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# Monolignol biosynthesis in microsomal preparations from lignifying stems of alfalfa (*Medicago sativa* L.)

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**Abstract**

Microsomal preparations from lignifying stems of alfalfa (*Medicago sativa* L.) contained coniferaldehyde 5-hydroxylase activity and immunodetectable caffeic acid 3-*O*-methyltransferase (COMT), and catalyzed the S-adenosyl L-methionine (SAM) dependent methylation of caffeic acid, caffeyl aldehyde and caffeyl alcohol. When supplied with NADPH and SAM, the microsomes converted caffeyl aldehyde to coniferaldehyde, 5-hydroxyconiferaldehyde, and traces of sinapaldehyde. Coniferaldehyde was a better precursor of sinapaldehyde than was 5-hydroxyconiferaldehyde. The alfalfa microsomes could not metabolize 4-coumaric acid, 4-coumaraldehyde, 4-coumaroyl CoA, or ferulic acid. No metabolism of monolignol precursors was observed in microsomal preparations from transgenic alfalfa down-regulated in COMT expression. In most microsomal preparations, the level of the metabolic conversions was independent of added recombinant COMT. Taken together, the data provide only limited support for the concept of metabolic channeling in the biosynthesis of S monolignols via coniferaldehyde.


*Keywords:* *Medicago sativa*; Leguminosae; Alfalfa; Lignification; *O*-Methyltransferase; Cytochrome P450; In vitro biosynthesis

## 1. Introduction

Our understanding of the biosynthesis of the monolignol building blocks of the cell wall polymer lignin has undergone several revisions in recent years (Dixon et al., 2001; Humphreys and Chapple, 2002). The initial premise, based on substrate specificity studies with compounds available at the time, was that the successive ring hydroxylation and *O*-methylation reactions necessary for formation of the guaiacyl (G) and syringyl (S) units of lignin take place at the level of hydroxycinnamic acid derivatives (Neish, 1968). In support of this model, the enzyme known as caffeic acid 3-*O*-methyltransferase (COMT) was first identified as converting caffeic acid (**1**) to ferulic acid (**2**), and was later shown also to convert 5-hydroxyferulic acid (**3**) to sinapic acid (**4**) (Shimada et al., 1970). The concept of a single pathway for ring methylation at the level of hydroxycinnamic acids received its first revision when it was demonstrated that a second and distinct *O*-methyltransferase, caffeoyl CoA *O*-methyltransferase (CCoAOMT), was involved in the methylation reactions of lignin biosynthesis in differentiating tracheary elements of *Zinnia elegans* (Ye et al., 1994). CCoAOMT converts caffeoyl CoA (**5**) to feruloyl CoA (**6**) and 5-hydroxyferuloyl CoA (**7**) to sinapoyl CoA (**8**), at least in vitro (Inoue et al., 1998; Grimmig et al., 1999).

The three successive ring hydroxylation reactions of monolignol biosynthesis are catalyzed by cytochrome P450 enzymes (Fig. 1). These are, respectively, the well-characterized cinnamate 4-hydroxylase (C4H) (Pierrel et al., 1994), a "4-coumarate 3-hydroxylase" that has recently been shown to be encoded by CYP98A3 and to operate at the level of quinate, shikimate, and possibly other esters of 4-coumarate (**15**) (Schoch et al., 2001; Franke et al., 2002), and the so-called ferulate 5-hydroxylase (F5H) (Meyer et al., 1996). As cytochrome P450s,

*Abbreviations:* CCoAOMT, caffeoyl coenzyme A 3-*O*-methyltransferase; COMT, caffeic acid 3-*O*-methyltransferase; F5H, ferulate/coniferaldehyde 5-hydroxylase; G, guaiacyl; S, syringyl; SAM, S-adenosyl L-methionine.

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all three enzymes are localized to the external surface of the endoplasmic reticulum by N-terminal hydrophobic membrane anchors (Chapple, 1998). The enzyme designated as F5H has a higher affinity for coniferaldehyde (**9**) than for ferulic acid (**2**), at least in sweet gum (Osakabe et al., 1999) and arabidopsis (Humphreys et al., 1999) and may not hydroxylate ferulic acid (**2**) itself in vivo. Likewise, COMT from aspen and alfalfa (*Medicago sativa* L.) has a significantly higher affinity for 5-hydroxyconiferaldehyde (**10**) than for caffeic acid (**1**) or 5-hydroxyferulic acid (**3**) (Osakabe et al., 1999; Parvathi et al., 2001).

The above in vitro substrate preferences are consistent with involvement of COMT primarily in the formation of S lignin via methylation of 5-hydroxyconiferaldehyde (**10**), a conclusion in agreement with the results of transgenic modification of COMT activity in vivo (Atanassova et al., 1995; Guo et al., 2000). Recombinant alfalfa COMT also exhibits high catalytic efficiency with caffeyl aldehyde (**11**) and caffeyl alcohol (**12**) (Parvathi et al., 2001), suggesting that COMT could be involved in both the 3- and 5-methylation reactions of S lignin biosynthesis at either the aldehyde or alcohol levels, and that caffeyl aldehyde (**11**) might be a previously unsuspected intermediate in S lignin biosynthesis.

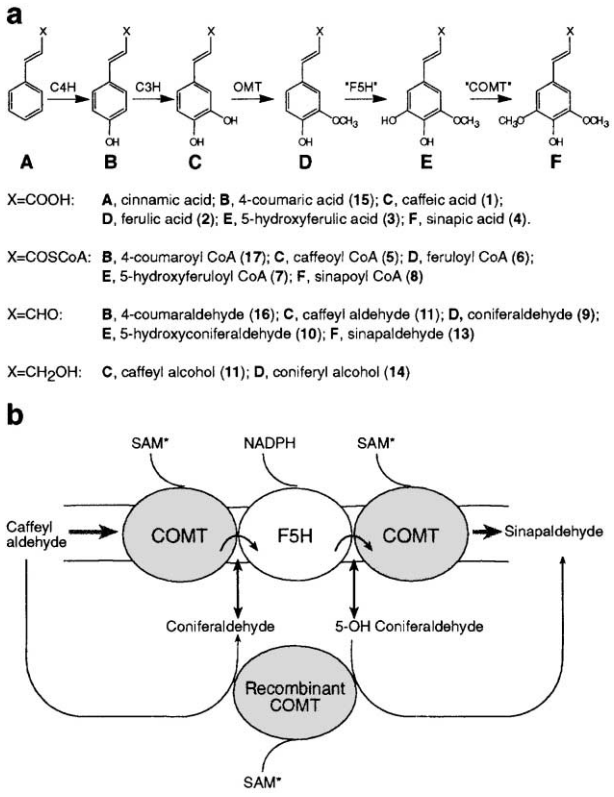


Fig. 1. Biosynthesis of syringyl monolignols. (a), Scheme for the successive ring hydroxylation and *O*-methylation reactions. X potentially represents a free acid, coenzyme A or other ester, aldehyde or alcohol function. The preferred substrates for each reaction based on current views of the lignin pathway are represented by the letters such that A = cinnamic acid, B = 4-coumaroyl ester, C = caffeic acid, caffeyl aldehyde/alcohol or caffeoyl ester; D = coniferaldehyde or coniferyl alcohol; E = 5-hydroxyferulic acid or 5-hydroxyconiferaldehyde/alcohol; F = sinapic acid or sinapyl aldehyde/alcohol. (b), A metabolic channel model for the hydroxylation and *O*-methylation reactions of monolignol biosynthesis at the aldehyde level of side chain oxidation. The interacting enzymes are envisaged as being co-localized to the external surface of the endoplasmic reticulum, and channeled reactions would proceed in such a way that the substrate of one reaction is passed to the active site of the next enzyme without equilibration with the bulk phase. The model also indicates potential accessibility of "unchanneled" substrates and products to exogenous recombinant COMT.

