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Flavonoid methylation: a novel 4′-*O*-methyltransferase from *Catharanthus roseus*, and evidence that partially methylated flavanones are substrates of four different flavonoid dioxygenases

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Abstract

Catharanthus roseus (Madagascar periwinkle) flavonoids have a simple methylation pattern. Characteristic are B-ring 5′ and 3′ methylations and a methylation in the position 7 of the A-ring. The first two can be explained by a previously identified unusual *O*-methyltransferase (CrOMT2) that performs two sequential methylations. We used a homology based RT-PCR strategy to search for cDNAs encoding the enzyme for the A-ring 7 position. Full-length cDNAs for three proteins were characterized (*CrOMT5*, *CrOMT6*, *CrOMT7*). The deduced polypeptides shared 59–66% identity among each other, with CrOMT2, and with CrOMT4 (a previously characterized protein of unknown function). The five proteins formed a cluster separate from all other OMTs in a relationship tree. Analysis of the genes showed that all *C. roseus* OMTs had a single intron in a conserved position, and a survey of *OMT* genes in other plants revealed that this intron was highly conserved in evolution. The three cDNAs were cloned for expression of His-tagged recombinant proteins. CrOMT5 was insoluble, but CrOMT6 and CrOMT7 could be purified by affinity chromatography. CrOMT7 was inactive with all compounds tested. The only substrates found for CrOMT6 were 3′-*O*-methyl-eriodictyol (homoeriodictyol) and the corresponding flavones and flavonols. The mass spectrometric analysis showed that the enzyme was not the expected 7OMT, but a B-ring 4′OMT. OMTs with this specificity had not been described before, and 3′,4′-dimethylated flavonoids had not been found so far in *C. roseus*, but they are well-known from other plants. The identification of this enzyme activity raised the question whether methylation could be a part of the mechanisms channeling flavonoid biosynthesis. We investigated four purified recombinant 2-oxoglutarate-dependent flavonoid dioxygenases: flavanone 3ß-hydroxylase, flavone synthase, flavonol synthase, and anthocyanidin synthase. 3′-*O*-Methyl-eriodictyol was a substrate for all four enzymes. The activities were only slightly lower than with the standard substrate naringenin, and in some cases much higher than with eriodictyol. Methylation in the A-ring, however, strongly reduced or abolished the activities with all four enzymes. The results suggested that B-ring 3′ methylation is no hindrance for flavonoid dioxygenases. These results characterized a new type of flavonoid *O*-methyltransferase, and also provided new insights into the catalytic capacities of key dioxygenases in flavonoid biosynthesis.

Keywords: *Catharanthus roseus*; Apocynaceae; Madagascar periwinkle; Homology based cDNA cloning; Recombinant protein expression; *O*-Methyltransferase; Flavanone 3ß-hydroxylase; Flavone synthase; Flavonol synthase; Anthocyanidin synthase

1. Introduction

Methylations are part of the biosynthesis of many flavonoids, and anthocyanin methyl groups play a role in flower colours (Harborne and Williams, 2000). Several enzyme activities have been described (see Dixon, 1999; Forkmann and Heller, 1999), but only a few have been characterized both at the functional and the molecular level (Maxwell et al., 1993; Gauthier et al.,

Abbreviations: 4′OMT, enzyme methylating the position 4′ in the B-ring of flavonoids; 7OMT, enzyme methylating the position 7 in the A-ring of flavonoids; ANS, anthocyanidin synthase; FHT, flavanone 3β-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; OMT, *O*-methyltransferase.

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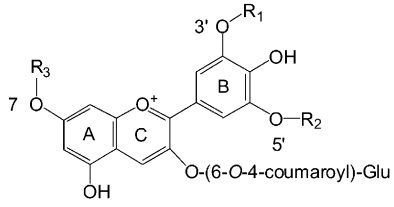
1995, 1998; Haga et al., 1997; Wu et al., 1997; He et al., 1998; Christensen et al., 1998; Akashi et al., 2003).

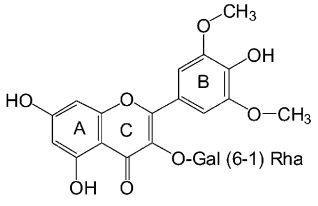
The flavonoids known from *Catharanthus roseus* (Madagaskar periwinkle) have a simple methylation pattern (Fig. 1), typically with methyl groups in the B-ring 3′ or both 3′ and 5′ positions, and only one anthocyanin (hirsutidin) carries an additional methyl group in the position 7 of the A-ring (Carew and Krueger, 1976; Knobloch et al., 1982; Milo et al., 1985; Piovan et al., 1998; Brun et al., 1999; Filippini et al., 2003). Previous experiments had shown that the two B-ring methylations, at least at the level of the flavonols, could be introduced by an unusual *O*-methyltransferase (OMT) that carried out two sequential methylations (Cacace et al., 2003). Theoretically it seemed possible that one additional enzyme, a 7-*O*-methyltransferase (7OMT) was sufficient to explain all methylations. It was also possible, however, that this was too simplistic, and that the 3′ and 5′ B-ring methylations in flavonols and anthocyanidins were carried out by different enzymes and with different intermediates.

