Biosynthesis of the major scent components 3,5-dimethoxytoluene and 1,3,5-trimethoxybenzene by novel rose *O*-methyltransferases

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Received 24 April 2002; revised 4 June 2002; accepted 4 June 2002

First published online 19 June 2002

Edited by Ulf-Ingo Flügge

Abstract In Chinese rose species and in many modern varieties, two methylated phenolic derivatives, 3,5-dimethoxytoluene and 1,3,5-trimethoxybenzene, are major scent components. We show that cell-free extracts of rose petals catalyse the synthesis of 3,5-dimethoxytoluene and 1,3,5-trimethoxybenzene by methylation of precursor molecules. An expressed sequence tag approach was used to identify four highly similar *O*-methyltransferase sequences expressed specifically in petals and anthers. Thin layer chromatography analysis showed that the activities of these enzymes with different substrates and the proportions of reaction products produced closely mimicked those observed using cell-free petal extracts, indicating that orcinol *O*-methyltransferases are responsible for the biosynthesis of 3,5-dimethoxytoluene and 1,3,5-trimethoxybenzene from un-methylated precursors in this organ.

Key words: Flower; *O*-Methyltransferase; Petal; Rose; Scent; *Rosa* spp.

1. Introduction

The rose is both the most economically important ornamental plant worldwide and the most diversified, with more than 25 000 cultivars [1]. This diversity has been derived, by intensive breeding activity, from a series of initial crosses that involved only seven or eight of the more than 120 species of the genus *Rosa*. The progenitors of modern roses included both European species (e.g. *Rosa gallica*, *Rosa phoenicia* and *Rosa moschata*), which contributed cold and disease resistance characters, and Chinese species (e.g. *Rosa chinensis* and *Rosa gigantea*), which contributed recurrent flowering. In addition to these traits, the European and Chinese progenitors each contributed distinctive scent characteristics. The major scent components of European roses include 2-phenylethanol and a number of monoterpenes, whereas Chinese roses principally produce lipid derived alcohols and esters (such as hexenol and hexenyl acetate) and aromatic compounds such as 3,5-dimethoxytoluene (DMT) and 1,3,5-trimethoxybenzene (TMB) [2]. The combination of molecules from these two lineages produces the characteristic 'tea' aroma found in many modern roses [3].

The aromatic compound DMT is used in the perfume industry, and its sedative properties [4] has led to its being used in aromatherapy as a 'relaxing fragrance'.

Little is known about the enzymes and genes responsible for the production of scent volatiles in flowers [5]. In particular, the biosynthetic pathways leading to DMT and TMB are unknown. Here we have investigated the potential role of *O*-methyltransferases (OMTs) in the production of these important scent components.

OMTs catalyse the transfer of methyl groups from the methyl donor *S*-adenosyl-L-methionine (AdoMet) to hydroxyl or carboxyl groups on a wide range of acceptor molecules. Several OMTs are involved in the biosynthesis of flower scent components. An (iso)eugenol *O*-methyltransferase (IEMT) is involved in the methylation of eugenol and isoeugenol in *Clarkia breweri* flowers, producing methyleugenol and isomethyleugenol, respectively [6]. Methyl benzoate, one of the major scent components of snapdragon flowers, is synthesised from benzoic acid by SAM-benzoic acid carboxyl methyltransferase [7]. OMTs have also been implicated in the production of fragrant molecules in non-floral organs of aromatic plants such as sweet basil. Recently, Gang et al. [8] reported the characterisation of two closely related OMTs from *Ocimum basilicum* which convert chavicol and eugenol to methylchavicol and methyleugenol, respectively.

We recently established a rose petal expressed sequence tag (EST) database with the aim of identifying genes involved in rose scent production [9]. Starting with the idea that OMTs may be involved in the synthesis of rose scent components, a search of the EST database led to the cloning of four closely related putative OMTs (OOMT1 to OOMT4; OOMT for orcinol OMT) from two different rose varieties. Enzymatic characterisation of OOMT1 and OOMT3 showed that they

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Abbreviations: AdoMet, *S*-adenosyl-L-methionine; CVOMT, chavicol OMT; DMT, 3,5-dimethoxytoluene; EOMT, eugenol OMT; EST, expressed sequence tag; GST, glutathione *S*-transferase; IOMT, isoflavone OMT; OMT, *O*-methyltransferase; OOMT, orcinol OMT; RT-PCR, reverse transcriptase-polymerase chain reaction; TLC, thin layer chromatography; TMB, 1,3,5-trimethoxybenzene

0014-5793/02/$22.00
PII: S0014-5793(02)02956-3

possess a novel substrate specificity and catalyse the synthesis of the major scent compounds of Chinese roses, DMT and TMB.

