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O-Methylation of benzaldehyde derivatives by "lignin specific" caffeic acid 3-*O*-methyltransferase☆

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Abstract

Although *S*-adenosyl-L-methionine (SAM) dependent caffeic acid/5-hydroxyferulic acid 3/5-*O*-methyltransferase (COMT) is one of the key enzymes in lignin biosynthesis, the present work demonstrates that alfalfa COMT methylates benzaldehyde derivatives more efficiently than lignin pathway intermediates. 3,4-Dihydroxy, 5-methoxybenzaldehyde and protocatechuic aldehyde were the best in vitro substrates for OMT activity in extracts from developing alfalfa stems, and these compounds were preferred over lignin pathway intermediates for 3-*O*-methylation by recombinant alfalfa COMT expressed in *Escherichia coli*. OMT activity with benzaldehydes was strongly reduced in extracts from stems of transgenic alfalfa down-regulated in COMT. However, although COMT down-regulation drastically affects lignin composition, it does not appear to significantly impact metabolism of benzaldehyde derivatives in alfalfa. Structurally designed site-directed mutants of COMT showed altered relative substrate preferences for lignin precursors and benzaldehyde derivatives. Taken together, these results indicate that COMT may have more than one role in phenylpropanoid metabolism (but probably not in alfalfa), and that engineered COMT enzymes could be useful for metabolic engineering of both lignin and benzaldehyde-derived flavors and fragrances.

Keywords: *Medicago sativa*; Leguminosae; Lignification; *O*-Methyltransferase; Site-directed mutants; Benzaldehydes

1. Introduction

In spite of the collective complexity of plant natural products, their biosynthesis is in large part brought about by the activities of a limited number of enzyme classes. Much of the diversity in many natural product groups arises from differential substitution of basic biosynthetic skeletons. Within phenylpropanoid biosynthesis, *O*-methylation and *O*-glycosylation are two of the most common substitution reactions, and many *O*-methyltransferases (OMTs) active against hydroxycinnamic acids, flavonoids and isoflavonoids have been reported (Gauthier et al., 1998; Frick and Kutchan, 1999; Maury et al., 1999; Chiron et al., 2000; Wein et al., 2002). It is becoming increasingly clear that plant small molecule OMTs can either show exquisite specificity (Zubieta et al., 2001), or have relatively promiscuous substrate preferences (Dixon, 2001; Parvathi et al., 2001; Wein et al., 2002; Zubieta et al., 2002). The latter observation has important implications for genomic annotation and the evolution of plant specialized (secondary) metabolism.

Caffeic acid 3-*O*-methyltransferase (COMT, EC. 2.1.1.68) was the first plant small molecule OMT to be described (Neish, 1968), its significance associated with its presumed role in the biosynthesis of lignin, the second most abundant polymer on earth. Lignin consists of hydroxylated and methoxylated phenylpropanoid units called monolignols; in dicots, these are primarily monomethylated guaiacyl (G) units derived from coniferyl alcohol, and dimethylated syringyl (S) units derived from sinapyl alcohol. The S and G units in lignin are joined through different types of ether and carbon–carbon

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linkages (Davin and Lewis, 1992). Until recently, most models of lignin biosynthesis presented the pathway as a metabolic grid, through which the side-chain reduction and successive ring hydroxylation/*O*-methylation reactions occur at several different levels (Whetten and Sederoff, 1995; Dixon et al., 2001) (Fig. 1). COMT was initially thought to be a bifunctional enzyme that used caffeic (**1**) and 5-hydroxyferulic (**2**) acids as substrates during monolignol biosynthesis. However, recent studies have shown that recombinant COMTs exhibit a higher preference for 5-hydroxyconiferaldehyde (**3**) than for caffeic acid (**1**) (Li et al., 2000; Parvathi et al., 2001). The preferred substrate for the enzyme originally known as ferulate 5-hydroxylase (F5H) is coniferaldehyde (**4**) rather than ferulic acid (**5**) (Humphreys et al., 1999; Osakabe et al., 1999), consistent with a pathway for monolignol formation in which COMT functions primarily to methylate 5-hydroxyconiferaldehyde (**3**) in the biosynthesis of S lignin (Li et al., 2000; Parvathi et al., 2001). Consistent with this model, COMT down-regulated transgenic plants show a stronger reduction in S lignin than in G lignin (Atanassova et al., 1995; Van Doorsselaere et al., 1995; Guo et al., 2000; Piquemal et al., 2002).

The substrate preferences of COMT enzymes might be even broader than initially realized. Within the context of monolignol biosynthesis, recombinant alfalfa COMT exhibits high catalytic efficiency not only with 5-hydroxyconiferaldehyde (**3**) but also with caffeyl aldehyde (**6**), caffeyl alcohol (**7**), and 5-hydroxyconiferyl alcohol (**8**) (Parvathi et al., 2001), suggesting the involvement of COMT in both 3- and 5-methylation reactions of S lignin biosynthesis at either the aldehyde or alcohol levels. These results are consistent with in vivo labeling studies