We used a homology-based RT-PCR strategy to identify and characterize additional OMTs, to look for a 7OMT and to gain insights into the diversity of OMTs expressed in various tissues. Three cDNAs for new OMTs were analyzed, and a functional identification was achieved with one of them. The result was unexpected: the enzyme very specifically used flavonoids methylated in the 3′-position of the B-ring to synthesize a 3′,4′-methylated product. Such enzyme had not been described before, and so far such flavonoids have not been identified from *C. roseus*. Interestingly, a methylated flavanone was the best substrate, raising the question whether the enzymes using flavanones to synthesize flavones, dihydroflavonols, or flavonols would be active with such substrates. The results with four 2-oxoglutarate-dependent enzymes showed that B-ring 3′-methylation was no serious obstacle for their activities.



Anthocyanins

	R_1	R_2	R_3
Petunidin	CH_3	H	H
Malvidin	CH_3	CH_3	H
Hirsutidin	CH_3	CH_3	CH_3



Flavonol glycoside
(Syringetin 3-*O*-robinobioside)

Fig. 1. Methylation patterns of known anthocyanins and flavonols in *C. roseus*. Syringetin 3-*O*-robinobioside = syringetin 3-*O*-α-L-rhamnopyranosyl-(1-6)-ß-D-galactopyranoside. Glu, glucose; Rha, rhamnose; Gal, galactose.

2. Results and discussion

2.1. Molecular characterization of three new OMTs

The RT-PCR approach with degenerate primers (Cacace et al., 2003) and RNAs from cell cultures, seedlings, or leaves from adult plants led to the identification of partial sequences for three new putative small molecule OMTs. Complete cDNAs were obtained by 5′ and 3′ RACE techniques with specific primers, and the sequences were designated *CrOMT5*, *CrOMT6*, and *CrOMT7*. Analysis by qualitative RT-PCR with gene-specific oligonucleotides revealed differential expression. *CrOMT6* expression was very low in dark-grown cell cultures, but strongly increased after induction by treatment with sucrose and irradiation, and it was also high in leaves. *CrOMT7* transcripts were present in seedlings, not detectable in dark-grown cell cultures, but high levels were observed after treatment with sucrose and irradiation. The expression was also high in leaves. Transcripts from *CrOMT5* were detected only in leaves, not in the other tissues investigated.

The overall relationships among the three deduced proteins and with the previously characterized CrOMT2 and CrOMT4 ranged from 59 to 74% (Table 1). The most closely related OMTs from other plants were the orcinol OMTs from *Rosa hybrida* (Lavid et al., 2002), with scores ranging from 48 to 52%. Blast searches with the proteins also detected 40–49% identity with an OMT of unknown function from *Prunus amygdalus* (almond) flowers (Suelves and Puigdomènech, 1998),

Table 1
Overall relationships (% identity) of the proteins encoded in the three new OMTs among each other and with the previously described CrOMT2 and CrOMT4 (Cacace et al., 2003)

	CrOMT2	CrOMT4	CrOMT5	CrOMT6
CrOMT4	74			
CrOMT5	66	60		
CrOMT6	63	60	65	
CrOMT7	64	59	64	64

The GenBank accession numbers of the new sequences are: *CrOMT5* gene = AY343487, *CrOMT5* cDNA = AY343488, *CrOMT6* gene = AY343489, *CrOMT6* cDNA = AY343490, *CrOMT7* gene = AY343491, *CrOMT7* cDNA = AY343492.

with isoflavonoid 3OMT (Wu et al., 1997), 4′OMTs (Akashi et al., 2003) and 7OMTs (He et al., 1998; Akashi et al., 2003). Similar scores were obtained with chavicol or eugenol OMTs (Gang et al., 2002; Scalliet et al., 2002). Of particular interest was the sequenced flavonoid 7OMT (Christensen et al., 1998), but the identity scores were not higher than with the other OMTs. These results did not permit a reasonable speculation on the function of the new genes, but the even lower values (30–33%) found with typical caffeic acid OMTs (COMTs) suggested that the new genes did not simply represent another set of such OMTs.

A more detailed analysis of the overall relationships with OMTs from other plants is presented with the relationship tree in Fig. 2. It shows that the *C. roseus* OMTs 2, 4, 5, 6, and 7 formed a cluster separate from all other small molecule OMTs, and that two subbranches could be distinguished (CrOMTs 6 and 7 versus CrOMTs 2,4, and 5).

2.2. A highly conserved intron

The *CrOMT2* and *CrOMT4* genes contained a single intron at the same position of the protein sequence (Cacace et al., 2003), and it was therefore of interest whether the gene structure would permit a more detailed differentiation than revealed by the relationship tree. The analysis was carried out with genomic fragments obtained after PCR with primers amplifying the genes from start to stop codon of the proteins. The results revealed that *CrOMT5*, *6*, and *7* also contained single introns, and protein alignments showed that they were precisely in the same position as in *CrOMT2* and *4* (Fig. 3). No significant similarity was detected between