Studies in which COMT or CCoAOMT activities have been down-regulated in transgenic alfalfa or tobacco indicate a clear requirement for COMT activity for formation of S lignin (Zhong et al., 1998; Guo et al., 2000). In tobacco, CCoAOMT appears to be involved in the formation of both G and S lignin (Zhong et al., 1998), whereas in alfalfa, CCoAOMT down-regulation leads to a reduction in the formation of G lignin but not of S lignin (Guo et al., 2000). Coordinated down-regulation of both COMT and CCoAOMT in transgenic alfalfa results in a complete loss of S lignin but no greater reduction in G lignin than observed following down-regulation of either enzyme alone (Guo et al., 2000). Taken together, these results suggest that there are independent pathways to G and S lignin in alfalfa, and that there may be other enzymes in addition to COMT and CCoAOMT involved in monolignol methylation (Dixon et al., 2001). The concept of independent pathways to G and S lignin is further supported by the recent demonstration of a form of cinnamyl alcohol dehydrogenase from sweetgum that is much more active with sinapaldehyde (**13**) than with coniferaldehyde (**9**) (Li et al., 2001).

Independent pathways to G and S lignin would nevertheless involve shared precursors, for example coniferaldehyde (**9**). This suggests a requirement for some form of metabolic compartmentation to facilitate independent regulation of the two pathways. Such regulation in plant natural product biosynthesis can occur by metabolic channeling, in which intermediates in the pathway are transferred through enzyme complexes localized to endomembranes (Wagner and Hrazdina, 1984; Hrazdina and Wagner, 1985b; Winkel-Shirley, 1999, 2001). Cytochrome P450 enzymes have been postulated to provide the membrane anchors necessary for the assembly of metabolic channels on the external surface of the endoplasmic reticulum (Hrazdina and Wagner, 1985a; Rasmussen and Dixon, 1999; Winkel-Shirley, 2001). We here describe experiments designed to test the hypothesis that the *O*-methylation and hydroxylation reactions of S-lignin biosynthesis can be carried out by a microsomal system comprising *O*-methyltransferase and the coniferaldehyde 5-hydroxylase ("F5H") cytochrome P450.

## 2. Results

### 2.1. Presence of COMT in alfalfa stem microsomes

Extracts from stems of mature alfalfa plants were fractionated by centrifugation into soluble and microsomal fractions, as outlined in Experimental. Protein gel blot analysis with monospecific antisera indicated that a small but reproducible proportion of the total immunodetectable COMT protein was present in washed microsomes (Fig. 2). In contrast, CCoAOMT protein was not detected in the microsomes under comparable conditions. Although the relative affinities of the COMT and CCoAOMT antisera may be different, these data provide semi-quantitative evidence for differential localization of OMTs in the microsomal preparation.

The total, soluble and microsomal fractions were assayed for S-adenosyl L-methionine (SAM) dependent OMT activity using caffeic acid (**1**), caffeyl aldehyde (**11**), and caffeyl alcohol (**12**) as substrates. All these compounds are readily methylated by recombinant alfalfa COMT in vitro, but at different rates (Fig. 3). The relative activities toward the three substrates were similar in the three fractions, with caffeyl aldehyde (**11**) and alcohol (**12**) being equally efficiently methylated, at about five times the rate of caffeic acid (**1**), similar to the substrate preference of the recombinant COMT. However, the total activity toward these substrates was at least 200 times lower in the microsomal compared to the soluble fraction when calculated on the basis of fresh weight of the original starting material. These data confirm that the small fraction of total OMT associated with the microsomes has the same substrate preference profile as the bulk COMT activity. That the microsomal methylation activity can be accounted for by COMT was further demonstrated by the observation that microsomal preparations from stems of COMT down-regulated transgenic alfalfa could not catalyze SAM-dependent methylation of caffeic acid (**1**) or caffeyl aldehyde (**11**) (data not shown).

### 2.2. Analysis of methylated monolignol precursor metabolism in alfalfa stem microsomes

The proposed sequence of reactions from caffeyl aldehyde (**11**) to sinapaldehyde (**13**) involves, successively, COMT-mediated *O*-methylation of caffeyl aldehyde (**11**) to coniferaldehyde (**9**), hydroxylation of **9** to 5-hydroxyconiferaldehyde (**10**) by the so-called "F5H" cytochrome P450, and methylation of **10** by COMT to yield sinapaldehyde (**13**) (Fig. 1). We first confirmed the presence of "F5H" activity by incubating microsomes with coniferaldhleyde (**9**) and NADPH, and monitoring production of 5-hydroxyconiferaldehyde (**10**) by GC/MS analysis after derivatization. No 5-hydroxyconiferaldehyde (**10**) was formed if NADPH was omitted from the reaction mixture. The average F5H activity in the stem microsome preparations was 12 pkat/mg protein. The two methylation steps in the pathway afforded opportunities for introduction of radiolabel through the use of [methyl-$^{14}$C]-labeled SAM as co-substrate. We therefore developed both TLC and HPLC

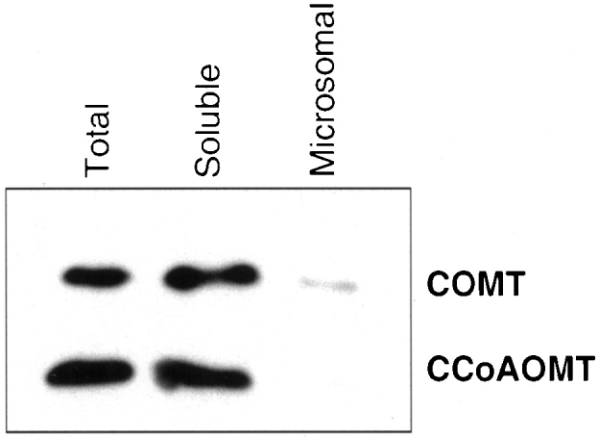


Fig. 2. Protein gel blot analysis of COMT and CCoAOMT in cell extracts from lignifying alfalfa stems. Proteins from total, soluble (130,000 *g* supernatant) and microsomal (washed 130,000 *g* pellet) fractions were separated by SDS-PAGE, blotted to a nitrocellulose membrane, and identified by hybridization with polyclonal antisera raised against recombinant alfalfa COMT and CCoAOMT (Kersey et al., 1999). The relative loading of the lanes in relation to the initial amount of tissue extracted is 1:1:2.5 (total: soluble: microsomal). This experiment was repeated three times with very similar results.

