenized proteins indicated that they have most likely not decisive roles in the substrate specificity because seven amino acid exchanges in other positions (130–131, 133–135, 164–165) were sufficient to change the substrate specificity (Wang and Pichersky, 1999). Those changes, however, were not sufficient to obtain the full catalytic activity, and it would be interesting to see whether the differences in the two motifs are important for that aspect.

(c) Other OMTs. Finally, we compared the typical COMT sequences in the two motifs with the corresponding regions in some other OMTs that use other substrates and cluster in distinctly different branches in the relationship tree (Fig. 1). The analysis of the similarities in the total sequences indicated that they were sufficiently closely related to the other OMTs to permit a reasonable overall alignment, including the regions of the two

motifs. Table 1 shows that these sequences are clearly different, as might be expected from enzymes with entirely different substrate specificities.

2.1. Concluding remarks

The example of the *C. roseus* cDNA discussed here indicated that a careful analysis of the deduced protein sequence can be of significant help in predicting potential substrates of the enzymes. It should be pointed out, however, that overall similarities are hardly sufficient. A more detailed consideration of specific regions, including the motifs defined in this work, appears to be essential for a reasonable prediction of the substrate specificities.

3. Experimental

3.1. Plant material

The cell suspension culture of Madagascar periwinkle (*Catharanthus roseus* L.G. Don, line CP3a) and its maintenance in MX growth medium in continuous dark with subcultures every week have been described (Vetter et al., 1992).

3.2. Homology based PCR and heterologous expression

The preparation of cDNA libraries followed established methods (Schröder et al., 1999). The initial PCR reactions for OMT sequences were carried out with the primers and techniques described previously (Frick and Kutchan, 1999), and overlapping clones for the complete coding region were obtained by PCR with specific primers designed from the known sequences and primers for phage sequences flanking the cDNA inserts.

For expression in *E. coli*, the protein coding region was amplified with a 5′-primer introducing a *Bam*HI site after the start AUG (5′ TA GGA TCC GCA AAT CCA GAC AAT AAA AAT TCG-3′) and a 3′ antisense primer introducing a *Hin*dIII site directly after the stop codon (5′-CC AAGCTT TTA TTT GAG CAA TTC CAT AAT CC-3′). The *Bam*HI/*Hin*dIII fragment was inserted into vector pHis8-3 which provided the expressed protein with a His-tag at the *N*-terminal (Ferrer et al., 1999). The protein was affinity purified with the His-trap™ purification kit from Pharmacia Biotech (Freiburg, Germany) as recommended by the manufacturer.

3.3. Enzyme assays

The assays contained 50 mM Tris–HCl (pH 7.5), 2 mM DTT, 0.1 mM substrate, 40 μM unlabelled *S*-adenosyl-L-methionine, and 9.3 μM *S*-adenosyl-L-[methyl-^{14}C] methionine (54 μCi/μmol, 55,000 dpm) in 50 μl. They were kept for 20 min at 30 °C. The reaction mixtures were then extracted with EtOAc (twice with 0.1 ml) after acidification. The solvent was removed by evaporation, the residue was dissolved in 10 μl methanol, and the radioactive products were analyzed by quantitative TLC (Vetter et al., 1992) on silica gel plates with fluorescence indicator ($60F_{254}$, Merck, Darmstadt, Germany). The reaction conditions were optimized with respect to linearity with time and protein concentration. Details for specific substrates: a) assays with the preferred substrates (caffeic acid, 5′-hydroxyferulic acid, caffeoyl-CoA, 5′-hydroxyferuloyl-CoA) were carried out with 0.3 μg recombinant enzyme. The CoA-esters were hydrolyzed (Ye et al., 1994) under alkaline conditions to the free acids after the incubations. The TLC solvent was toluol-HOAc-H_2O (57,5:36:1.5); b) the incubations with flavonoids were performed with 1 and 3 μg recombinant protein. The TLC analysis used several solvents to check whether more than one radioactive product was produced. The solvent described for the phenylpropanoids could be used in most cases. Other solvents, used in particular with flavonols, were toluol–ethyl formiate–HCO_2H (5:4:1) and $CHCl_3$–MeOH (75:5). The results are averages from at least three determinations.

3.4. Relationship tree

The protein sequences were aligned with CLUSTAL W built into the sequence analysis program OMIGA 2.0 (Oxford Molecular, England), and the alignment was improved by visual inspection. The relationship tree was developed with the program TREECON for Windows (Van de Peer and De Wachter, 1994), using the matrix for amino acid sequences and the neighbour-joining method. The outgroup for the plant sequences was the *O*-demethylpuromycin-*O*-methyltransferase from *Streptomyces anulatus* (SwissProt DMPM_STRLP).

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