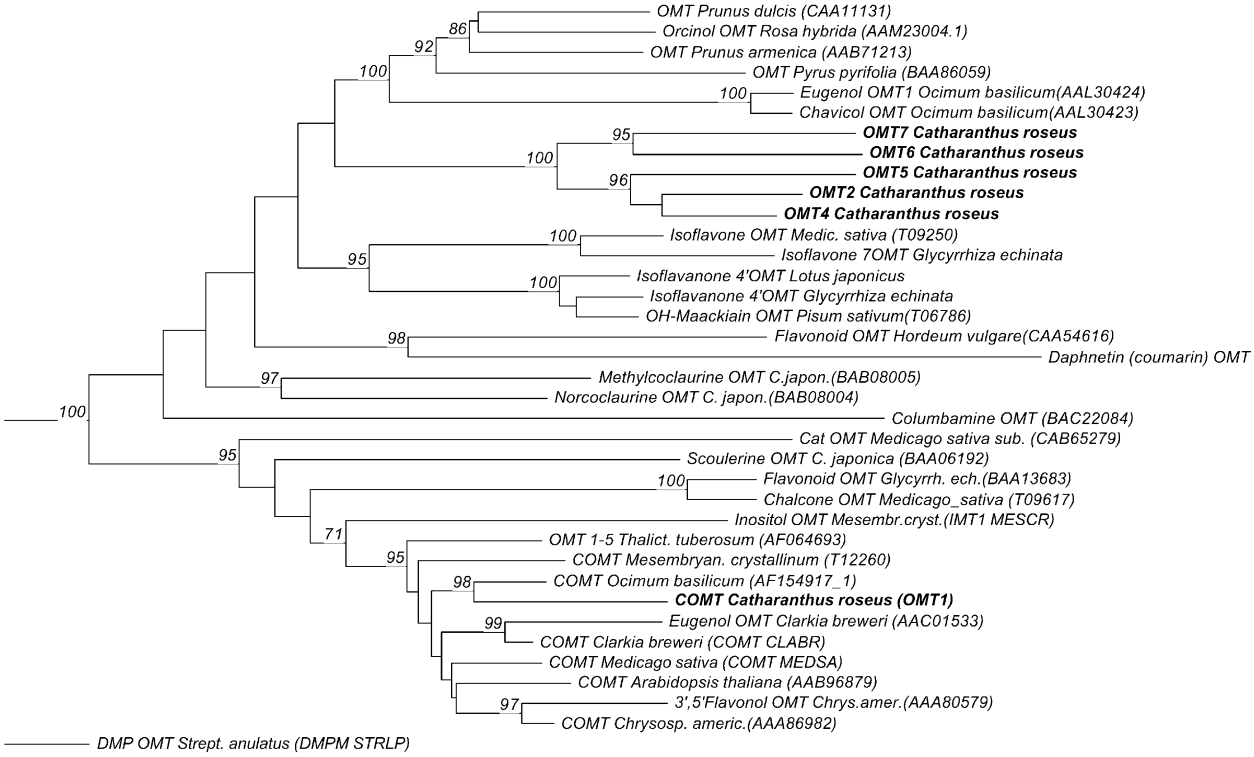


Fig. 2. Relationship tree of selected plant OMTs. The numbers at the forks are bootstrap values that indicate the percent values for obtaining this particular branching in 1000 repetitions of the analysis; only the values above 60% are shown. DMPM_STRLP, the *O*-demethylpuromycin-*O*-methyltransferase from *Streptomyces anulatus* that was used as outgroup for the development of the tree.

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                intron position and size (bp)
CrOMT2   GGDMFEKIPSANAILLK  (380) WILHDWKDEECVKVLKMCR
CrOMT4   GGDMFEKIPS ANAILLK  (369) SVLHDWKDEDSVKILKNCK
CrOMT5   GGDMFERIPNANAILLK  (652) IVLHNWNDEDCMKILKKCK
CrOMT6   GGDMFEKLPPSNAILLK (1135) WILHDWNDEDCVKILKNCK
CrOMT7   GGNMFEKIPPAHVVFLK (1224) MVLHDWNDEDCVKILKNCR
CrOMT1   GGDMFVSVPKGDAIFMK  (737) WICHDWSDAHCLKFLKNCH
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Fig. 3. Conserved position of the single intron in *C. roseus OMT* genes. The positions of the conserved lysine (boxed) in the protein sequences are: CrOMT2 and CrOMT4 = 246; CrOMT5 = 248; CrOMT6 = 259; CrOMT7 = 254; CrOMT1 = 265.

the intron sequences, and large differences were also observed in their sizes (Fig. 3).