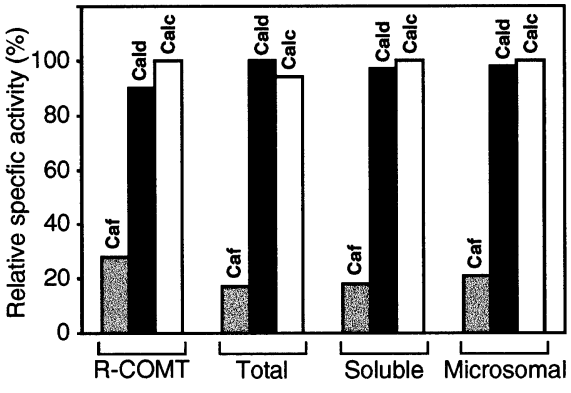


Fig. 3. COMT activity in cell extracts from lignifying alfalfa stems. The bars show the relative substrate preferences of recombinant alfalfa COMT (R-COMT) and the OMT activity in the total, soluble (130,000 *g* supernatant) and microsomal (washed 130,000 *g* pellet) protein fractions from stem material. The fractions were assayed for OMT activity with $^{14}$C-SAM and either caffeic acid (**1,** Caf), caffeyl aldehyde (**11,** Cald) or caffeyl alcohol (**12,** Calc), and results expressed as a% of the activity with the substrate having the highest conversion (100%). Absolute activities based on starting plant material were in the ratio of 100:70:0.4 (total: soluble: microsomal). COMT activity of the total fraction with **11** as substrate was 87 pkat/g fr.w.

methods to resolve methyl-$^{14}$C-labeled coniferaldehyde (**9**), 5-hydroxyconiferaldehyde (**10**) and sinapaldehyde (**13**), monitoring the separations either by phosphorimager analysis (for TLC) or fraction collection and liquid scintillation counting (for HPLC).

Stem microsomes were incubated with caffeyl aldehyde (**11**) and $^{14}$C-SAM to produce radiolabeled coniferaldehyde (**9**); with **11**, $^{14}$C-SAM and NADPH, for production of **9** and further metabolism to 5-hydroxyconiferaldehyde (**10**); and with **10** and $^{14}$C-SAM for production of labeled sinapaldehyde (**13**). Fig. 4 shows the separation of these reaction products by TLC from a typical experiment. Fig. 5a shows the HPLC separation of authentic standards of the various intermediates, and Fig. 5b–d shows the chromatographic separations of the various $^{14}$C-labeled monolignol precursors generated as described above. It was possible to separate and quantify the various labeled intermediates that are formed by successive methylation and hydroxylation reactions of monolignol precursors in alfalfa microsomes.

In the HPLC system used in Fig. 5, 5-hydroxyconiferaldehyde (**10**) elutes within a retention time only one minute different from that of coniferyl alcohol (**14**). Since contamination of the alfalfa microsomes with cinnamyl alcohol dehydrogenase (CAD) activity could result in conversion of coniferaldehyde (**9**) to coniferyl alcohol (**14**) rather than or in addition to conversion by F5H to 5-hydroxyconiferaldehyde (**10**), we utilized TLC (see Experimental) to clearly separate the two potential products (Rfs 0.11 and 0.48 for the **10** and **14** respectively). The results indicated that, following *O*-methylation of caffeyl aldehyde (**11**), labeled coniferaldehyde (**9**) was converted, in the presence of NADPH, to 5-hydroxyconiferaldehyde (**10**), with no conversion to coniferyl alcohol (**14**) (500–800 dpm in 5-hydroxyconiferaldehyde (**10**), 0 dpm in coniferyl alcohol (**14**) using the same experimental conditions as for Fig. 5). This is consistent with the presence of F5H activity in the microsomes, and argues against the presence of residual CAD activity.

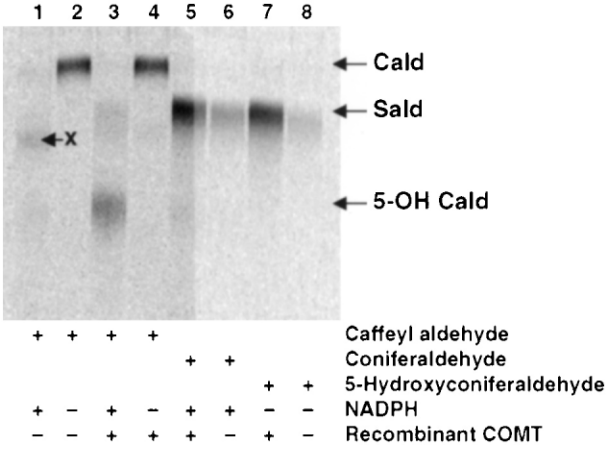


Fig. 4. TLC analysis of labeled metabolites from alfalfa microsomal preparations fed monolignol precursors and $^{14}$C-SAM. The autoradiograph shows the separation of labeled coniferaldehyde (**9**, Cald) from sinapaldehyde (**13**, Sald) and 5-hydroxyconiferaldehyde (**10**, 5-OH Cald). X is an unidentified compound that was reproducibly detected in preparations fed caffeyl aldehyde (**11**), $^{14}$C-SAM and NADPH. The experiment shows the effects of addition of NADPH and recombinant COMT to the microsomal extracts. Numerical data from the same experiment were computed following separation of the products by HPLC and determination of radioactivity by scintillation counting, and are presented in Table 2.

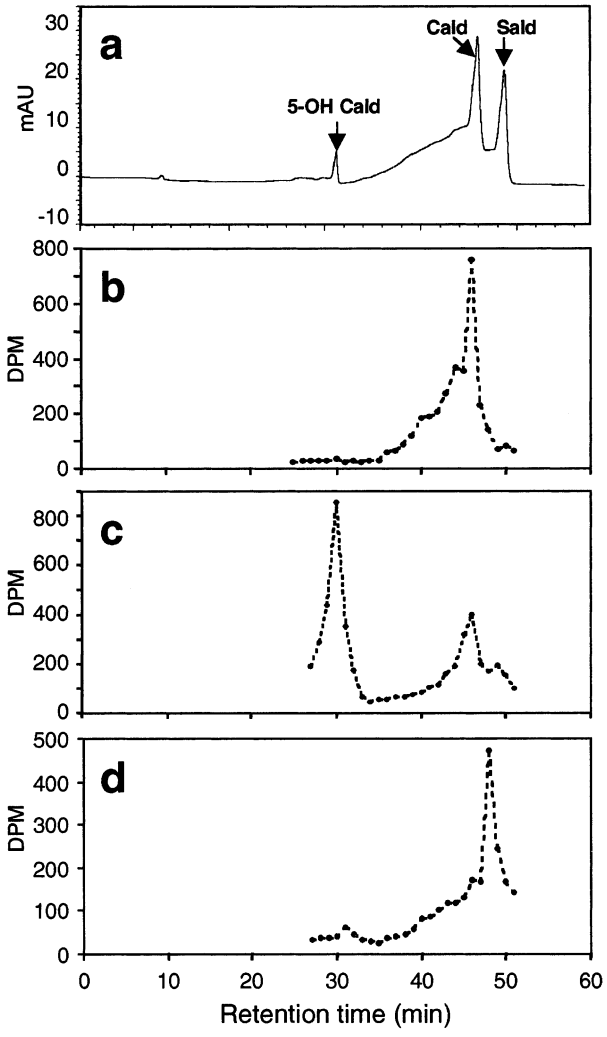


Fig. 5. HPLC analysis of labeled metabolites from alfalfa microsomal preparations fed monolignol precursors and $^{14}$C-SAM. (a), HPLC trace showing the separation of authentic standards of coniferaldehyde (**9**, Cald), sinapaldehyde (**13**, Sald) and 5-hydroxyconiferaldehyde (**10**, 5-OH Cald). (b), Radio-HPLC trace showing coniferaldehyde (**9**) produced in alfalfa microsomes from caffeyl aldehyde (**11**) and $^{14}$C-SAM. (c), Radio-HPLC trace showing coniferaldehyde (**9**), 5-hydroxyconiferaldehyde (**10**) and sinapaldehyde (**13**) produced in alfalfa microsomes from caffeyl aldehyde (**11**) and $^{14}$C-SAM in the presence of NADPH. (d), Radio-HPLC trace showing sinapaldehyde (**13**) produced in alfalfa microsomes from 5-hydroxyconiferaldehyde and $^{14}$C-SAM.