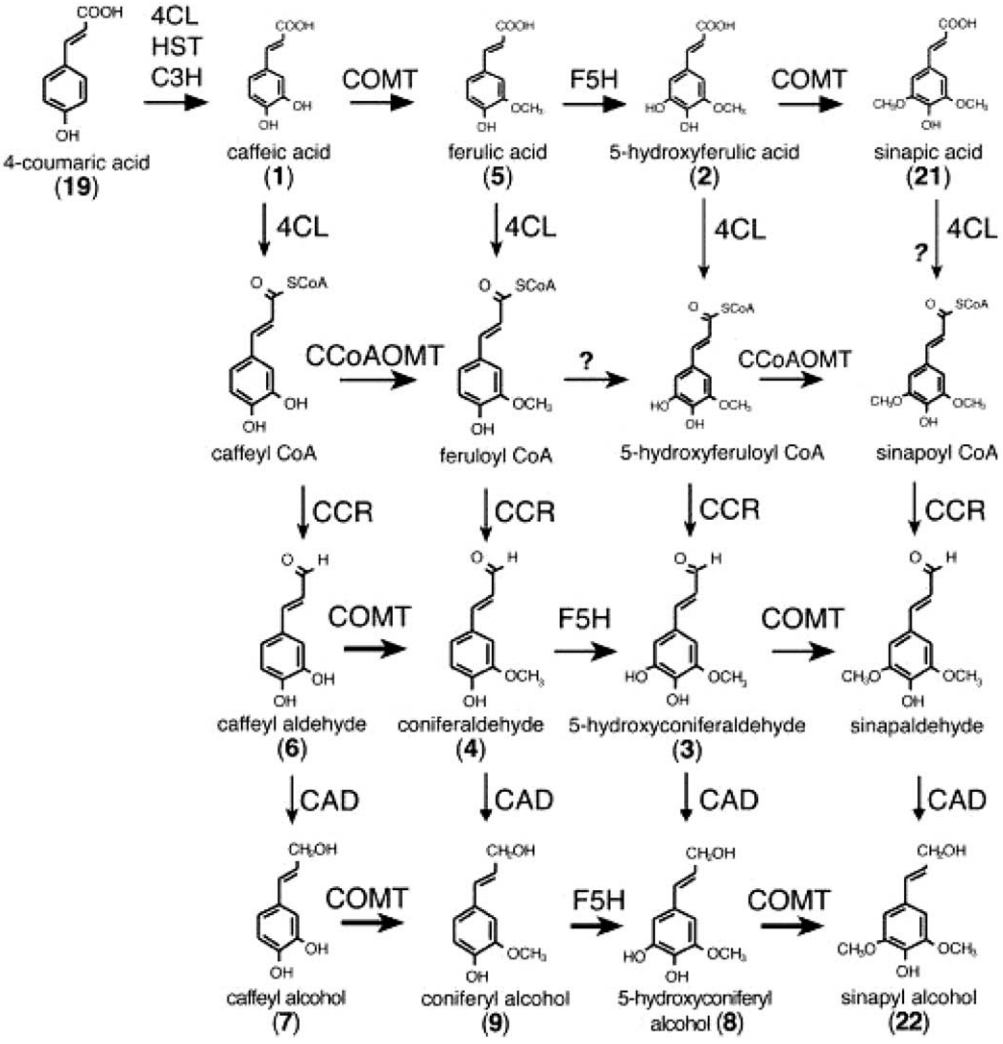


Fig. 1. Hypothetical metabolic grid for the biosynthesis of the monolignols coniferyl alcohol (**19**) and sinapyl alcohol (**22**) from 4-coumaric acid (**19**). This scheme has undergone significant revision in recent years (Humphries and Chapple, 2002). The enzymes are: C3H, "coumarate hydroxylase", now known to function at the level of the corresponding shikimate ester (Schoch et al., 2001); 4CL, 4-coumarate: CoA ligase; HST, hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase; COMT, caffeic acid 3-*O*-methyltransferase (more accurately termed 5-hydroxyconiferaldehyde 3-*O*-methyltransferase); F5H, ferulate 5-hydroxylase (more accurately termed coniferaldehyde 5-hydroxylase); CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; CCR, cinnamyl CoA reductase; CAD, coniferyl alcohol dehydrogenase.

in *Magnolia kobus* in which S lignin can be derived from coniferyl alcohol (**9**) (Matsui et al., 1994; Chen et al., 1999).

The recently determined three-dimensional crystal structure of alfalfa COMT reveals an unusually spacious catalytic site (Zubieta et al., 2002) and provides an explanation for the broad specificity of the enzyme for a range of hydroxycinnamic acid, aldehyde and alcohol derivatives. Furthermore, enzymes with COMT activity from some species may accept substrates other than monolignol precursors. For example, tobacco COMT I and COMT II have also been reported to be active against protocatechuic aldehyde (**10**) (Maury et al., 1999), OMT from *Chrysosplenium americanum* catalyzes methylation of both caffeic acid (**1**) and flavonoids (Gauthier et al., 1998), OMT from *Thalictrum tuberosum* shows activity toward both alkaloids and phenylpropanoid substrates (Frick and Kutchan, 1999), and an OMT from strawberry fruit exhibits broad substrate specificity against monolignol precursors and furanone aroma compounds (Wein et al., 2002).

We here report that benzaldehyde derivatives are the most efficient in vitro substrates for alfalfa COMT determined to date. We describe the pattern of OMT activity with benzaldehydes in extracts from alfalfa stem internodes harvested at various stages of development, and the kinetic properties of recombinant alfalfa COMT for benzaldehydes. Structurally-targeted site directed mutagenesis of alfalfa COMT can alter the relative substrate preference of the enzyme for monolignol precursors and benzaldehyde derivatives. We evaluate the significance of the broad in vitro substrate preference of COMT for natural product biosynthesis in vivo.