The conservation of the position confirmed the close relationships in this subgroup, and it seemed possible that it might be specific for these OMTs. This was investigated by analyzing the gene structure of the *COMT* (*CrOMT1*) from *C. roseus* that locates to quite a different branch of the relationship tree (Fig. 2). The results revealed a single intron in the same position (Fig. 3), and thus the conserved intron position was not a property specific for a particular OMT subgroup in *C. roseus*. A further check of *OMT* genes in other plants revealed that the intron position was not even specific for *C. roseus*, but highly conserved throughout widely different plant families. For example, the 17 genes in the genome of *A. thaliana* predicted to code for *OMTs* (11 on chromosome 1, one on chromosome 3, two on chromosome 4, and three on chromosome 5) contained 1 to 4 predicted introns, but there was always an intron in the position corresponding to that in *C. roseus*, and it was always the last with respect to the 3′ end of the protein-coding region. It was also conserved in caffeic acid *OMTs* from *Pinus radiata* (Moyle et al., 1999), various *Populus* species (Hayakawa et al., 1996; Hu and Chiang, 1997), and *Eucalyptus globulus* (GenBank accession AF168777). It was also described for the gene of the inositol *OMT* from *Mesembryanthemum crystallinum* (Vernon and Bohnert, 1992), and in a putative *COMT* from *Hordeum vulgare* (Lee et al., 1997). This intron was apparently highly conserved in evolution. However, there were also exceptions. It was absent in the caffeic acid *OMT* genes from *Zea mays* (Collazo et al., 1992), *Sorghum bicolor* (Bout and Vermerris, 2003), and in a genomic sequence described from *Oryza sativa* (GenBank accession AAAA01004407). The conservation of this intron had not been noticed before. Not many OMT genes in this family have been investigated, but further sequences could provide some interesting insights into the evolution of these genes.

2.3. Identification of a novel flavonoid O-methyltransferase

CrOMT5, *6*, and *7* were recloned for expression of His-tagged recombinant proteins in *E. coli* (details in Experimental). SDS-PAGE of cells lysed in the presence of SDS showed that all three proteins were expressed. Analysis of extracts prepared with non-denaturing buffers showed that CrOMT6 and CrOMT7 were soluble proteins. However, CrOMT5 was always obtained only as insoluble protein and thus could not be tested for enzyme activities. CrOMT6 and CrOMT7 were purified to near homogeneity by affinity chromatography based on the His-tag.

Assays with the collection of flavonoids (flavanones, flavones, dihydroflavonols, and flavonols) investigated in previous work (Schröder et al., 2002; Cacace et al., 2003, see also Table 2) failed to reveal any activity with either CrOMT6 or CrOMT7. Of particular interest with respect to a 7OMT was syringetin because it carried the B-ring dimethylation pattern that could be a precursor configuration for a final methylation to hirsutidin (see Fig. 1), and, although it was not likely to be a precursor to anthocyanidins, it seemed possible that it might be accepted with low efficiency by a 7OMT. However, no activity was detectable. In the context of looking for potential substrates with partial methylations we also investigated 3′-*O*-methyl-eriodictyol (homoeriodictyol) because it resembled a possible partially methylated flavanone intermediate for a 7OMT activity, except for the missing 5′-substitution. CrOMT7 was inactive, but with CrOMT6 the standard TLC analysis revealed a new radioactive substance in incubations with ^{14}C-labelled SAM. Large-scale incubations with unlabelled substrates were carried out for product identification, and the results are shown in Fig. 4. These flavonoids revealed a subfragmentation pattern permitting an unambiguous conclusion whether the introduced methyl

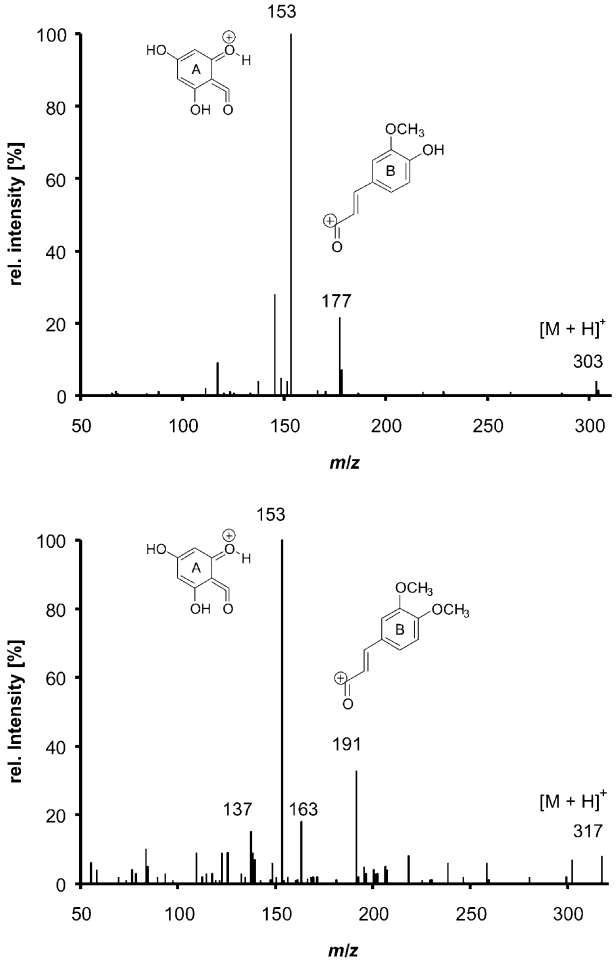
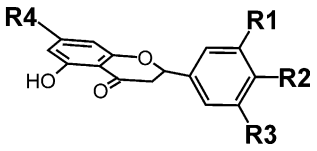
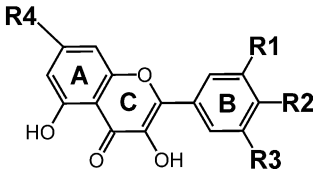
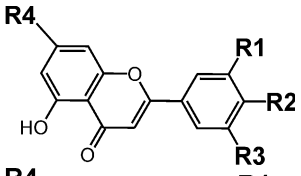
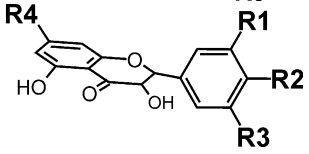


Fig. 4. MS/MS analysis of homoeriodictyol (top) and its reaction product in vitro (bottom).