### 2.3. Coupled monolignol O-methylation and hydroxylation in alfalfa stem microsomes

Exogenously added caffeyl aldehyde (**11**) was converted to $^{14}$C-coniferaldehyde (9) on incubation of alfalfa stem microsomes with $^{14}$C-SAM (Fig. 5b, Table 1). When **11** was incubated with SAM in the presence of NADPH, the reductant for the P450 catalyzed hydroxylation of coniferaldehyde (**9**), the amount of label in **9** decreased compared to that in incubations lacking NADPH, and $^{14}$C-5-hydroxyconiferaldehyde (**10**) was formed (Fig. 5c, Table 1). In one of the two independent experiments reported in Table 1, a small amount of label was also detected in sinapaldehyde (**13**) following incubation of caffeyl aldehyde (**11**) with $^{14}$C-SAM and NADPH, indicating the coupled operation of the three successive reactions for 3-*O*-methylation, 5-hydroxylation and 5-*O*-methylation.

Alfalfa microsomes effectively converted coniferaldehyde (**9**) to sinapaldehyde (**13**), but only in the presence of NADPH. This conversion was as effective as the direct conversion of exogenously added 5-hydroxyconiferaldehyde (**10**) to **13** (Table 1), suggesting close coupling of the F5H and 5-OMT reactions. No **13** was formed from coniferaldehyde (**9**) in microsomes from stems of COMT down-regulated alfalfa (data not shown).

The above in vitro labeling experiments were repeated several times, with similar results but different overall extents of label incorporation. Thus, the experiments reported in Table 2 show a similar pattern of label incorporation as reported in Table 1 for the same experimental conditions, but with about 10–25% of the overall incorporation. In these two latter experiments, coniferaldehyde (**9**) was a somewhat better precursor of sinapaldehyde (**13**) than was 5-hydroxyconiferaldehyde (**10**).

Based on the specific activity of the labeled SAM, it was possible to estimate concentrations for the labeled monolignol pathway intermediates from the amount of $^{14}$C incorporated. These may be underestimates because of the potential for dilution by endogenous unlabeled SAM. All initial unlabeled substrates were supplied at a concentration of 1 mM, a value much higher than the $K_m$ values for alfalfa COMT (6.9 μM for caffeyl aldehyde (**11**) and 1.8 μM for 5-hydroxyconiferaldehyde

Table 1
Metabolic conversions in alfalfa stem microsomes

| Substrate | NADPH | Coniferaldehyde | 5-OH-Coniferaldehyde | Sinapaldehyde |
|---|---|---|---|---|
| | | dpm [Conc$^n$] | dpm [Conc$^n$] | dpm |
| 4-Coumaric acid (**15**) | + | 0 | 0 | 0 |
| 4-Coumaraldehyde (**16**) | + | 0 | 0 | 0 |
| 4-Coumaroyl CoA (**17**) | + | 0 | 0 | 0 |
| Caffeyl aldehyde (**11**) | – | 3936/2160 [2.4 μM] | 0 | 0 |
| Caffeyl aldehyde (**11**) | + | 1212/515 | 1044/1389 [0.9 μM] | 191/0 |
| Coniferaldehyde (**9**) | – | 0 | 0 | 0 |
| Coniferaldehyde (**9**) | + | 0 | 0 | 1273/644 |
| 5-OH-Coniferaldehyde (**10**) | – | 0 | 0 | 1308/872 |

The substrates listed (final concentration 1 mM) were incubated with microsomal proteins and $^{14}$C-SAM, in the presence or absence of NADPH, for 30 min. Reactions were stopped, and the products partitioned into ethyl acetate prior to work up for HPLC analysis and scintillation counting of the separated products as described in Experimental. Results are given for two independent experiments with different microsome preparations. Values in square brackets are minimum estimates (average values) for the overall concentrations of the intermediates formed.

Table 2
Effects of recombinant alfalfa COMT (1.25 pkat) on metabolic conversions in alfalfa stem microsomes

| Substrate | NADPH | R-COMT | Coniferaldehyde | 5-OH Coniferaldehyde | Sinapaldehyde |
|---|---|---|---|---|---|
| | | | dpm [Conc$^n$] | dpm [Conc$^n$] | dpm |
| Caffeyl aldehyde (**11**) | – | – | 398/506 [0.35 μM] | 0 | 0 |
| Caffeyl aldehyde (**11**) | + | – | 0/63 | 312/563 [0.34 μM] | 0 |
| Caffeyl aldehyde (**11**) | – | + | 606/473 [**50,000**] | 0 | 0 |
| Caffeyl aldehyde (**11**) | + | + | 0/31 | 1,109/1,617 [1.1 μM] | 88/0 |
| Coniferaldehyde (**9**) | + | – | 0 | 0 | 647 |
| Coniferaldehyde (**9**) | + | + | 0 | 0 | 1027 |
| 5-OH Coniferaldehyde (**10**) | – | – | 0 | 0 | 368 |
| 5-OH Coniferaldehyde (**10**) | – | + | 0 | 0 | 2457 (**12,250**) |

Reaction conditions and analysis are as listed in the legend to Table 1. Results with caffeyl aldehyde (**11**) are given for two independent experiments. Values in square brackets are minimum estimates (average values) for the concentrations of the intermediates formed. Bold numbers in parentheses indicate dpm in product obtained with recombinant COMT in the absence of microsomes.

(**10**) (Parvathi et al., 2001) or for F5H [1.2–2.77 μM for coniferaldehyde (**9**)] (Humphreys et al., 1999; Osakabe et al., 1999). In the experiments summarized in Tables 1 and 2, coniferaldehyde (**9**) formed from caffeyl aldehyde (**11**) reached average concentrations of between 0.35 and 2.4 μM, below and within the reported $K_m$ range for F5H. Concentrations of 5-hydroxyconiferaldehyde (**10**) were from 0.34 to 1.1 μM, just below the $K_m$ for alfalfa COMT.

To test whether intermediates in the conversion of caffeyl aldehyde (**11**) to sinapaldehyde (**13**) might be channeled in the microsomal preparations, we included several sets of parallel incubations in which an excess of recombinant alfalfa COMT was added to the assays. The rationale behind this experiment was that channeled substrates or intermediates would not be available for methylation by recombinant COMT, whereas intermediates that were freely diffusible in the aqueous medium would be methylated (Fig. 1). Depending upon the particular microsome preparation, the amount of recombinant COMT activity added [measured with caffeyl aldehyde (**11**)] was from 12 to approximately 100 times higher than the endogenous COMT activity measured in the microsomal extracts (Table 2). Channeling would be indicated by $^{14}$C-SAM incorporation into products at levels significantly below those expected from the activity of the added recombinant COMT that was confirmed for each experiment.

In the two independent experiments shown in Table 2, production of coniferaldehyde (**9**) from caffeyl aldehyde (**11**) in the absence of NADPH was essentially unaffected by addition of recombinant COMT to a level that would produce 50,000 dpm/30 min in coniferaldehyde (**9**) in the absence of the microsomal preparation. As before, addition of NADPH to reactions containing caffeyl aldehyde (**11**) and $^{14}$C-SAM resulted in disappearance of label in coniferaldehyde (**9**) and accumulation of label in 5-hydroxyconiferaldehyde (**10**); production of **10** increased approximately three-fold in the presence of recombinant COMT, and a small amount of sinapaldehyde (**13**) was detected in one experiment. However, the conversion of coniferaldehyde (**9**) to sinapaldehyde (**13**) in the presence of NADPH and $^{14}$C-SAM was only slightly stimulated in the presence of recombinant COMT. In contrast, when 5-hydroxyconiferaldehyde (**10**) was used as substrate, addition of recombinant COMT increased conversion to sinapaldehyde (**13**) by nearly seven-fold, although this was still approximately five-fold lower than the predicted conversion by recombinant COMT in the absence of the microsomes.