2. Results and discussion

2.1. Developmental changes in O-methyltransferase activities against benzaldehyde derivatives

OMTs from alfalfa stem extracts have broad substrate preference against monolignol precursors (Parvathi et al., 2001). Lignin levels and OMT activities against all potential monolignol precursors increase with the development of the stem in alfalfa, preceding the increase in lignin methoxyl content (S/G ratio) with advanced maturity (Inoue et al., 1998, 2000). Following a serendipitous observation that crude extracts from alfalfa stems are also able to methylate protocatechuic aldehyde (**10**), as has also been reported for tobacco COMT (Maury et al., 1999), crude protein extracts from individual internodes (first to tenth) were assayed for their ability to catalyze methylation of a range of benzaldehyde derivatives.

Typical developmental profiles of OMT activities are shown in Fig. 2. The highest activities for all substrates were recorded in the sixth to eighth internodes. Surprisingly, the highest activities were obtained with protocatechuic aldehyde (**10**) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**). The extracts catalyzed methylation of these compounds to a greater extent than the preferred monolignol precursor 5-hydroxyconiferaldehyde (**3**) (Fig. 2).

2.2. O-Methyltransferase activities in stem extracts of transgenic alfalfa down-regulated in COMT

COMT activity and protein level are strongly down-regulated in alfalfa by expression of homologous sense or antisense COMT sequences driven by the vascular tissue-specific bean *PAL2* promoter (Guo et al., 2000; Parvathi et al., 2001). Control alfalfa cv Regen SY plants transformed with an empty vector that exhibit wild-type COMT expression have also been produced (Guo et al., 2000). We took advantage of the transgenic alfalfa lines to demonstrate that the above activities with benzaldehyde derivatives in crude extracts were indeed due to the activity of COMT rather than a second OMT specific for benzaldehyde derivatives. It is very unlikely that the antisense strategy used targets other OMT enzymes based on the known sequences of COMT-like enzymes in the model legume *Medicago truncatula* (information available at the TIGR website, http://www.tigr.org/tdb/tgi.shtml), genes from which share very high sequence identity to their orthologs in alfalfa.

OMT activities against benzaldehyde derivatives and monolignol precursors in stem extracts from the sixth to eighth internodes of COMT down-regulated and empty vector control lines are shown in Table 1. The stem samples were collected from plants at the same developmental stage grown together under the same environmental conditions. Down-regulation of COMT had a

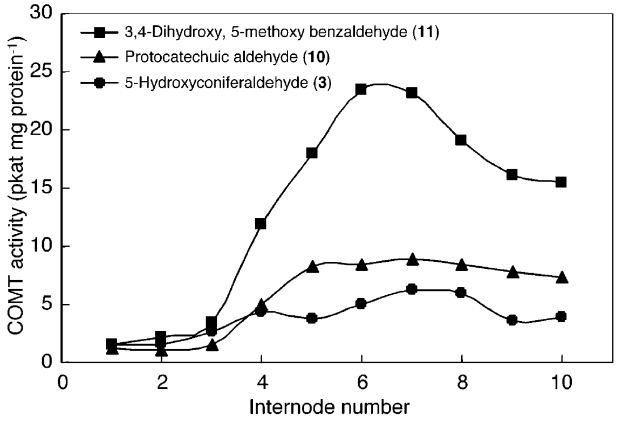


Fig. 2. Developmental changes in *O*-methyltransferase activities in alfalfa stem internodes (cultivar Apollo). Enzyme activities with the indicated substrates (50 μM) and ^{14}C-SAM were determined in crude stem protein extracts from internodes 1–10. Duplicate assays were performed (maximum analytical variation less than ±5%).

Table 1
OMT activities (pkat mg^{-1} protein) in stem material from wild-type (empty vector control) and COMT down-regulated (line 310) transgenic alfalfa

Substrate	Wild type	COMT down-regulated	Reduction in COMT activity (-fold)
Protocatechuic aldehyde (**10**)	7.1±0.04	0.5±0.01	14.2
3,4-Dihydroxy, 5-methoxybenzaldehyde (**11**)	14.6±0.9	1.0±0.06	14.6
Protocatechuic acid (**12**)	0.18±0.01	0.1±0.04	1.8
Caffeic acid (**1**)	3.8±0.06	0.7±0.14	5.7
5-Hydroxy coniferaldehyde (**3**)	4.5±0.30	1.7±0.003	2.6

Values are from duplicate assays with pooled stem material from multiple plants, expressed as average±spread of values.

larger impact on the methylation of protocatechuic aldehyde (**10**) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) than on the monolignol precursors. However, the low level of activity against protocatechuic acid (**12**) was much less affected. These results indicate that the major activity catalyzing methylation of benzaldehydes in alfalfa stems is the enzyme known as COMT.

2.3. Kinetic properties of recombinant alfalfa COMT with benzaldehyde derivatives

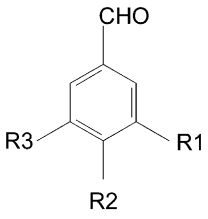
The alfalfa COMT cDNA was cloned in pET15b and expressed in *E. coli* (Gowri et al., 1991; Inoue et al., 1998) as an N-terminally hexahistidine tagged protein. COMT expression was induced by IPTG and the enzyme purified to homogeneity from the soluble protein fractions by nickel column affinity chromatography (Parvathi et al., 2001; Zubieta et al., 2002). This recombinant "wild-type" enzyme was used to study the catalytic behavior of COMT towards benzaldehyde derivatives. Kinetic analyses were performed by measuring the initial velocity against a range of substrate concentrations at a fixed concentration (60 μM) of ^{14}C-labeled SAM. The substrate preferences and kinetic constants are presented in Tables 2 and 3. The smallest K_m values obtained were for protocatechuic aldehyde (**10**) and 5-hydroxy-coniferaldehyde (**3**), the latter of which is the favored substrate (expressed as Km) for COMT in the monolignol biosynthetic pathway. The highest V_{max}/K_m values observed were for protocatechuic aldehyde (**10**) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**), whereas protocatechuic acid (**12**) was a much poorer substrate in terms of catalytic efficiency expressed as V_{max}/K_m (Table 3). The difference in catalytic efficiency between aldehydes and their corresponding acids was much greater for the benzaldehyde derivatives than for the phenylpropanoid derived monolignol precursors.