2. Materials and methods

2.1. Plant material

R. chinensis cv. Old Blush, *Rosa damascena* (Summer Damask) and *Rosa×hybrida* cv. Lady Hillingdon were from the Lyon Botanical Garden.

2.2. Chemicals and radiochemicals

3-Methoxy,5-hydroxytoluene was purified, using preparative thin layer chromatography (TLC), from a commercial preparation of DMT (Lancaster Synthesis, Bischheim, France) which contained 2% 3-methoxy,5-hydroxytoluene as an impurity. Purity was assessed by gas chromatography and mass spectrometry. Other phenolic substrates were from Fluka (St. Quentin Fallavier, France). *S*-Adenosyl-L-methyl [^{14}C]methionine (55 mCi/mmol) was from Amersham (Uppsala, Sweden). All other chemicals and reagents were from Sigma (St. Quentin Fallavier, France).

2.3. Gas chromatography and mass spectrometry

Gas chromatography and mass spectrometry were carried out as in Antonelli et al. [10].

2.4. Preparation of cell-free rose petal extracts

One gram of fresh rose petals was ground in a chilled mortar with 0.2 g of sand, 0.1 g insoluble polyvinylpyrrolidone and 1 ml of extraction buffer (100 mM Tris–HCl, pH 7.5, 10% glycerol, 2 mM dithiothreitol). After filtration through gauze and centrifugation at 15 000×*g* for 10 min, the supernatant was used for enzyme assays.

2.5. Cloning of OMT cDNAs

Full-length *OOMT1* and *OOMT2* cDNAs were identified using the rose petal EST database. *OOMT3* and *OOMT4* cDNA sequences were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from Lady Hillingdon petal cDNA using OMT2SBam (5′-GCGGGATCCATGGAAAGGCTAAACAGCTTTAGACACCTTA-3′) and OMT2ASBam (5′-CGCGGATCCTCAAGGATAAACCTCAATGAGAGACCTTAAA-3′). Note that the first and last 10 codons of the open reading frames corresponded to oligonucleotide sequence. The ends of the deduced proteins were, therefore, removed for sequence comparisons. An internal fragment of the *COMT1* coding sequence was amplified from Old Blush petal cDNA using two oligonucleotides based on the *Fragaria×ananassa OMT* gene sequence (accession number AF220491). Overlapping fragments corresponding to the 5′ and 3′ ends of the *COMT1* cDNA were then obtained by rapid amplification of cDNA ends-PCR using the Marathon protocol (Clontech, Palo Alto, CA, USA).

Multiple sequence alignments were constructed using the Megalign program (Lasergene, DNAstar, Madison, WI, USA). Neighbour joining trees were constructed from these alignments using ClustalW [11] and NJplot [12].

2.6. RNA gel blots

RNA was extracted using the method described by Cock et al. [13] and RNA gel blots were carried out as described [9].

2.7. Expression and purification of recombinant OMTs

For protein expression, OMT coding regions were cloned into either pGEX-4T1 for OOMT1 and OOMT3 or into pQE30 for COMT1 and transformed into *Escherichia coli* strain JM110. Recombinant proteins were affinity purified on either glutathione–agarose or Ni-NTA resin according to the manufacturer's instructions (Qiagen, Courtaboeuf, France; Pharmacia, Uppsala, Sweden).

2.8. Measurement of enzyme activity

The protocol for measuring OMT activity was adapted from Wang et al. [6]. Cell-free rose petal extracts or purified recombinant OMTs were incubated in a final volume of 50 μl with 20 μM AdoMet and varying concentrations (200 μM to 1 mM) of phenolic substrates in extraction buffer. Ranges of phenolic substrate concentrations of between 10 μM and 1 mM were used for K_m determination. K_m and V_{max} values were calculated from Lineweaver–Burk plots. Reaction products were analysed by TLC on silica gel (Merck, Darmstadt, Germany) with chloroform as the solvent. Enzyme reaction products were visualised by autoradiography using a Storm 860 phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA) and identified by comparison with co-migrating standards.