To determine whether the apparent lack of effect of addition of recombinant COMT in these experiments could result from limitation of SAM availability, we measured the recovery of labeled SAM in the supernatants after incubation with microsomes for 30 min. Values were reduced to around 25% of the initial concentration, suggesting considerable sequestration or metabolism of SAM by the microsomes. Nevertheless, the concentration at the end of the incubation was still more than seven times the $K_m$ value of COMT for SAM.

In one preparation of microsomes, the production of coniferaldehyde (**9**) from caffeyl aldehyde (**11**) increased more than 12-fold, and production of sinapaldehyde (**13**) from 5-hydroxyconiferaldehyde (**10**) nearly 20-fold, on incubation with recombinant COMT. However, in the same microsomes, production of sinapaldehyde (**13**) from coniferaldehyde (**9**) via microsomal F5H only increased 2.6-fold following addition of recombinant COMT, suggestive of channeling of endogenously formed 5-hydroxyconiferaldehyde (**10**). This experiment showed the greatest effect of addition of recombinant COMT among the seven independent microsome preparations assayed. However, in this preparation, the levels of methylation of **11** and **10** in microsomes incubated with recombinant COMT were still significantly less (by 62% and 66% respectively) than the theoretical values.

### *2.4. 4-Coumaraldehyde is not an intermediate in monolignol biosynthesis*

There has been much discussion as to the nature of the reaction that introduces the 3-hydroxyl group into the caffeate moiety (reviewed in Kojima and Takeuchi, 1989; Dixon et al., 2001; Humphreys and Chapple, 2002). Although polyphenol oxidase can convert 4-coumaric acid (**15**) to caffeic acid (**1**) (Bolwell and Butt, 1983), a cytochrome P450 activity is generally favored for the 3-hydroxylation reaction, and such an activity has been demonstrated to act at the level of esters of 4-coumaric acid (Heller and Kühnl, 1985; Schoch et al., 2001; Franke et al., 2002). 4-Coumaraldehyde (**16**) would also be a logical substrate for 3-hydroxylation if caffeyl aldehyde (**11**) were a *bona fide* intermediate in monolignol biosynthesis. Because of the effective coupling of the hydroxylation of coniferaldehyde (**9**) to methylation to yield sinapaldehyde (**13**), we reasoned that a similar coupled reaction (involving COMT-mediated *O*-methylation of the newly introduced 3-hydroxyl group) could be used to provide a radiometric assay for a microsomal activity catalyzing 3-hydroxylation of 4-coumaraldehyde (**16**). We therefore incubated microsomes with NADPH, $^{14}$C-SAM, and **16**. No $^{14}$C was incorporated into any compound resolved by the HPLC system. Similar negative results were obtained when 4-coumaric acid (**15**) or 4-coumaroyl CoA (**17**) replaced 4-coumaraldehyde (**16**) (Table 1).

An alternative source of caffeyl aldehyde (**11**) would be from caffeoyl CoA (**5**) by the action of cinnamoyl CoA reductase (CCR). Although feruloyl CoA (**6**) has

previously been shown to be the best hydroxycinnamoyl CoA substrate for CCR (Wengenmayer et al., 1976; Luderitz and Grisebach, 1981; Sarni et al., 1984; Goffner et al., 1994), CCR from soybean and eucalyptus is nevertheless active with caffeoyl CoA (**5**) (Wengenmayer et al., 1976; Goffner et al., 1994). We therefore assayed crude extracts from alfalfa stems for CCR activity against 4-coumaroyl CoA (**17**), caffeoyl CoA (**5**), feruloyl CoA (**6**), 5-hydroxyferuloyl CoA (**7**) or sinapoyl CoA (**8**). In assays in which each substrate was present at a final concentration of 100 mM, 5-hydroxyferuloyl CoA (**7**) was reduced the most effectively, but activity with caffeoyl CoA (**5**) was approximately 65% of that with feruloyl CoA (**6**).

### *2.5. Monolignol O-methylation and hydroxylation at the free acid level*

The involvement of ferulic acid (**2**) as an intermediate in monolignol biosynthesis has been questioned in view of the very poor activity of the microsomal ferulate 5-hydroxylase with **2** (Humphreys et al., 1999; Osakabe et al., 1999). However, it is possible that **2** formed in vivo from caffeate (**1**) might be a better substrate for F5H than is exogenously added **2**, in a similar manner to the way in which phenylalanine is a better precursor of 4-coumarate (**15**) than is cinnamate in tobacco stem microsomes (Rasmussen and Dixon, 1999). To test this, we fed caffeic acid (**1**) plus $^{14}$C-SAM to alfalfa microsomes in the presence or absence of NADPH. Equal amounts of ferulic acid (**2**) (approximately 1100 dpm/30 min) were produced in each case, with no evidence for formation of 5-hydroxyferulic acid (**3**) (Fig. 6). Addition of recombinant COMT increased production of **2** nearly seven-fold, but again no 5-hydroxyferulic acid (**3**) was formed, and ferulic acid (**2**) itself was not metabolized to sinapic acid (**4**) in microsomes incubated with NADPH in the presence or absence of recombinant COMT.

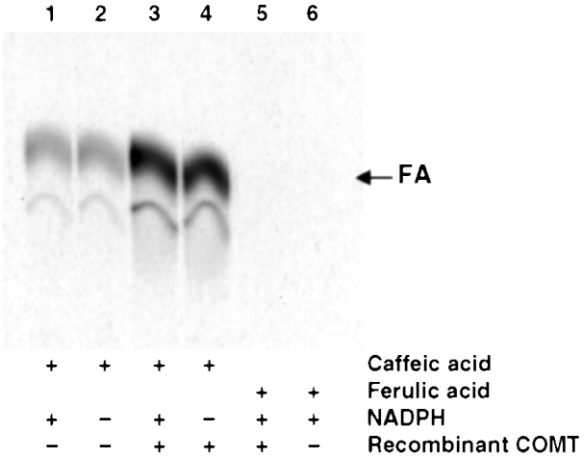


Fig. 6. TLC analysis of labeled metabolites from alfalfa microsomal preparations fed potential hydroxycinnamic acid precursors of monolignol and $^{14}$C-SAM. Reaction components are indicated under the TLC images. The experiment shows the effects of addition of NADPH and recombinant COMT to the microsomal extracts. The autoradiograph shows the production of ferulic acid (**2**) from caffeic acid (**1**), but no formation of 5-hydroxyferulic acid (**3**).

## 3. Discussion

We have analyzed the metabolism of monolignol precursors in alfalfa stem microsomes as an approach to address the potential for metabolic channeling in S monolignol biosynthesis. Tight metabolic channeling between caffeyl aldehyde (**11**) and sinapaldehyde (**13**) would be supported by: (1) association of OMT with microsomal membranes, reflecting potential interactions between OMT and the "F5H" cytochrome P450; (2) more efficient incorporation of upstream precursors than downstream intermediates into the end product sinapaldehyde (**13**); (3) coupling of methylation and hydroxylation activities to provide efficient conversion of upstream substrates to end products with reduced kinetic constraints; (4) relative ineffectiveness of externally added COMT to compete with endogenous microsomal OMT for substrates or intermediates. Our results indicate that some, but not all, of these criteria were met in the alfalfa stem microsome system.