To confirm the nature of the products formed by the COMT-mediated methylation of protocatechuic aldehyde (**10**) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) with ^{14}C-labeled SAM, HPLC/diode-array analysis with parallel radiodetection was performed. HPLC/UV absorption traces of the reaction products from reaction mixtures of recombinant COMT with protocatechuic aldehyde (**10**) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) are presented in Fig. 3. The terminated reaction was spiked with the potential methylated products, vanillin (**13**) and syringaldehyde (**14**) respectively. In Fig. 3A, 3,4-dimethoxybenzaldehyde (**15**) was also added to the reactions after termination, to check whether COMT could methylate both hydroxyls on protocatechuic

Table 2
Activities of recombinant alfalfa COMT against benzaldehyde derivatives and protocatechuic acid (100 μM)

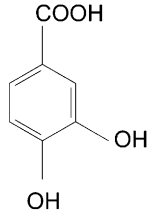
Substrate (100 μM)	COMT activity pkat mg^{-1}
3-Hydroxy benzaldehyde (**16**)	0
4-Hydroxy benzaldehyde (**17**)	0
Protocatechuic aldehyde (**10**)	949
Isovanillin (**18**)	10
Vanillin (**13**)	0
3,4-Dihydroxy, 5-methoxybenzaldehyde (**11**)	1277
Syringaldehyde (**14**)	0
Protocatechuic acid (**12**)	20

A



10, 11, 13-18

B



12

A.

16	3-Hydroxybenzaldehyde:	R1 = OH, R2 = H, R3 = H
17	4-Hydroxybenzaldehyde:	R1 = H, R2 = OH, R3 = H
10	Protocatechuic aldehyde:	R1 = OH, R2 = OH, R3 = H
18	Isovanillin:	R1 = OH, R2 = OCH3, R3 = H
13	Vanillin:	R1 = OCH3, R2 = OH, R3 = H
11	3,4-Dihydroxy 5-methoxy benzaldehyde:	R1 = OH, R2 = OH, R3 = OCH3
14	Syringaldehyde:	R1 = OCH3, R2 = OH, R3 = OCH3
15	3,4-Dimethoxybenzaldehyde:	R1 = OCH3, R2 = OCH3, R3 = H

B.

12 Protocatechuic acid

Table 3
Kinetic properties of purified recombinant alfalfa COMT (WT) and a series of site-directed mutants

COMT mutants	V_{max}/K_m				
	Caffeic acid (**1**)	5-OH Coniferaldehyde (**3**)	Protocatechuic aldehyde (**10**)	3,4-Dihydroxy, 5-methoxy benzaldehyde (**11**)	Protocatechuic acid (**12**)
WT	833/43 (19)	500/5 (100)	476/5 (102)	2000/16 (122)	128/515 (0.3)
L136Y	303/10 (29)	278/13 (21)	909/15 (60)	1250/31 (31)	196/52 (52)
A162T	476/12 (39)	455/11 (44)	556/10 (57)	1667/39 (43)	ND
M130L	ND	909/28 (32)	43/19 (2)	250/12 (21)	ND
F172Y	ND	333/10 (33)	526/153 (3)	1429/27 (52)	ND
N131L	250/335 (0.8)	500/13 (40)	179/10 (18)	1000/13 (79)	ND
N131K	556/9 (60)	417/12 (34)	3333/55 (61)	2500/57 (44)	250/352 (1)
N131D	278/121 (2)	5000/120 (42)	ND	ND	ND
N131E	9/214 (0.04)	68/1 (60)	ND	ND	ND
N324Y	83/133 (0.6)	714/17 (42)	57/6 (10)	370/5 (71)	ND
N324HM130L	250/32 (8)	1000/41 (24)	222/6 (37)	3333/87 (39)	667/357 (2)
H183K	167/140 (1)	455/8 (56)	ND	ND	ND

The mutations are given as single-letter amino acid codes. For each mutant, values are given for V_{max} and K_m and numbers in parentheses show the V_{max}/K_m ratio. V_{max} values are given as pkat/mg COMT, and K_m values are expressed in μM. ND, no activity determined. Assays were performed in duplicate or triplicate (maximum analytical variation less than ±5%).

aldehyde (**10**). The elution profiles of incorporation of radioactivity in the products from ^{14}C-labeled SAM are superimposed on the UV traces. The collective results indicate that the only methylation product of protocatechuic aldehyde (**10**) was, vanillin (**13**), and that syringaldehyde (**14**) was the product formed from 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**). Because of the potential volatility of benzaldehydes, total radioactivity was determined before and after enzymatic incubation. No loss was observed.

Table 2 shows the extent of methylation of different benzaldehyde derivatives by recombinant alfalfa COMT. 3-Hydroxybenzaldehyde (**16**) and 4-hydroxybenzldehyde (**17**) were not methylated. Similarly, vanillin (**13**) and syringaldehyde (**14**) could not be methylated by COMT. In contrast, protocatechuic acid (**12**) and isovanillin (**18**) were methylated, although to a lesser degree than protocatechuic aldehyde (**10**) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**). Together with the results presented in Fig. 3, it is clear that COMT can effectively catalyze the SAM-dependent methylation of 3,4-dihydroxy-substituted benzaldehydes at the 3-OH (*meta*) position, but not at the 4-OH (*para*) position.