3. Results and discussion

3.1. Methylated phenolic derivatives in rose petals

As a starting point for the characterisation of TMB and DMT biosynthesis, we carried out an analysis of scent compounds in petals of three genetically diverse rose varieties which have been reported to differ markedly in their abilities to accumulate methylated phenolic derivatives [2]. The three varieties were *R. chinensis* cv. Old Blush, a rose of Chinese origin that was a progenitor of modern roses, *R. damascena* (Summer Damask), a rose that is widely used in the perfume industry and which is thought to be entirely of European origin [14] and *R.×hybrida* cv. Lady Hillingdon, a member of the Tea group of roses, descended from both oriental and

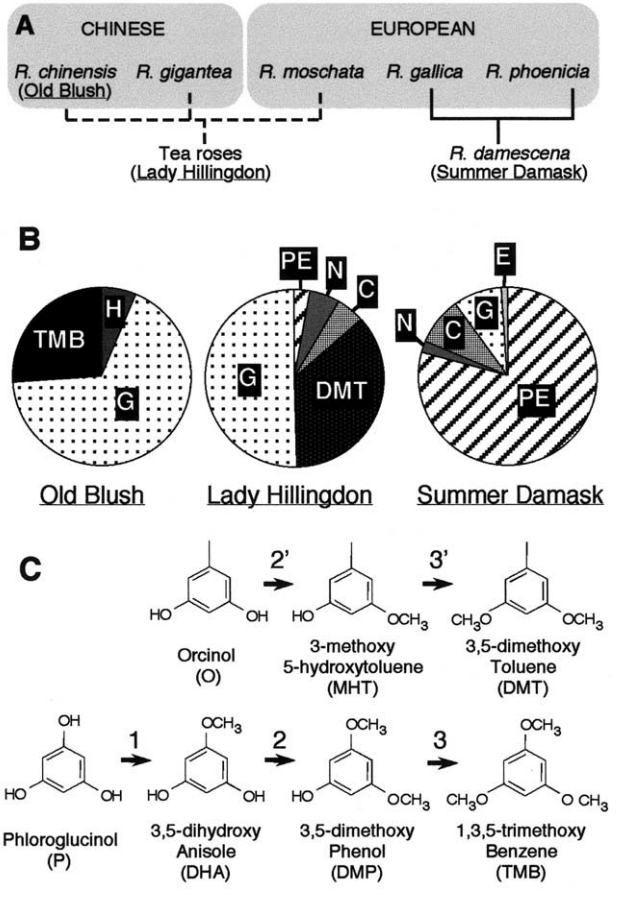


Fig. 1. DMT and TMB accumulation in rose petals. A: Simplified genealogical tree showing the relationships between the roses used in this study. The dotted lines leading to the Tea roses indicate that this group was derived from the progenitor species by a series of several crosses. B: Representation of the percentages of different scent compounds in petals of Old Blush, Lady Hillingdon and Summer Damask. C, citronellol; E, eugenol; G, geraniol; H, cis-3-hexenol; N, nerol; PE, phenylethanol. C: Proposed pathways for biosynthesis of DMT and TMB. The abbreviations shown in brackets correspond to those used in Fig. 3.

European progenitors (Fig. 1A). Gas chromatography was used to identify the major compounds present in pentane/diethyl ether extracts of petals. TMB was a major component of the Old Blush extract but was not detected in Lady Hillingdon which, in contrast, produces high levels of DMT (Fig. 1B). Neither TMB nor DMT was detected in Summer Damask petals, which contain high levels of phenylethanol. Petals of all three varieties contained monoterpenes. These observations are consistent with the proposition that TMB and DMT are characteristic of the Chinese group of roses and their descendants [2]. The analyses carried out here are consistent with published studies using headspace gas chromatography, except that headspace analysis indicates that methylated phenolic derivatives constitute an even greater proportion of the volatiles actually emitted from petals [2].