Table 2
Substrate preferences of purified recombinant OMT6 from *C. roseus*

Structures	Compound	R1	R2	R3	R4	pmol/min/mg	rel. act. (%)[a]
	*Flavanones*						
	Naringenin	H	OH	H	OH	⩽5	<2
	Eriodictyol	OH	OH	H	OH	20±2	7
	Pentahydroxyflavanone	OH	OH	OH	OH	⩽1	<1
	Homoeriodictyol	O-CH_3	OH	H	OH	275±10	100
	Hesperetin	OH	O-CH_3	H	OH	⩽1	⩽1
	*Flavonols*						
	Kaempferol	H	OH	H	OH	15±5	5
	Quercetin	OH	OH	H	OH	35±5	13
	Myricetin	OH	OH	OH	OH	<1	<1
	Isorhamnetin	O-CH_3	OH	H	OH	100±10	36
	7,3′-*O*-Dimethyl-quercetin	O-CH_3	OH	H	O-CH_3	<1	<1
	7-*O*-Methyl-quercetin	OH	OH	H	O-CH_3	<1	<1
	Syringetin	O-CH_3	OH	O-CH_3	OH	<1	<1
	*Flavones*						
	Apigenin	H	OH	H	OH	⩽1	<1
	Luteolin	OH	OH	H	OH	⩽1	<1
	Tricetin	OH	OH	OH	OH	⩽1	<1
	Chrysoeriol	O-CH_3	OH	H	OH	102±10	37
	Velutin	O-CH_3	OH	H	O-CH_3	⩽1	⩽1
	*Dihydroflavonols*						
	Dihydrokaempferol	H	OH	H	OH	⩽1	<1
	Dihydroquercetin	OH	OH	H	OH	⩽1	<1
	Dihydromyricetin	OH	OH	OH	OH	⩽1	<1
	3′-*O*-Methyl-dihydroquercetin	O-CH_3	OH	H	OH	⩽1	<1

[a] Average from at least four independent incubations.

group was in the A- or the B-ring. The MS/MS analysis clearly showed that the methylation was not, as had been expected, in the A-ring, but in the B-ring, and it must have been in the 4′ position because this was the only free hydroxyl group in the substrate.

This unexpected result prompted a more detailed investigation, and the data are summarized in Table 2. The enzyme was specific for the B-ring configuration of homoeriodictyol, because no or little activity was observed with naringenin, eriodictyol, pentahydroxyflavanone, and importantly, with hesperetin (4′-*O*-methyl-eriodictyol) that carries a B-ring methyl group, but in the 4′ position. Low activities were detected with the flavonols kaempferol and quercetin, but appreciable rates were found only with isorhamnetin (3′-*O*-methyl-quercetin) that had the same substitution pattern as homoeriodictyol. As noted before, no activity was detected with syringetin (3′,5′-*O*-dimethyl-myricetin), indicating that the additional *O*-methylation in the 5′-position was not tolerated. The specificity was even more pronounced with flavones, because only chrysoeriol (3′-*O*-methyl-luteolin) revealed good activities. Interestingly, the corresponding dihydroflavonol (3′-*O*-methyl-dihydroquercetin) was not accepted (Table 2). The structural requirements for the A-ring appeared similarly rigid, as suggested by incubations with the few available substrates. An *O*-methyl group in position 7 of the A-ring (e.g. 7-*O*-methyl-quercetin) was not acceptable, even in combination with an optimal B-ring configuration, e.g. with the flavonol 7,3′-*O*-dimethyl-quercetin or with the flavone velutin (7,3′-*O*-dimethyl-luteolin) (Table 2).

Flavonoids with the substitution pattern of homoeriodictyol or of the 4′-methylated derivative have not yet been described from *C. roseus*. It seemed therefore possible that the physiological substrate was not a flavonoid, but another molecule containing a substitution pattern corresponding to the B-ring of homoeriodictyol. Such compounds are well known, e.g. from lignin biosynthesis (e.g. ferulic acid, coniferylalcohol) or in scent production (Lewinsohn et al., 2000; Gang et al., 2001; Lavid et al., 2002). Fig. 5 summarizes the compounds tested. The recombinant CrOMT6 had no detectable activity with any of them. These results indicated that not only the B-ring configuration, but also the size and shape of the A-ring were critical parts of the substrate specificity.