2.4. Kinetic discrimination of COMT by site-directed mutagenesis

The crystal structure of COMT explains the broad substrate specificity of the enzyme (Zubieta et al., 2002). The active site of COMT is more spacious than that of other small molecule OMTs (Zubieta et al., 2001, 2002) and accommodates both 3- and 5-substituted C6–C3 substrates (phenylpropanoids), with either acid or aldehyde groups at the end of the 3-carbon propanoid tail. Whether C6–C3 or C6–C1, the aldehydes would in general undergo more facile deprotonation of the targeted hydroxyl moiety in preparation for *O*-methylation than the corresponding acids. Indeed, the preference would be for the *meta*-position deprotonation/methylation (3- and 5-hydroxyl) rather than *para* directed deprotonation/methylation (4-hydroxyl). Owing to the versatile structure of the binding site, aromatic compounds smaller than phenylpropanoids will fit albeit with a possibly loose arrangement in the active site cavity near the putative histidine general base (His 269) and the reactive methyl group of a firmly bound SAM molecule. However, their binding might lack the constraints contributed by residues surrounding the propanoid tail. On the other hand, aldehydes lack a negative charge that would clash sterically and electronically with residues including I316, M130, I319, and M180 that surround the propanoid tail (Zubieta et al., 2002). In total, the difference in the K_m values between aldehydes and acids can be explained by effects due to the pK_a shift of the –OHs and/or loss of repulsive interactions between the acid tail and M180, I316, M130, and I319.

Based on the crystal structure of alfalfa COMT, a series of mutations was designed to alter residues that contact the aromatic ring and side chain of natural COMT substrates. They were then constructed by site-directed mutagenesis to facilitate the functional determination of the catalytic and substrate recognition roles of the residues lining the active site surface. These studies ultimately will help resolve the relative importance of key active site residues in the kinetic discrimination between monolignol precursors and various benzaldehydes. The mutant enzymes were expressed in *E. coli* and purified to homogeneity. Table 3 shows the kinetic