3.2. Cloning of rose OMTs

We used a functional genomics approach to identify enzymes potentially involved in the synthesis of DMT and TMB in rose petals. We have recently described an EST survey of the transcripts present in Old Blush petals [9]. Analysis of this EST database identified cDNAs corresponding to two putative OMT genes, designated *OOMT1* and *OOMT2,* which together accounted for 0.89% of the 1794 ESTs. The deduced polypeptide products of these cDNAs were closely related (96.0% amino acid identity) and were most similar to an OMT from *Prunus dulcis* (Fig. 2A,B).

Using a PCR approach and oligonucleotides based on the Old Blush OOMT sequences we identified two additional OOMT sequences expressed in petals of Lady Hillingdon, a rose that produces large quantities of DMT (Fig. 1B). The deduced amino acid sequences of these cDNAs, designated OOMT3 and OOMT4, were highly similar to OOMT1 and OOMT2 (the four sequences were between 96.0 and 97.4% identical in pairwise comparisons; Fig. 2A). These data indicate that there is a significant level of polymorphism between OOMT sequences in the genus *Rosa*. It is not yet clear whether the four rose OOMT sequences we identified represent distinct genes or alleles of the same gene. Note, however, that the 3′ untranslated regions of *OOMT1* and *OOMT2* were highly similar (95.7% identity).

The recent characterisation of the three-dimensional structures of alfalfa chalcone and isoflavone *O*-methyltransferases identified residues important for the function of these enzymes, including catalytic residues and residues involved in substrate binding [15]. Analysis of an alignment of the four rose OOMTs with *M. sativa* isoflavone *O*-methyltransferase and three other closely related OMTs indicated that the rose enzymes contain all the conserved sequences necessary for OMT activity and that the differences between these four proteins are mostly conservative (Fig. 2A).

Phylogenetic analysis indicates that OMTs fall into two distinct groups: the first group includes mainly COMTs while the second includes enzymes with a more diverse range of specificities [8]. OOMT1 and OOMT2 were more similar to sequences of the latter group, suggesting that their preferred substrate was not caffeic acid. Further support for this hypothesis was obtained by cloning a cDNA corresponding to the class of COMTs from the rose (Rc COMT1, Fig. 2). A phylogenetic tree, constructed using the amino acid sequences of COMT1, OOMT1 and OOMT2, confirmed that the former is closely related to COMTs, whereas the latter two sequences belong to a distinct group that includes flavone, isoflavone, chavicol and eugenol OMTs (Fig. 2B).

RT-PCR analysis showed that *COMT1* transcripts accumulated in all the organs analysed, indicating that it does not have a function specific to the flower. In contrast, transcripts of *OOMT1*/*OOMT2* were only detected in petals and anthers (Fig. 2C), organs that are known to be sites of scent production in the rose [16]. *OOMT1*/*OOMT2* transcripts were more abundant in old than in young petals. RNA gel blot analysis of *OOMT1*/*OOMT2* transcript abundance confirmed the results obtained by RT-PCR analysis (data not shown).

3.3. Synthesis of DMT and TMB by rose OMTs

To characterise the enzymatic activities of the rose OMTs, the coding sequences of Old Blush *OOMT1* and *COMT1* and Lady Hillingdon *OOMT3* were cloned into expression vectors and the corresponding proteins were expressed in *E. coli* (Fig. 2D). The activity of these enzymes was then tested in vitro in the presence of a number of potential precursors of TMB and DMT. Recombinant OOMT1 and OOMT3 exhibited similar relative activities for a panel of different substrates (Fig. 3A) despite the fact that these proteins are derived from rose varieties that produce predominantly either TMB (OOMT1 from Old Blush) or DMT (OOMT3 from Lady Hillingdon).

For comparison, we assayed OMT activities in cell-free extracts of Old Blush, Summer Damask and Lady Hillingdon petals. OMT activities potentially involved in the biosynthesis of DMT and TMB were detected in Old Blush and Lady Hillingdon petal extracts (Fig. 3A) but not in extracts of Summer Damask petals (data not shown), which do not contain detectable levels of either DMT or TMB (Fig. 1B). Hence, the presence of DMT or TMB in petals correlated with the presence of OMT activities capable of synthesising these molecules from precursors in vitro.