A small proportion of the total cellular OMT activity against potential monolignol precursors associates with microsomal membranes from alfalfa stem tissues. The observations that the substrate preference profile for microsomal OMT activity matches that of recombinant alfalfa COMT, and that this activity is absent from microsomes from transgenic alfalfa plants down-regulated in COMT, indicate that the OMT activity that is being measured in the microsomal extracts is due to the operationally soluble alfalfa COMT that has been previously characterized (Gowri et al., 1991; Parvathi et al., 2001) and not to some other novel, specifically microsomal OMT. That this is not simply cytoplasmic contamination is suggested by the apparent lack of CCoAOMT protein or CAD activity in the microsomes, although it is nevertheless possible that COMT sticks to membranes during fractionation more than other enzymes of the pathway. It is also, however, possible that a much greater proportion of OMT is microsomally localized in the intact cells, but is lost to the soluble fraction during the physical processes of cell disruption and fractionation. However, the relative proportion of microsomal as compared to total OMT is clearly less than for phenylalanine ammonia-lyase or isoflavone *O*-methyltransferase, two other operationally soluble enzymes of phenylpropanoid metabolism recently shown to be associated with microsomal cytochrome P450 enzymes in metabolic complexes (Rasmussen and Dixon, 1999; Liu et al., 2001). The

localization studies do not therefore directly support the metabolic channel model. If physical associations do exist between OMT and "F5H" in vivo, they are probably quite weak.

In the in vitro microsomal system, coniferaldehyde (**9**) (in the presence of NADPH) appears to be equally good or better as a substrate for formation of sinapaldehyde (**13**) than does the direct precursor 5-hydroxyconiferaldehyde (**10**), in spite of the fact that the concentration of exogenous **10** in the reaction mixture will be much greater than the concentration of **10** produced in situ from coniferaldehyde (**9**) by the action of F5H. This is suggestive of metabolic channeling between F5H and COMT in the direction of sinapaldehyde (**13**) production. Furthermore, addition of an excess of recombinant COMT to the microsomes only increases sinapaldehyde (**13**) production from coniferaldehyde (**9**) approximately 1.7-fold, whereas there was nearly a seven-fold increase in sinapaldehyde (**13**) production from 5-hydroxyconiferaldehyde (**10**) following addition of recombinant COMT. This might also indicate channeling, with endogenously produced 5-hydroxyconiferaldehyde (**10**) unable to contact the exogenous COMT. However, this effect could also be explained by substrate limitation, i.e. the concentration of 5-hydroxyconiferaldehyde (**10**) produced in situ from exogenous coniferaldehyde (**9**) will be low and may not exceed the amount that can be converted by the endogenous COMT in the microsomes. Nevertheless, assuming that most, if not all, of the cellular F5H activity is present in the microsomes due to its membrane targeting, and that the flux through that reaction in the in vitro system reflects the flux in vivo, our data would suggest that the small amount of membrane associated COMT present in the microsomes is fully sufficient for subsequent 5-*O*-methylation of the 5-hydroxyconiferaldehyde (**10**), even though this COMT activity is such a small percentage of the total cellular activity.

The levels of 5-hydroxyconiferaldehyde (**10**) generated in situ from caffeyl aldehyde (**11**) in the presence of NADPH and SAM reached approximately 50% of the $K_m$ value of COMT for 5-hydroxyconiferaldehyde (**10**). Under these conditions, however, only approximately five percent, at most, could be further converted to sinapaldehyde (**13**). Thus, assuming that the overall concentrations of substrates and products in the microsomal reaction mixture reflect the localized concentrations near the enzyme active sites, any potential metabolic channel between F5H and COMT in the direction of sinapaldehyde (**13**) production would not appear to be able to overcome kinetic constraints on the 5-*O*-methylation reaction. Increasing the efficiency of enzymatic reactions by delivery of the product of one enzyme to the active site of the next enzyme is regarded as one potential advantage of metabolic channeling (Srere, 1987; Winkel-Shirley, 1999). Thus, the alfalfa membrane system does not meet the "improved catalytic efficiency test" for metabolic channeling.

In several independent experiments, addition of excess recombinant COMT had no significant effect on metabolism of caffeyl aldehyde (**11**) by alfalfa stem microsomes. This suggests that this substrate might be rapidly sequestered into the microsomal membranes, a process that might initiate channeled reactions to sinapaldehyde (**13**) in vivo. In contrast, caffeic acid (**1**) was converted to ferulic acid (**2**) to a much greater extent in the presence of recombinant COMT. Ferulic acid (**2**) does not now appear to be a true intermediate in monolignol biosynthesis (Humphreys et al., 1999).

The present results do not rule out the operation of a membrane-associated metabolic channel for interconversion of S monolignol precursors, but such a conclusion requires several important qualifications. First, the experiments were performed using an in vitro microsomal system that will contain membrane fragments and vesicles from all the cell types present within the stem, whereas monolignol biosynthesis only takes place in a subset of cells within the vascular tissue (Lewis et al., 1999). Second, entrapment of COMT within membrane vesicles might result in artifactual metabolic compartmentation if the substrates are readily able to pass into the vesicles. However, the highly charged NADPH molecule should not to be able to enter microsomal vesicles, and the NADPH-dependent hydroxylation of coniferaldehyde (**9**) formed by the action of microsomal COMT suggests that a significant portion of the COMT/F5H activity is on the external surface of the microsomal membranes, in direct contact with exogenous reagents. Finally, it is not yet possible to assess the proportion of COMT that might be associated with microsomal membranes in undisturbed cells in vivo. Previous results using immunogold labeling have revealed both COMT and CCoAOMT to be present in the cytosol of alfalfa vascular parenchyma cells, but provide no evidence for an ordered location suggestive of binding to the surface of the endoplasmic reticulum (Kersey et al., 1999). However, fixation of the tissues could disturb loose associations, and this approach may not have the necessary resolving power to detect protein localization associated with metabolic channeling.

The effects of separate down-regulation of COMT and CCoAOMT in transgenic alfalfa suggest that COMT can catalyze both the 3- and 5-*O*-methylation reactions in the biosynthesis of S lignin, and that the hydroxycinnamyl aldehydes are the preferred substrates. This raises the question of the metabolic origin of caffeyl aldehyde (**11**). A direct route from 4-coumaraldehyde (**16**) appears to be ruled out by the inability of this compound to be hydroxylated by the alfalfa stem microsomes in assays that should provide a sensitive measure of activity through coupling to COMT activity,

and which readily measure hydroxylation of coniferaldehyde (**9**) when coupled to methylation. An alternative route is by the action of CCR on caffeoyl CoA (**5**). Most studies on the substrate specificity of CCR (e.g. Luderitz and Grisebach, 1981; Sarni et al., 1984; Goffner et al., 1994) have not investigated caffeoyl CoA (**5**) as a potential substrate, although the enzyme from soybean is reported to have a $K_m$ value of around 200 μM for **5**, a value less than three-fold higher than the $K_m$ for the preferred substrate, feruloyl CoA (**6**), and the $K_m$ for **5** of the enzyme from *Eucalyptus gunnii* was 45 μM, similar to that for **6** (Goffner et al., 1994). In alfalfa, preliminary results suggest that caffeoyl CoA (**5**) might be a reasonable substrate for CCR, consistent with the potential involvement of caffeyl aldehyde (**11**) in monolignol biosynthesis. More work is now needed on the detailed substrate profiles of the several *CCR* gene products revealed in the TIGR Gene Index for *Medicago* species (Quackenbush et al., 2000).