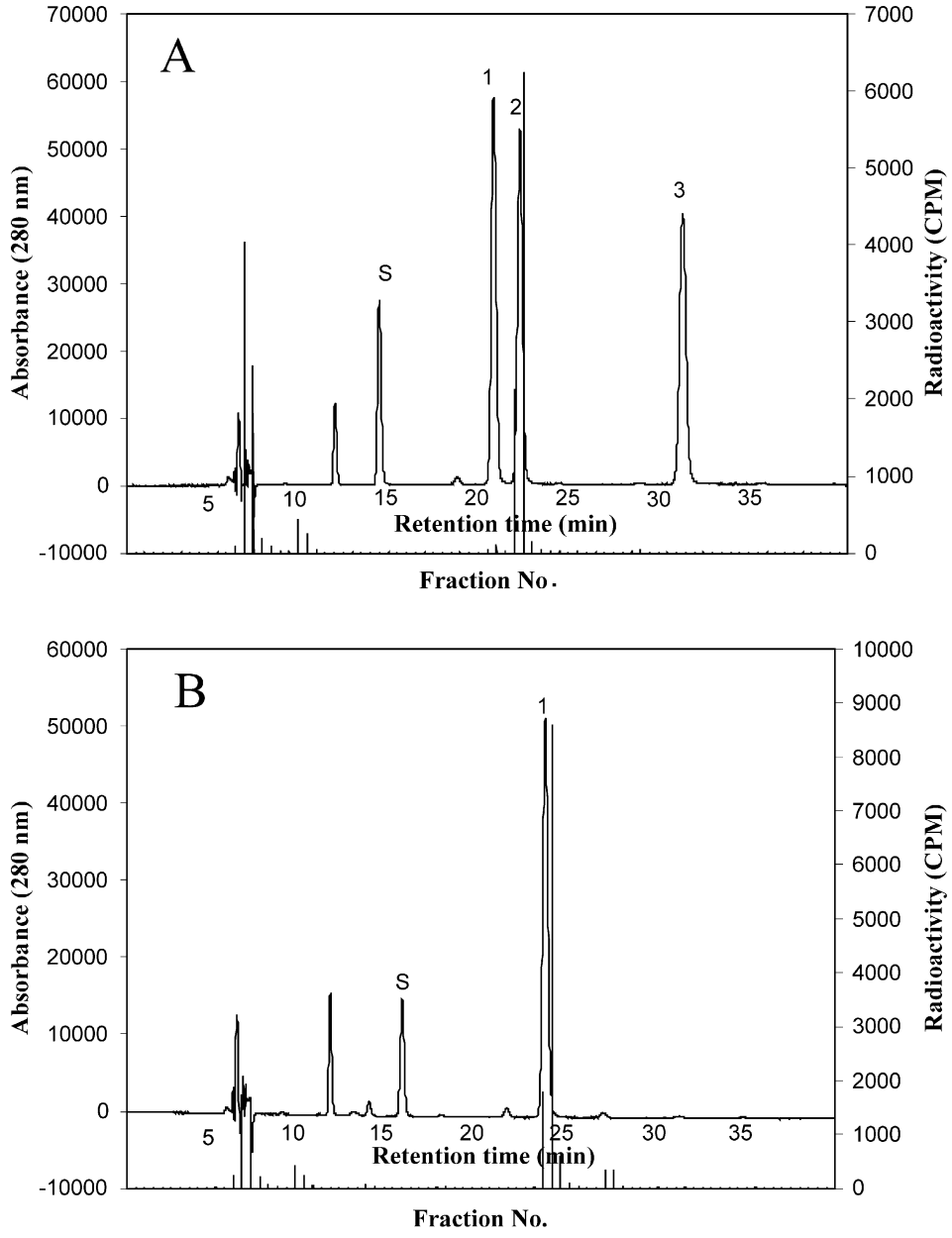


Fig. 3. Radio-HPLC analysis of the products of recombinant alfalfa COMT with protocatechuic aldehyde (**10**) and 3, 4-dihydroxy 5-methoxybenzaldehyde (**11**). COMT were incubated with ^{14}C-SAM and each of the two substrates and reactions were stopped by addition of TCA. Each reaction was then spiked with the predicted product, and substrates/products separated by HPLC monitored by UV absorption at 280 nm. Eluate passing through the diode-array detector was collected (0.5 ml fractions) and analyzed for radioactivity by liquid scintillation counting. The substrates (S) analyzed were protocatechuic aldehyde (**10**) (Fig. 2A) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) (Fig. 2B). Potential products in A were 1, isovanillin (**18**); 2, vanillin (**13**); 3,(3,4)-dimethoxylbenzaldehyde (**15**). Product 1 in B is syringaldehyde (**14**).

constants of recombinant wild-type and mutant COMTs against two monolignol precursors (caffeic acid (**1**) [poor] and 5-hydroxyconiferaldehyde (**3**) [preferred]), as well as protocatechuic aldehyde (**10**), 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) and protocatechuic acid (**12**). Wild-type COMT exhibited higher steady state kinetic preferences (expressed as V_{max}/K_m) for all aldehydes over their respective acids. The L136Y mutant displayed little substrate discrimination with a moderate preference for protocatechuic aldehyde (**10**) and protocatechuic acid (**12**), whereas A162T lost the ability to discriminate between the various phenylpropanoid acids and aldehydes, and was inactive when assayed with protocatechuic acid (**12**). M130L displayed a complete loss of activity against all acids and had very low residual activity with protocatechuic aldehyde (**10**). A similar overall substrate preference pattern was seen for the F172Y mutant, although this was due to an increased

K_m value in contrast to the reduced V_{max} for M103L. N131K behaved kinetically like A162T, except for its detectable but very weak activity with protocatechuic aldehyde (**10**). N131D catalyzed turnover of monolignol precursors, but had reduced preference for caffeic acid (**1**) and was not active with the benzaldehyde derivatives. N131E likewise did not methylate benzaldehyde derivatives, and had an even lower preference for caffeic acid (**1**) than did N131D. N324Y preferred 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) over the other substrates, and was not active with protocatechuic acid (**12**). The double mutant N324H/M130L exhibited activity against caffeic acid (**1**) that was not seen in the M130L single mutant. It also exhibited high V_{max} values for 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) and protocatechuic acid (**12**), although the K_m values for these substrates were also high.

In conclusion, the kinetic results obtained using structurally designed point mutants of alfalfa COMT demonstrate that it is possible to engineer alfalfa COMT to retain high V_{max} and low K_m values for the preferred monolignol precursor 5-hydroxyconiferaldehyde (**3**) while at the same time strongly reducing methylation activity with benzaldehyde derivatives. In contrast, none of the present set of mutants contained selective activity for only benzaldehyde derivatives.

Further mutagenesis studies will be required to determine whether it is possible to generate COMT with a marked or total preference for benzaldehydes. These experiments will likely invoke the use of the high resolution crystal structure of alfalfa COMT (Zubieta et al., 2002) to direct introduction of mutations in and around the propanoid tail binding region. The net effect of such mutations would be to sterically occlude this region of the active site thus preventing COMT from sequestering the C3 tail of C6–C3 phenylpropanoid substrates while at the same time retaining the ability to sequester the C6 aromatic rings of benzaldehyde containing substrates. Non-engineered alfalfa COMT can clearly be used for genetic engineering of benzaldehyde-derived flavors or fragrances such as vanillin (**13**), but it is nevertheless important to determine whether an OMT distinct from COMT is ever used by plants for the biosynthesis of benzaldehyde derivatives. Future studies on structure/activity relationships and engineering of enzymes of plant secondary metabolism should lead to development of new classes of biocatalysts for the rational synthesis of bioactive molecules both in vivo and in vitro.

2.5. *Effects of down-regulation of COMT on soluble and wall-bound phenolic compounds*

To address the in vivo significance of the activity of alfalfa COMT with benzaldehyde derivatives, we profiled phenolic metabolites in the soluble and wall-bound fractions from empty vector control and COMT down-regulated transgenic alfalfa lines (Fig. 4). A small decrease in the levels of 4-coumaric (**19**) and ferulic (**5**) acids was found in the cell-wall esterified phenolic fractions from both young (1–3 internodes) and old (6–7 internodes, possibly not significant) stem tissues of COMT down-regulated compared with wild-type lines (Fig. 4). There was no difference between young and old internodes in the extent of decrease in these compounds. COMT down-regulation led to a small increase in the levels of vanillyl alcohol (**20**) in the soluble fraction of extracts from young internodes, and in 4-coumaric acid (**19**) levels in extracts from old internodes. Overall 4-coumaric acid (**19**) levels in the soluble plus wall-bound fractions did not appear to be affected by COMT down-regulation. We could not detect any accumulation of non-methylated benzaldehyde derivatives in extracts from COMT down-regulated plants.