In order to determine whether the recombinant OMT enzymes were responsible for the activities measured in cell-free petal extracts, we compared profiles of relative activities with different substrates. Fig. 3A shows that the relative activity profiles of recombinant OOMT1 and OOMT3 resembled those of the petal extracts much more closely than did the profile obtained with COMT1 for most of the substrates tested. There were some differences. For example, petal extracts exhibited higher relative activities with phloroglucinol and 3-methoxy,5-hydroxytoluene than did the recombinant OOMTs. However, TLC analyses indicated that these differences were not due to the presence of additional OMT activities involved in the biosynthesis of DMT and TMB because neither these molecules nor reaction intermediates accumulated to high levels in these samples (Fig. 3B). It is likely that these differences were due to the incorporation of radioactivity into non-phenolic compounds by additional biosynthetic pathways (i.e. using substrates present in the petal extracts rather than the added substrate). We suggest that these compounds were highly volatile and, therefore, were not detected by TLC. Note, however, that this does not rule out the presence of additional OMT activities in the petal extracts that use other substrates such as eugenol and isoeugenol (Fig. 3A).

TLC analysis showed that the proportions of the different reaction products that accumulated when OOMT1 or OOMT3 were combined with different substrates were highly similar to those obtained in the presence of cell-free petal

extracts (Fig. 3B). Taken together, the data presented in Fig. 3 provide strong support for the hypothesis that the closely related group of enzymes represented by OOMT1/OOMT3 account for the majority of OMT activity in rose petals and catalyse DMT and/or TMB synthesis in this organ via the biosynthetic pathway proposed in Fig. 1C. Note also that small amounts of compounds that most probably corresponded to 3-methoxy,5-hydroxytoluene and DMT accumulated after incubation of Lady Hillingdon petal extract with [^{14}C]AdoMet in the absence of an added phenolic substrate (Fig. 3B). This suggests that orcinol was present in the petal extract and supports the hypothesis that the pathway shown in Fig. 1C operates in vivo. Further work will be required to confirm the presence of orcinol in petals and to identify the biosynthetic pathway that produces it. Very little is known about the biosynthesis of compounds such as orcinol, which