In addition to addressing the potential for metabolic channeling in the biosynthesis of S lignin at the monolignol aldehyde level, our results confirm the lack of involvement of ferulic acid (**2**) as an intermediate in lignin biosynthesis, consistent with the now appreciated substrate preference of F5H for 5-hydroxyconiferaldehyde (**10**) (Humphreys et al., 1999; Osakabe et al., 1999). Ferulic acid (**2**) generated in situ by microsomal COMT can not be converted to 5-hydroxyferulic acid (**3**) by F5H, ruling out any metabolic channeling model that might allow **2** to function in monolignol biosynthesis. The recent report of the substrate preference of CYP98A3 (''4-coumarate hydroxylase'') for 4-coumarate esters rather than for free 4-coumaric acid (**15**) (Schoch et al., 2001; Franke et al., 2002) completes a picture in which, beyond cinnamic acid, free acids can no longer be viewed as in vivo intermediates in the hydroxylation or methylation reactions of monolignol biosynthesis in spite of their acting as substrates in vitro.

## 4. Experimental

### 4.1. Chemicals

Caffeyl aldehyde (**11**) and 5-hydroxyconiferaldehyde (**10**) were synthesized by a Wittig reaction from the corresponding hydroxybenzaldehydes, as described elsewhere (Chen et al., 2001). 5-Hydroxyferulic acid (**3**) was from our laboratory collection. Other phenolic compounds were obtained from Sigma (St. Louis, MO).

### 4.2. Plant material

Alfalfa (*Medicago sativa* cv. Regen SY) was grown in the greenhouse under standard conditions and harvested just before flowering. Stem samples were taken from internodes two to nine (counting from the top of the stem), frozen in liquid nitrogen, and ground in a tissue grinder. Details of transgenic alfalfa lines with drastically reduced COMT expression as a result of post-transcriptional gene silencing have been given elsewhere (Guo et al., 2000).

### 4.3. Preparation of membrane fractions

Frozen (−80 °C) ground stem tissue was homogenized for 2 × 30 s in 5 ml 0.1 M potassium phosphate buffer (pH 7.5) containing 0.4 M sucrose and 28 mM 2-mercaptoethanol using an Ultraturrax blender (Brinkmann Instruments, Inc., Westbury, NY). The homogenate was centrifuged (10,000 *g* for 15 min) and filtered through a syringe filled with glass wool. The filtrate was ultracentrifuged (130,000 *g* for 1 h), the supernatant decanted, and the microsome pellet washed by re-suspending in 5–10 ml of the same buffer and re-centrifuging at 130,000 *g* for 20 min. The pellet was blot dried and re-suspended in 200 μl of assay buffer (0.1 M phosphate with 14 mM 2-mercaptoethanol, pH 7.0). All steps were conducted on ice at 4 °C.

### 4.4. Protein gel blot analysis

Proteins were extracted, separated on 8–12% gradient SDS-polyacrylamide gels, and electroblotted onto nitrocellulose membranes. The membranes were incubated in blocking buffer (PBS containing 0.05% Tween 20 and 5% skimmed milk) for 2 h and then in blocking buffer with anti-(alfalfa COMT) and anti-(alfalfa CCoAOMT) antisera (Kersey et al., 1999) for 2 h. The signals were detected with enhanced chemiluminescence protein gel-blotting detection reagents (Amersham) according to the manufacturer's protocol.

### 4.5. In vitro enzyme/channeling assays

For assay of F5H activity, microsomes (85 μl) were incubated with coniferaldehyde (**9**) (1 mM) and NADPH (1 mM) in a total volume of 110 μl. Reaction products were partitioned into EtOAc, and the solvent was then evaporated and the residue silylated with MSTFA and subjected to GC/MS analysis. This was performed on a Hewlet Packard 5890 series II gas chromatograph with a 5971 series mass selective detector (column: HP-1, 60 m × 0.25 mm × 0.25 μm film thickness) using electron ionization (70 eV). 5-Hydroxyconiferaldehyde (**10**) was detected as the product (*m*/*z* 338), and no product was formed in the absence of NADPH.

For measurement of coupled methylation and hydroxylation reactions, washed microsomes (20 μl) were preincubated with assay buffer (70 μl ) for 5 min, and

reactions were started by addition of substrate, [methyl-$^{14}$C]-SAM co-substrate (7.15 nmol, 93.6 nCi) and 120 nmol of NADPH, to give a total reaction volume of 120 μl. Five to 10 microlitres of recombinant COMT (1.25–2.5 pkat with caffeyl aldehyde (**11**) as substrate) was added to some assays for estimation of substrate/intermediate availability. The reactions were incubated at 30 °C and stopped after 30 min with 10 μl of 6 N HC1, and extracted with EtOAc (200 μl). The organic phase (75 μl) was used directly for TLC analysis, and a further 75 μl was taken to dryness and dissolved in MeOH for HPLC analysis. Products were separated by reversed phase HPLC as described below, monitoring by UV absorbance at 254 and 310 nm, and fractions were collected. The radioactivity in the fractions was determined by liquid scintillation counting, using the automatic quench compensation for $^{14}$C label counting on an LS 1701 scintillation counter (Beckman Instruments, Fullerton, CA).

### *4.6. Separation of monolignol pathway intermediates by reversed phase HPLC and TLC*

Organic extracts from enzyme assays were applied to an ODS reversed phase HPLC column (5-μm particle size, 4.6 × 250 mm; Metachem Technologies, Inc., Torrance, CA) and eluted in 1% phosphoric acid with an increasing acetonitrile concentration gradient (0–5 min, 5% [v/v] acetonitrile; 5–10 min, 5–10% acetonitrile; 10–25 min, 10 to 17% acetonitrile; 25–65 min, 17–19% acetonitrile; 65–74 min, 100% acetonitrile) at a constant flow rate of 1 ml min$^{-1}$). UV absorbance was monitored with a photodiode array detector (Hewlett Packard, Waldbronn, Germany).

For TLC analysis, organic extracts were subjected to TLC on silica gel plates. The plates were developed with EtOAc: hexane (2:1), dried, and exposed overnight in a phosphorimager (Molecular Dynamics, Storm 820). For improved separation of 5-hydroxyconiferaldehyde (**10**) and coniferyl alcohol (**14**), the solvent was changed to EtOAc: hexane (3:2).

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## References

Atanassova, R., Favet, N., Martz, F., Chabbert, B., Tollie, M.-T., Monties, B., Fritig, B., Legrand, M., 1995. Altered lignin composition in transgenic tobacco expressing *O*-methyltransferase sequences in sense and antisense orientation. Plant J. 8, 465–477.

Bolwell, G.P., Butt, V.S., 1983. Photoinduced changes in *o*-diphenol oxidase and *p*-coumarate hydroxylase activities in spinach beet seedlings and leaves. Phytochemistry 22, 37–45.

Chapple, C., 1998. Molecular-genetic analysis of plant cytochrome P450-dependent monooxygenases. Annu. Rev. Plant Physiol. Plant Mo.l Biol. 49, 311–343.

Chen, F., Kota, P., Blount, J.W., Dixon, R.A., 2001. Chemical synthesis of caffeoyl and 5-OH coniferyl aldehydes and alcohols and the determination of lignin *O*-methyltransferase activities in dicot and monocot species. Phytochemistry 58, 1035–1042.

Dixon, R.A., Chen, F., Guo, D., Parvathi, K., 2001. The biosynthesis of monolignols: a "metabolic grid", or independent pathways to guaiacyl and syringyl units? Phytochemistry 57, 1069–1084.