Taken together, these experiments identified an OMT activity that to the best of our knowledge has not been described before with flavonoids. The role in *C. roseus* remains open as 3′,4′-*O*-methylated compounds have not been described from this plant. It should be noted, however, that *C. roseus* has not been characterized very extensively with respect to flavonoids, and it would be

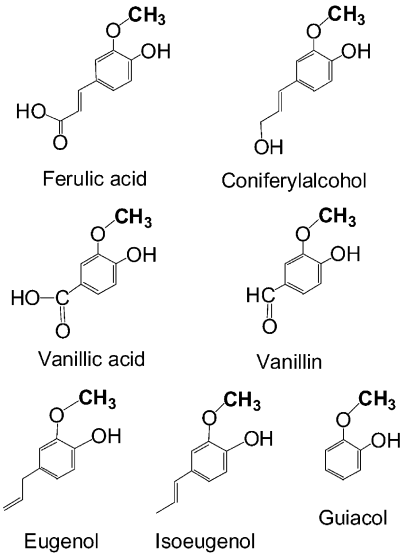


Fig. 5. Potential substrates of CrOMT6, containing the same methylation configuration as in homoeriodictyol, isorhamnetin, and chrysoeriol (Table 1).

useful to initiate a metabolic profiling specifically searching for such methylation patterns. Our studies also provide some helpful information. The enzyme activity with homoeriodictyol was present in dark-grown *C. roseus* seedlings, and it was induced slightly by irradiation. As noted already, irradiation also induced *CrOMT6* transcription in the cell suspension cultures, and the presence of the protein in these cells was confirmed in a proteomics approach using mass spectrometric protein identification (S. Cacace and J. Schröder, unpublished results).

2.4. Homoeriodictyol is a substrate for several 2-oxoglutarate-dependent flavonoid dioxygenases

The identification of this OMT specificity is of interest to many other plants as flavonoids carrying a 3′,4′-*O*-dimethylation are known from various plant families, e.g. Asteraceae, Fabiaceae, Lamiaceae, Rutaceae, Zingiberaceae, Scrophulariaceae (see Harborne and Baxter, 1999, for overview). There is little information at which stage the *O*-methyl groups are introduced (Heller and Forkmann, 1994; Forkmann, 1994; Forkmann and Heller, 1999), and CrOMT6 could be an example for 4′OMTs in their biosynthesis. In this context it is important to note that a flavanone was the best substrate (homoeriodictyol). Flavanones are the basis for the formation of all other classes, and, although this needs to be confirmed with other plants, the substrate preference of CrOMT6 raised the question whether such methylation would be tolerated by downstream activities, e.g. the enzymes synthesizing flavones, flavonols, or dihydroflavonols. To the best of our knowledge this had not been investigated before, and the information was expected to be of general interest because acceptance or non-acceptance could provide some new clues on potential channelling points in the pathways to the final products.

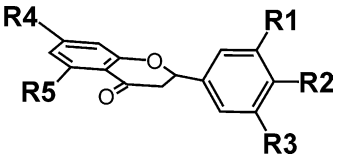
We investigated four enzymes involved in key reactions converting flavanones into further products: flavanone 3β-hydroxylase (FHT, product dihydroflavonol), flavone synthase (FNS, product flavone); and two enzymes (flavonol synthase, FLS, and anthocyanidin synthase, ANS) that have recently been shown to synthesize both dihydroflavonols and flavonols from flavanones (Lukačin et al., 2003; Martens et al., 2003). Clones for these enzymes are not yet available from *C. roseus*, but these enzymes appear to have similar properties in all plants investigated (Forkmann and Heller, 1999), and thus it seemed justified to use purified recombinant proteins from other plants for first experiments investigating these new questions.

The results, summarized in Table 3, revealed substrate specificities that had not been suspected before. Homoeriodictyol (3′-*O*-methyleriodictyol) was a good substrate for all four proteins, and with FNS and FLS it was even a much better substrate than eriodictyol. The position of the methyl group was important because hesperetin (4′-*O*-methyleriodictyol) had much lower (FHT, FNS) or almost no activity (FLS, ANS). Additional methyl groups were not tolerated as all four enzymes were inactive with the flavanone carrying *O*-methyl groups in all three positions of the B-ring (3′,4′,5′-*O*-trimethyl-flavanone).

Compounds methylated in the A-ring were not available in the eriodictyol series, but pinocembrin and a few derivatives carrying A-ring substitutions could be tested. Methylation in position 7 of the A-ring abolished the activity with all four enzymes, and, except for a low activity found with FHT for 5-*O*-methylpinocembrin, the same effect was observed with methylations in the position 5 or both 5 and 7.

The sum of these results showed that an *O*-methyl group in B-ring position 3′ (as in homoeriodictyol) had no large effect on the capacities of FHT, FNS, FLS, or ANS to carry out their reactions. Such modification thus would not present a hindrance for the further conversion into the other flavonoid classes, i.e. such methylation was not likely to provide a channelling point. That was clearly different with any of the other modifications investigated. Methylations at the neighbouring 4′ position (hesperetin) or in more than one position (e.g. 3′,4′,5′-*O*-trimethyl-flavanone) reduced or abolished the enzyme activities. The same was found with *O*-methylations in the A-ring because single methylations in either 5 or 7 position, or double methylations, completely blocked the further conversions by any of the four flavonoid hydroxylases investigated. The data were compatible with ideas that these modifications might occur later in the complex pathway

Table 3
Methylated flavanones as substrates of recombinant 2-oxoglutarate-dependent flavonoid dioxygenases: FHT from *Petunia hybrida*, FNS from *Petroselinum crispum,* FLS from *Citrus unshiu*, and ANS from *Gerbera hybrida*