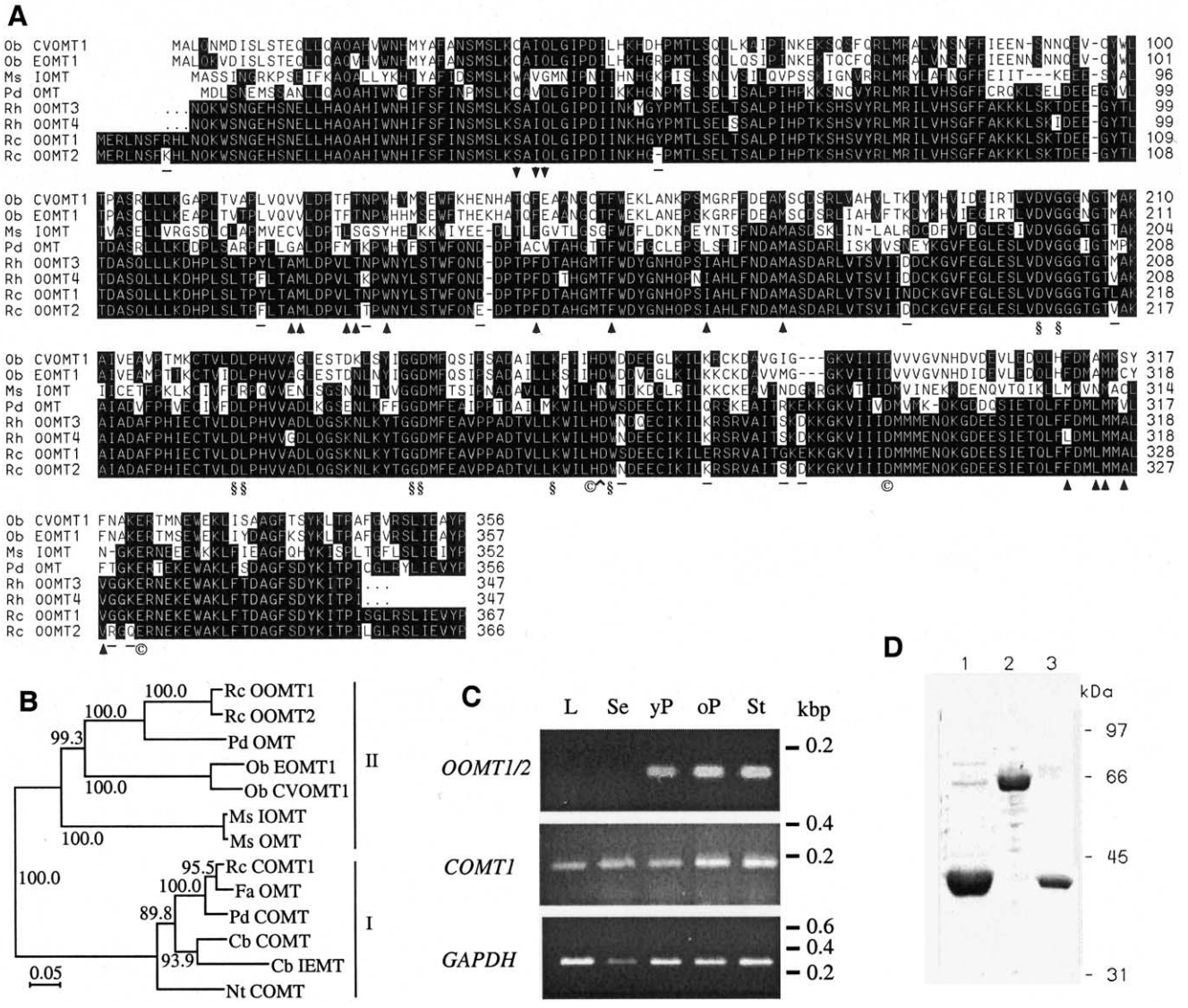
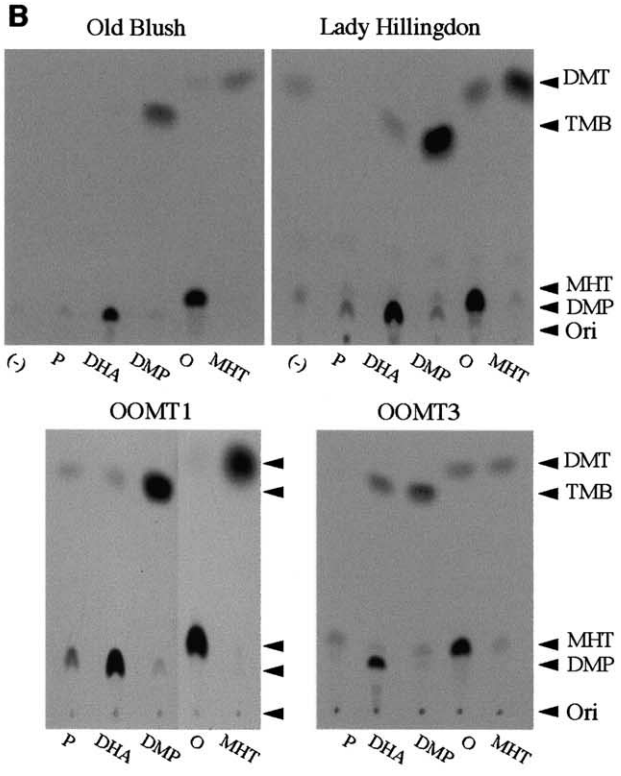