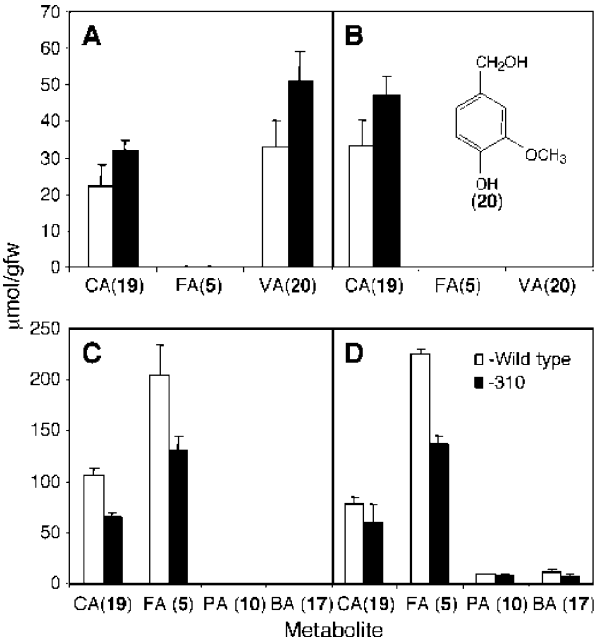


Fig. 4. HPLC analysis of soluble and wall-bound phenolic compounds in stem extracts from wild-type and COMT down-regulated alfalfa. Powdered stems from young (internodes 1–3) and old (internodes 6–7) empty vector control (WT) and COMT down-regulated (310) plants were extracted in acetone and re-suspended in methanol. Twenty microliter aliquots were analyzed by HPLC with diode array detection at 310 nm. Residues were analyzed for wall bound phenolics after alkaline hydrolysis. Soluble phenolics from young (A) and old (B) internodes and wall-bound phenolics from young (C) and old (D) internodes are represented as µmol of metabolite per g fresh weight. CA, 4-coumaric acid (**19**); FA, ferulic acid (**5**); VA, vanillyl alcohol (**20**); PA, protocatechuic aldehyde (**10**); BA, 4-hydroxybenzaldehyde (**17**). Values are from duplicate assays with duplicate tissue samples consisting of pooled stem material from multiple plants, expressed as average ± standard deviation.

2.6. Biological significance of the in vitro activities of alfalfa COMT

Based on the availability of the substrates at the time, the OMT identified as converting caffeic acid (**1**) to ferulic acid (**5**) was named caffeic acid OMT (Neish, 1968). COMT was later shown to be bifunctional and able to methylate 5-hydroxyferulic acid (**2**) to sinapic acid (**21**) (Shimada et al., 1970). However, the accepted pathway for biosynthesis of monolignols has recently undergone several revisions (Dixon et al., 2001; Anterola and Lewis, 2002; Humphreys and Chapple, 2002), based in part on new discoveries concerning OMT substrate specificity in vitro and in vivo (Li et al., 2000; Matsui et al., 2000; Parvathi et al., 2001). It is probably true to say that the in vivo significance of the in vitro substrate preferences of COMT for the various monolignol precursors still remains to be determined, a major problem being that the steady state kinetic constants determined in vitro may or may not be relevant criteria for assessing in vivo routes to monolignols through a potential metabolic grid if substrate channeling occurs (Dixon et al., 2001; Guo et al., 2002).

The broad substrate spectrum of COMT suggests that COMT may be involved in more than one pathway of phenylpropanoid biosynthesis. However, whereas COMT down-regulation drastically reduces *S*-lignin biosynthesis in alfalfa (Guo et al., 2000), we could see no evidence for either reduction in levels of methylated benzaldehyde derivatives (in fact, levels of vanillyl alcohol (**20**) were increased), or accumulation of non-methylated benzaldehyde derivatives, in COMT down-regulated plants. Thus, it is possible that the activity of COMT with protocatechuic aldehyde (**10**) and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) has no physiological significance in alfalfa. The activity of COMT for 5-hydroxyferulic acid (**2**) may likewise have no physiological significance for lignin biosynthesis (Dixon et al., 2001; Humphreys and Chapple, 2002). The broad substrate specificity of COMT may simply reflect active site chemistry that is integral to the ability of the enzyme to efficiently methylate its physiological substrate. Tobacco COMT is also active with protocatechuic acid (**12**), although, unlike alfalfa COMT, 5-hydroxyconiferaldehyde (**3**) is the preferred substrate in vitro (Maury et al., 1999). A similar multifunctional role for COMT has been proposed in strawberry (Wein et al., 2002), where an enzyme with all the attributes of lignin-specific COMT appears to also be involved in formation of the flavor compound 2,5-dimethyl-4-methoxy-3(2*H*)-furanone. The in vivo function of COMT may therefore differ between different cells of a plant, or between different species, depending upon available substrates.

Plant small molecule OMTs might have evolved from a common ancestral gene and have divided into groups based on their substrate preferences (Ibrahim et al., 1998; Pichersky and Gang, 2000). Substitution of as few as seven amino acids can convert an isoeugenol *O*-methyltransferase (IEMT) from *Clarkia breweri* to a COMT (Wang and Pichersky, 1999) and even a single amino acid change in *T. tuberosum* OMTs can quantitatively alter substrate preference between alkaloids and phenylpropanoids (Frick and Kutchan, 1999). Such flexibility is, however, usually seen within the context of conserved regio-specificity, e.g. the hydroxylation of C6–C1 and C6–C3 compounds by alfalfa COMT is at the same position on the aromatic ring. Where *O*-methylation occurs at multiple positions on a polyhydroxylated substrate, a number of distinct enzymes are usually involved, as seen in the biosynthesis of polymethylated flavonoids in *Chrysosplenium americanum* (De Luca and Ibrahim, 1985; Gauthier et al., 1996).