Substrate 						Relative activity[a]			
	R1	R2	R3	R4	R5	FHT	FNS	FLS[b]	ANS[b]
Naringenin	H	OH	H	OH	OH	100	100	100/100	100/100
Eriodictyol	OH	OH	H	OH	OH	95	24	7	75/167
Homoeriodictyol	O-CH_3	OH	H	OH	OH	65	97	82/47	42/130
Hesperetin	OH	O-CH_3	H	OH	OH	34	26	7	8
5,7-Dihydroxy-3′,4′,5′-*O*-trimethyl-flavanone	O-CH_3	O-CH_3	O-CH_3	OH	OH	<1	<1	<1	<1
Pinocembrin	H	H	H	OH	OH	38	94	18	8
7-*O*-Methyl-pinocembrin	H	H	H	O-CH_3	OH	<1	<1	<1	<1
5-*O*-Methyl-pinocembrin	H	H	H	OH	O-CH_3	9	<1	<1	<1
5,7-*O*-Dimethyl-pinocembrin	H	H	H	O-CH_3	O-CH_3	<1	<1	<1	<1

[a] Values normalized with respect to the activity with naringenin; 100 corresponds to the following specific activities (mkat/kg): FHT = 1.49; FNS = 0.34; FLS = 0.28 (product dihydroflavonol), 0.15 (product flavonol); ANS = 0.12 (product dihydroflavonol), 0.03 (product flavonol). The data are the average from triplicate incubations, with variations <15% between the assays.

[b] FLS and ANS can synthesize both dihydroflavonols (first figure) and flavonols (second figure) from flavanones (Lukačin et al., 2003)

to the end products. It will be interesting to see whether the specificities elucidated in our work are actually contributing to metabolite channeling in vivo.

2.5. Concluding remarks

The results from the previous and the present work show that *C. roseus* expresses at least six proteins belonging to the family of small molecule OMTs that emerged in the last years, and the functions of three have been identified (caffeic acid OMT: Schröder et al., 2002; 3′,5′OMT, CrOMT2: Cacace et al., 2003; 4′OMT, CrOMT6: this work). The functional diversity within this family is large; it ranges from caffeic acid OMTs to enzymes methylating various other phenylpropanoid derivatives, flavonoids, isoflavonoids, chalcones, orcinols, inositol, coumarins, and various alkaloids. It should be noted that the protein family is much larger than shown in Fig. 2, because most of the members identified only by protein similarity were left out in this presentation. Crystal structures of a few OMTs have been published (Zubieta et al., 2001, 2002), but the structural reasons for the functional diversity are still not easily elucidated, and the same applies to attempts for predictions of functions for new members of the family. It is further complicated by the finding that some proteins with high similarity to caffeic acid OMTs did not only catalyze the enzyme reaction typical for such proteins, but also had high activities with other substrates (Gauthier et al., 1998; Frick and Kutchan, 1999; Chiron et al., 2000; Wein et al., 2002). Heterologous expression and enzyme assays still appear to be essential for functional identifications. Our present work highlights another challenging aspect: linking sequences and functions is in many cases severely hampered by the fact that most plants have not been investigated thoroughly with respect to the range of natural products they contain. One good example for this is that the major anthocyanin in *Arabidopsis thaliana* has been identified only recently (Bloor and Abrahams, 2002).

3. Experimental

3.1. Plant material

The cell suspension culture of Madagascar periwinkle (*Catharanthus roseus* L.G. Don, line CP3a), its maintenance in MX growth medium in continuous dark with subcultures every week, and the induction with sucrose and/or irradiation have been described (Vetter et al., 1992; Kaltenbach et al., 1999; Schröder et al., 1999).

3.2. Homology based PCR

The preparation of cDNA and libraries in phage lambda NM1149 followed published methods (Schröder et al., 1999). The PCR reactions for OMT specific sequences were carried out with a degenerate primer (5′ T-[TG]-G-[AC]-I-[CT]-A-T-G-T-T-G-G-[AT]-G-G-I-G-A-T-A-T-G-T-T-T-G 3′) designed from the motif 3 conserved in OMTs (Ibrahim, 1997; Ibrahim et al., 1998). The PCR reactions were carried out either with phage lysates with phage primers flanking the cDNA inserts, or with a 5′/3′-RACE kit (Roche Diagnostics, Germany). Overlapping clones for the complete coding

regions were obtained by 5′ and 3′-RACEs with specific primers designed from the known sequences. The DNAs were sequenced on both strands.

3.3. 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 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).

3.4. Expression and purification of recombinant protein

For expression in *E. coli*, the protein coding regions were amplified with a 5′-primer providing a *Bam*HI site directly before the second codon, and a 3′-primer inserting a *Sal*I site after the stop codon. CrOMT5: 5′ AA GGATCCGAA GTT CAA TCA GTC GAG TTT TG 3′ and 5′ TA GTCGAC ATAATAA TTA AGG ATA AAC CTC 3′; CrOMT6: 5′ AA GGATCC GAT TTG CAA ACT GCC GAG TTT CG 3′ and 5′ TT GTCGACTCA AGG ATA AAC CTC AAT GAT ACT TC 3′; CrOMT7: 5′ AA GGATCCGAA GTT CAA TCA GCC GAG CTC CG 3′ and 5′ TT GTCGAC TCA AGG ATA AAC TTC AAT AAG ACT CC 3′. The *Bam*HI/*Sal*I fragments were inserted into vector pHis8-3 (Ferrer et al., 1999) which provided the protein with a His-tag at the *N*-terminal. The proteins were affinity purified with the His-trap™ purification kit from Pharmacia Biotech (Freiburg, Germany) as recommended by the manufacturer. The elution was in most cases with 0.5 M imidazole that was subsequently removed by passing the proteins through a PD10 column (Amersham Biosciences). The SDS gelelectrophoretic analysis of fractions from typical preparations of CrOMT6 and CrOMT7 is shown in Fig. 6.