Fig. 2. Sequence and expression analysis of rose OMTs. A: Alignment of *R. chinensis* cv. Old Blush OOMT1 (accession number AJ439741) and OOMT2 (AJ439742) with *R. hybrida* cv. Lady Hillingdon OOMT3 (AJ439743) and OOMT4 (AJ439744) and three other closely related OMTs. Residues identical with the corresponding residue of OOMT1 are highlighted. The 14 differences between OOMT1 and OOMT2 are underlined. Symbols indicating catalytic residues (©), SAM binding residues (§), a binding pocket residue in chavicol OMT (CVOMT; ^) and substrate binding residues in isoflavone OMT (IOMT; ▲) and in the dyad polypeptide active site (▼) use the same notation as Gang et al. [8]. B: Unrooted neighbour joining tree based on an alignment of OMT sequences. The tree was constructed using the PAM250 amino acid substitution matrix, numbers next to the branches are bootstrap values expressed as percentage confidence level and based on 1000 repeats. The polypeptide sequences compared were *R. chinensis* OOMT1 (Rc OOMT1), *R. chinensis* OOMT2 (Rc OOMT2), *P. dulcis* OMT (Pd OMT; CAA11131), *O. basilicum* CVOMT1 (Ob CVOMT1; AF435007), *O. basilicum* eugenol OMT1 (Ob EOMT1; AF435008), *Medicago sativa* OMT (Ms OMT; T09299), *M. sativa* IOMT (Ms IOMT; T09254), *F.* x *ananassa* OMT (Fa OMT; AAF28353). *R. chinensis* COMT1 (Rc COMT1; AJ439740), *P. dulcis* COMT (Pd COMT; CAA58218), *C. breweri* IEMT (Cb IEMT AAC01533), *C. breweri* COMT (Cb COMT; AAB71141) and *Nicotiana tabacum* COMT (Nt COMT; S36403). C: RT-PCR analysis of the expression patterns of rose *OOMT1*/*OOMT2* (*OOMT1/2*) and *COMT1*. The expression pattern of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analysed as a control. The positions of DNA size markers are shown to the right in kbp. L: leaf, Se: sepal, yP: young petals from unopened buds, oP: mature petals at all stages after full opening of the flower, St: stamens. D: Purification of rose COMT1 and OOMT1 expressed in *E. coli*. The lanes show purified COMT1 (1), purified glutathione S-transferase (GST)::OOMT1 fusion protein (2) and purified OOMT1 after thrombin cleavage (3). The positions of protein size markers are shown to the right in kDa.

A

substrate	relative activity				
	OB	LH	OOMT1	OOMT3	COMT
phloroglucinol	75	49	4	3	1
3,5-dihydroxyanisol	100	84	77	63	4
3,5-dimethoxyphenol	27	61	17	12	1
orcinol	100	100	100	100	15
3-methoxy,5-hydroxytoluene	100	51	15	14	2
eugenol	23	1	3	2	1
isoeugenol	28	14	4	5	1
caffeic acid	6	2	5	3	100

B



C

substrate	OOMT1 / OOMT3		
	Km (μM)	Kcat ($.10^{-3}$ s^{-1})	Kcat/Km ($M^{-1}.s^{-1}$)
phloroglucinol	254 / 270	3 / 0.5	12 / 2
3,5-dihydroxyanisol	107 / 135	33 / 29	308 / 215
3,5-dimethoxyphenol	371 / 379	8 / 7	22 / 18
orcinol	75 / 86	45 / 43	600 / 500
3-methoxy,5-hydroxytoluene	280 / 320	8 / 7	29 / 22

Fig. 3. Rose petal OMTs catalyse the synthesis of DMT and TMB. A: Comparison of the relative activities of Old Blush (OB) and Lady Hillingdon (LH) cell-free petal extracts and purified Old Blush OOMT1, Lady Hillingdon OOMT3 and Old Blush COMT1 enzymes after addition of different substrate molecules. Relative activity was calculated as a percentage of the activity with either orcinol (for petal extracts, OOMT1 and OOMT3) or with caffeic acid (for caffeic acid OMT) as substrate. B: TLC analysis of reaction products produced following incubation of petal extracts or purified OOMT with various phenolic substrates. Old Blush (upper left panel) or Lady Hillingdon (upper right panel) petal extracts or purified OOMT1 (lower left panel) or OOMT3 (lower right panel) were incubated with the indicated phenolic substrates in the presence of [^{14}C]AdoMet. For the petal extracts, control incubations were carried out without addition of a phenolic substrate (−). Arrows indicate the positions of the origin (Ori) and the reaction products 3,5-dimethoxyphenol (DMP), 3-methoxy,5-hydroxytoluene (MHT), TMB and DMT. C: Kinetic parameters for OOMT1 (left) and OOMT3 (right) with different phenolic substrates.
←

are hydroxylated in positions 3 and 5, in vivo and it is not yet clear whether orcinol is synthesised by the monolignol biosynthetic pathway or by an alternative pathway [17].