Other potentially multifunctional small molecule OMTs exist in addition to COMT. These include pinosylvin *O*-methyltransferase from Scots pine (Chiron et al., 2000), OMTs from *Chrysosplenium americanum* active against both phenylpropanoids and flavonoids (Gauthier et al., 1998), and recombinant OMTs from *Thalictrum tuberosum* with specificity for both phenylpropanoid and alkaloid substrates (Frick and Kutchan, 1999). The in vivo significance of these findings remains to be assessed and, as a note of caution, differences in substrate specificities between the enzymes isolated from their natural source and expressed in *E. coli* have been reported for Scots pine, aspen and tobacco (Martz et al., 1998; Meng and Campbell, 1998; Chiron et al., 2000). Whatever its in vivo significance, the apparent catalytic promiscuity of some plant small molecule OMTs is a significant problem for gene annotation in sequencing projects.

3. Experimental

3.1. Plant material

Non-transgenic (cv. Apollo) and transgenic (cv. Regen SY) alfalfa (*Medicago sativa* L.) plants were grown under standard greenhouse conditions. Plants with shoots consisting of at least 10 internodes were selected for analysis. Transgenic plants were either control (harboring an empty pCAMBIA 3300 binary vector) or COMT down-regulated, as described previously (Guo et al., 2000).

3.2. Enzyme extraction and assay

Separate internodes from 1 to 10 (counting from the first fully opened leaf at the top) from non-transgenic alfalfa, and internodes five to eight (combined) from transgenic alfalfa, were collected and ground under

liquid N_2. Samples were extracted, and enzyme activity determined, as described previously (Parvathi et al., 2001). Monolignol precursors and benzaldehyde derivatives were included at 50 μM for assay of developmental changes in OMT activities.

3.3. HPLC analysis of reaction products

The enzyme reaction was terminated with 10 μl TCA and potential products added to a final concentration of 100 μM. After centrifugation at 10,000*g* for 5 min, 50 μl supernatant was mixed with 60 μl methanol and directly subjected to HPLC on a Beckman System Gold HPLC system consisting of a programmable solvent module 126, a System Gold 508 autosampler and a System Gold 168 diode array detector. A Waters XTerra 5μ reverse phase column (5 μm particle, 250 × 4.6 mm) was used, with the mobile phase consisting of 10 mM ammonium formate in water (pH 3.7) (A), and acetonitrile/water (95:5) (B). The gradient used was 91% A, 9% B for 2 min, a linear gradient to 85% A, 15% B in 30 min, hold at 15% B for 5 min then a linear gradient to 100% B in 5 min. A flow rate of 1 ml min^{-1} was used in all experiments.

3.4. Kinetic studies

For the determination of V_{max} and apparent K_m values, caffeic acid (**1**), protocatechuic acid (**12**) (5–100 μM), 5-hydroxyconiferaldehyde (**3**), protocatechuic aldehyde (**10**), and 3,4-dihydroxy, 5-methoxybenzaldehyde (**11**) (all at 0.25–100 μM) were used as substrates with recombinant alfalfa COMT. All incubations contained 60 μM (30 times the K_m value) [Me^{14}C]-*S*-adenosyl-L-methionine as methyl group donor. Radiolabeled products were extracted into hexane: ethyl acetate (1:1) and quantified by liquid scintillation counting. V_{max} and K_m values were determined from Lineweaver–Burk plots of initial rate data.

3.5. Expression, purification and mutagenesis of COMT

The COMT open reading frame was excised from the previously described cDNA (Parvathi et al., 2001) and re-cloned between the *Bam*H1 and *Nde*I sites of the pET15b expression vector. *E. coli* BL21 (DE3) cells harboring pCOMT were grown to an optical density of 0.6 (at 600 nm), and expression was induced by addition of IPTG to a final concentration of 0.5 mM. Cells were collected by centrifugation 3 h after addition of IPTG. The enzyme was extracted from the *E. coli* cells by sonication. After centrifugation the enzyme was purified from the soluble fraction using Ni-NTA His.bind resin (Novagen, Madison, WI, USA) according to the manufacturer's protocol. The purified enzyme was concentrated by Centricon 10 filtration, equilibrated to 100 mM Tris–HCl, pH 7.5, 2 mM DTT, 0.2 mM $MgCl_2$ and 30% glycerol, and stored at −80 °C until used for enzyme activity assay.

Site-directed mutants were generated using the QuickChange protocol (Stratagene, La Jolla, CA) and purified from *E. coli* cultures as described previously (Zubieta et al., 2002).

3.6. Determination of soluble and wall-bound phenolic compounds

Stem tissues (young internodes 1–3 and old internodes 6–7) were ground in liquid N_2. Residues previously extracted for soluble phenolics (Howles et al., 1996) were washed three times with absolute EtOH, dried under N_2 and subjected to base hydrolysis for 18 h in 10 ml of 1 M NaOH at room temperature. After centrifugation (8000 *g* at 4 °C for 15 min), 60% of the supernatant was removed, acidified to pH 1.0–2.0 with 2 M HCl, and extracted three times with an equal vol of EtOAc. The organic phases were combined, taken to dryness, and re-suspended in HPLC grade MeOH to a final conc. equivalent to 200 mg dry wt of original stem tissue per μl MeOH. Twenty microlitres of solution were analyzed by HPLC as described (Howles et al., 1996; Guo et al., 2000), monitoring at 270 and 310 nm. Soluble phenolics were analyzed by HPLC as described (Howles et al., 1996).

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