3.5. OMT enzyme assays

The incubations with the various flavonoids, caffeic acid, or caffeoyl-CoA were performed as described (Schröder et al., 2002) with appropriate amounts of the recombinant protein or plant enzyme preparations. Standard assays contained 50 mM Tris–HCl (pH 7.5), 2 mM DTT, 0.1 mM substrate, 40 μM unlabelled SAM, 9.3 μM *S*-adenosyl-L-[methyl-^{14}C]methionine (55,000 dpm, 54 μCi/μmol), and 5–10 μg purified recombinant protein in a final volume of 50 μl. The reactions were stopped after 30 or 60 min by acidification (2 μl 1 M HCl), and the EtOAc-extracted products were quantified after TLC separation (Schröder et al., 2002; Cacace et al., 2003).

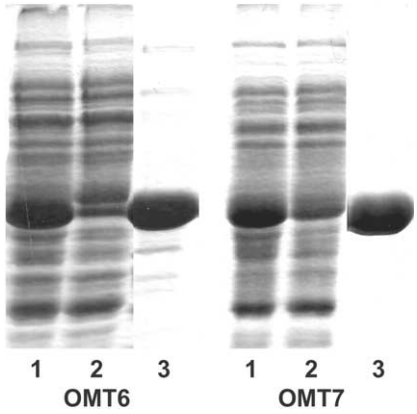


Fig. 6. SDS gelelectrophoretic analysis of recombinant proteins. Lanes: 1, crude extracts; 2, flow-through from Ni affinity columns; 3, after elution from the affinity columns with 0.5 M imidazole. The proteins were stained with Coomassie Brilliant Blue.

3.6. MS analysis of the CrOMT6 in vitro reaction product from homoeriodictyol

High pressure liquid chromatography- electrospray ionisation- tandem mass spectrometry (HPLC-ESI-MS/MS) was performed utilizing a TSQ 7000 tandem mass spectrometer system equipped with an electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany) and an Applied Biosystems (BAI, Bensheim, Germany) 140b pump. Data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany) using Finnigan MAT ICIS 8.1 software. HPLC separation with MS detection was carried out on a Knauer Eurospher-100 C18 column (length 100×2 mm i.d., particle size 5 μm) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. HPLC was programmed from 50% B to 100% B within 10 min at a flow rate of 200 μl/min. The injection volume was 5 μl and mass spectra were acquired in the positive ion mode. For pneumatic assisted ESI, the spray voltage was set to 4 kV, the temperature of the heated capillary was 210 °C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 units). Product ion scanning was performed at a collision gas pressure of 2.0 mtorr and a collision energy of 30 eV with a total scan duration of 1.0 s for a single spectrum.

3.7. Recombinant FHT, FNS, FLS, and ANS

The expression of recombinant FHT (Lukačin et al., 2000) and FLS (Wellmann et al., 2002) in *E. coli* and the purification by ammonium sulfate precipitation and size exclusion chromatography on fractogel EMD BioSEC (S) has been described (Lukačin et al., 2000). Recombinant

FNS and ANS were expressed in yeast (Martens et al., 2001, 2003). Growth of the transformants and protein extraction were carried out as described (Urban et al., 1997), and the proteins were purified as outlined above for FHT and FLS.

3.8. FHT, FNS, FLS, and ANS enzyme assays

The incubations (0.36 ml total volume) contained 0.1 ml buffer (0.2 M sodium acetats, pH 5.0), 0.277 mM flavonoid substrate, 83 μM 2-oxoglutarate, 42 μM ammonium iron(II) sulfate, 2.5 mM sodium ascorbate, 2 mg/ml bovine catalase, and 200 μl protein (FHT and FLS: 0.25 μg/μl, FNS: 0.3 μg/μl, ANS: 0.4 μg/μl). The incubations were for 30 min at 37 °C in open vials with gentle shaking, and they were terminated by the addition of 15 μl saturated aqueous EDTA solution. The flavonoids were isolated by repeated extraction with EtOAc (100 μl and 50 μl) and separated by reversed-phase HPLC (Shimadzu, Tokyo, Japan) on a Nucleodur C18-column (250×4 mm, 5 μm; Macherey-Nagel, Düren, Germany). The column equilibration was with solvent A (20% aqueous methanol), and the elution was in a linear linear gradient of solvent A and solvent B (100% methanol) at 1 ml/min for 3 min, followed by solvent B for 7 min. The substances were quantified by their absorption at the appropriate wavelengths (280, 308, 320, and 360 nm). More details have been described (Wellmann et al., 2002).

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