Different phenolic derivatives accumulate in petals of Old Blush and Lady Hillingdon (TMB and DMT, respectively; Fig. 1B). The presence of enzymes with very similar activities in petals of Old Blush and Lady Hillingdon (OOMT1 and OOMT3, respectively) indicates that it is not the substrate specificity of the OOMT that directs the metabolism towards synthesis of DMT or TMB. Differences in the abundance of DMT compared with TMB are more likely to be due to the differences in the accumulation of precursor molecules.

Recombinant OOMT1 and OOMT3 enzymes had very similar K_m values for several different substrates (Fig. 3C). The K_m values for orcinol, which were around 80 μM, were comparable to those of other OMTs for their corresponding substrates. For example, IEMT, which is involved in scent production in *C. breweri*, has a K_m of 74 μM for isoeugenol [18]. Turnover rates (K_{cat}) for OOMT1 and OOMT3 with orcinol were also comparable to the IEMT K_{cat} for isoeugenol [17]. In contrast, the related enzymes CVOMT and eugenol OMT (EOMT) from *O. basilicum* exhibit lower K_m values for their substrates chavicol (6 μM) and eugenol (3 μM), respectively, but the K_{cat} values for these enzymes were also lower than those measured for OOMT1 and OOMT3 [8].

Orcinol was the best substrate for OOMT1 and OOMT3; all other substrates tested gave higher K_m and lower K_{cat} values. Comparison of K_{cat}/K_m values (which reflect catalytic efficiency with the different substrates) showed that the various putative DMT or TMB precursors were not equivalent substrates and that successive methylation reactions proceeded with different kinetics (Fig. 3C). For example, methylation of phloroglucinol (Fig. 1C, reaction step 1) was less efficient in vitro than later steps in the pathway in the presence of either petal extracts or recombinant enzymes, suggesting that another substrate might be used in vivo (Fig. 3C). In contrast, methylation of orcinol and 3,5-dihydroxyanisole (Fig. 1C, reaction steps 2 and 2′) was very efficient in vitro and high K_{cat}/K_m values were measured for these reactions (Fig. 3C). Orcinol and 3,5-dihydroxyanisole are, therefore, highly likely to be precursors of DMT or TMB in vivo.

Interestingly, Fig. 3 shows that orcinol and 3,5-dihydroxyanisole were better substrates for recombinant OOMT1/OOMT3 and rose petal extracts (Fig. 1C, reaction steps 2 and 2′) than were the putative reaction intermediates 3-methoxy,5-hydroxytoluene and 3,5-dimethoxyphenol (Fig. 1C, reaction steps 3 and 3′). This contrasts with the substrate preference of COMT, which also methylates hydroxyl groups at positions 3 and 5, but which is more active with the reaction intermediate 5-hydroxyferulic acid than with its precursor, caffeic acid [19]. This may reflect structural differences in the active sites of these two enzymes.

The observation that the OOMTs metabolise a biochemical intermediate less rapidly than their initial substrate is unusual. This may indicate that substrate channelling is occurring in vivo to prevent accumulation of the reaction intermediate, as

has been proposed for enzymes of the phenylpropanoid pathway [15,20,21]. Future work will be aimed at determining whether a similar channelling process occurs during the synthesis of methylated phenolic volatiles in rose petals.

4. Note added in proof

Similar OMTs were recently characterized in modern rose varieties by Lavid et al. [22].

Acknowledgements: We would like to thank Michel Legrand, Pierrette Geoffroy, Danièle Werck-Reichhart (IBMP, Strasbourg, France) and Jacques Gentili (ENS, Lyon, France) for helpful discussions, Alexis Lacroix and Roseraies Orard for help with plant material and Marion Moly, Hervé Leyral, Claudia Bardoux, Christophe Ferry, Gérard Boulon and Henri Fourcade for technical assistance. This work was supported by an ACI Jeunes Chercheurs contract (Ministère de la Recherche, France) and by the Région Rhône-Alpes, France, the Institut National de la Recherche Agronomique and the Centre National de la Recherche Scientifique.

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