Franke, R., Humphreys, J.M., Hemm, M.R., Denault, J.W., Ruegger, M.O., Chapple, C., 2002. The Arabidopsis *REF8* gene encodes the 3-hydroxylase of phenylpropanoid metabolism. Plant J. 30, 33–45.

Goffner, D., Campbell, M.M., Campargue, C., Clastre, M., Borderies, G., Boudet, A., Boudet, A.M., 1994. Purification and characterization of cinnamoyl-coenzyme A: NADP oxidoreductase in *Eucalyptus gunnii*. Plant Physiol. 106, 625–632.

Gowri, G., Bugos, R.C., Campbell, W.H., Maxwell, C.A., Dixon, R.A., 1991. Stress responses in alfalfa (*Medicago sativa* L.) X. Molecular cloning and expression of S-adenosyl-L-methionine: caffeic acid 3-*O*-methyltransferase, a key enzyme of lignin biosynthesis. Plant Physiol. 97, 7–14.

Grimmig, B., Kneusel, R.E., Junghanns, K.T., Matern, U., 1999. Expression of bifunctional caffeoyl-CoA 3-*O*-methyltranferase in stress compensation and lignification. Plant Biol. 1, 299–310.

Guo, D., Chen, F., Inoue, K., Blount, J.W., Dixon, R.A., 2000. Down-regulation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in transgenic alfalfa (*Medicago sativa* L.): impacts on lignin structure and implications for the biosynthesis of G and S lignin. Plant Cell 13, 73–88.

Heller, W., Kühnl, T., 1985. Elicitor induction of a microsomal 5-*O*-(4-coumaroyl)shikimate 3′-hydroxylase in parsley cell suspension cultures. Arch. Biochem. Biophys. 241, 453–460.

Hrazdina, G., Wagne, G.J., 1985a. Compartmentation of plant phenolic compounds; sites of synthesis and accumulation. Annu. Proc. Phytochem. Soc. Eur 25, 119–133.

Hrazdina, G., Wagner, G.J., 1985b. Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes. Arch. Biochem. Biophys. 237, 88–100.

Humphreys, J.M., Chapple, C., 2002. Rewriting the lignin roadmap. Current Opinion Plant Biol. 5, 224–229.

Humphreys, J.M., Hemm, M., 1999. R, Chapple, C. New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proc. Natl. Acad. Sci. USA 96, 10045–10050.

Inoue, K., Sewalt, V.J.H., Balance, G.M., Ni, W., Stürzer, C., Dixon, R.A., 1998. Developmental expression and substrate specificities of alfalfa caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in relation to lignification. Plant Physiol. 117, 761–770.

Kersey, R., Inoue, K., Schubert, K.R., Dixon, R.A., 1999. Immunolocalization of two lignin *O*-methyltransferases in stems of alfalfa (*Medicago sativa* L.). Protoplasma 209, 46–57.

Kojima, M., Takeuchi, W., 1989. Detection and characterization of *p*-coumaric acid hydroxylase in mung bean, *Vigna mungo*, seedlings. J. Biochem. 105, 265–270.

Lewis, N.G., Davin, L.B., Sarkanen, S., 1999. The nature and function of lignins. In: Barton, D.H.R. (Ed.), Comprehensive Natural Products Chemistry, Vol. 3. Elsevier, Oxford, pp. 617–745.

Li, L., Cheng, X.F., Leshkevich, J., Umezawa, T., Harding, S.A., Chiang, V.L., 2001. The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell 13, 1567–1585.

Liu, C.-J., He, X.-Z., Dixon, R.A., 2001. Elicitor-induced association of isoflavone *O*-methyltransferase with endomembranes prevents formation and 7-*O*-methylation of daidzein during isoflavonoid phytoalexin biosynthesis. Plant Cell 13, 2643–2658.

Luderitz, T., Grisebach, H., 1981. Enzymic synthesis of lignin precursors. Comparison of cinnamoyl-CoA reductase and cinnamoyl alcohol: NADP+ dehydrogenase fron spruce (*Picea abies* L.) and soybean (*Glycine max* L.). Eur. J. Biochem. 119, 115–124.

Meyer, K., Cusumano, J.C., Somerville, C., Chapple, C.C.S., 1996. Ferulate-5-hydroxylase from *Arabidopsis thaliana* defines a new family of cytochrome P450-dependent monooxygenases. Proc. Natl. Acad. Sci. USA 93, 6869–6874.

Neish, A.C., 1968. Monomeric intermediates in the biosynthesis of lignin. In: Freudenberg, K., Neish, A.C. (Eds.), Constitution and Biosynthesis of Lignin. Springer-Verlag, Berlin, pp. 2–43.

Osakabe, K., Tsao, C.C., Li, L., Popko, J.L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi, C.P., Chiang, V.L., 1999. Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proc. Natl. Acad. Sci. USA 96, 8955–8960.

Parvathi, K., Chen, F., Guo, D., Blount, J.W., Dixon, R.A., 2001. Substrate preferences of *O*-methyltransferases in alfalfa suggest new pathways for 3-*O*-methylation of monolignols. Plant J. 25, 193–202.

Pierrel, M.A., Batard, Y., Kazmaier, M., Mignotte-Vieux, C., Durst, F., Werck-Reichhart, D., 1994. Catalytic properties of the plant cytochrome P450 CYP73 expressed in yeast. Eur. J. Biochem. 224, 835–844.

Quackenbush, J., Liang, F., Holt, I., Pertea, G., Upton, J., 2000. The TIGR gene indices: reconstruction and representation of expressed gene sequences. Nucleic Acids Res. 28, 141–145.

Rasmussen, S., Dixon, R.A., 1999. Transgene-mediated and elicitor-induced perturbation of metabolic channeling at the entry point into the phenylpropanoid pathway. Plant Cell 11, 1537–1551.

Sarni, R., Grand, C., Boudet, A.M., 1984. Purification and properties of cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase from poplar stems (*Populus* X *euramericana*). Eur. J. Biochem. 139, 259–265.

Schoch, G., Goepfert, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., Werck-Reichart, D., 2001. CYP98A3 from *Arabidopsis thaliana* is a 3′-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J. Biol. Chem. 276, 36566–36574.

Shimada, M., Ohashi, H., Higuchi, T., 1970. *O*-Methyltransferases involved in the biosynthesis of lignins. Phytochemistry 9, 2463–2470.

Srere, P.A., 1987. Complexes of sequential metabolic enzymes. Annu. Rev. Biochem. 56, 89–124.

Wagner, G.J., Hrazdina, G., 1984. Endoplasmic reticulum as a site of phenylpropanoid and flavonoid metabolism in*Hippeastrum*. Plant Physiol. 74, 901–906.

Wengenmayer, H., Ebel, J., Grisebach, H., 1976. Enzymic synthesis of lignin precursors. Purification and properties of a cinnamoyl-CoA: NADPH reductase from cell suspension cultures of soybean (*Glycine max*). Eur. J. Biochem. 65, 529–536.

Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126, 485–493.

Winkel-Shirley, B., 1999. Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. Physiol. Plant 107, 142–149.

Ye, Z.H., Kneusel, R.E., Matern, U., Varner, J.E., 1994. An alternative methylation pathway in lignin biosynthesis in *Zinnia*. Plant Cell 6, 1427–1439.

Zhong, R., Morrison, I.W.H., Negrel, J., Ye, Z.H., 1998. Dual methylation pathways in lignin biosynthesis. Plant Cell 10, 2033–2045.