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Chemical syntheses of caffeoyl and 5-OH coniferyl aldehydes and alcohols and determination of lignin *O*-methyltransferase activities in dicot and monocot species

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Abstract

To investigate the substrate preferences of *O*-methyltransferases in the monolignol biosynthetic pathways, caffeoyl and 5-hydroxy coniferyl aldehydes were synthesized by a new procedure involving a Wittig reaction with the corresponding hydroxybenzaldehydes. The same procedure can also be used to synthesize caffeoyl and 5-hydroxyconiferyl alcohols. Relative *O*-methyltransferase activities against these substrates were determined using crude extracts and recombinant caffeic acid *O*-methyltransferase from alfalfa (*Medicago sativa*), and crude extracts from the model legume *Medicago truncatula*, tobacco, wheat and tall fescue. Extracts from all these species catalyzed methylation of the various monolignol aldehydes and alcohols more effectively than the corresponding hydroxycinnamic acids.

Keywords: *Medicago sativa*; *Medicago truncatula*; *Nicotiana tabacum*; *Triticum aestivum*; *Festuca arundinacea*; Leguminosae; Solanaceae; Gramineae; Syntheses; Caffeoyl aldehyde; 5-Hydroxyconiferaldehyde; *O*-Methyltransferase; Lignin

1. Introduction

The current concept of monolignol biosynthesis is a metabolic grid, through which side chain reduction and ring hydroxylation/methylation can occur at several different levels (reviewed in Lewis and Yamamoto 1990, Whetten and Sederoff 1995, Dixon et al., 2001). This concept was first proposed following the discovery of apparently independent pathways for monolignol *O*-methylation at the levels of hydroxycinnamic acids and their coenzyme A esters, catalyzed by caffeic acid *O*-methyltransferase (COMT) and caffeoyl CoA *O*-methyltransferase (CCoAOMT), respectively (Ye et al., 1994; Higuchi 1996). However, in recent years, increasing evidence has pointed to the hydroxylation/methylation of cinnamyl aldehydes or cinnamyl alcohols in lignin biosynthesis (Chen et al., 1999; Osakabe et al., 1999; Li et al., 2000).

Our recent studies (Guo et al., 2001) have centered on genetic manipulation of the *O*-methylation reactions of guaiacyl (G) and syringyl (S) lignin biosynthesis in alfalfa (*Medicago sativa*). Alfalfa is the world's major forage legume and a species in which lignification has important implications for forage digestibility and therefore utilization efficiency (Jung et al., 1997). To fully understand the opportunities for, and consequences of, genetically modifying lignin *O*-methyltransferases (OMTs), it is important to determine relative OMT activities against all potential substrates in the monolignol pathway. Unfortunately, caffeoyl- and 5-hydroxyconiferyl aldehydes and alcohols are not commercially available, and present some challenges in synthesis due to their *o*-dihydroxy ring substitution. In the past, 4-hydroxycinnamyl alcohol was synthesized by reducing 4-hydroxycinnamic acid esters with lithium aluminum hydride or diisobutylaluminum hydride (DIBALH) (Quideau and Ralph, 1992; Terashima et al., 1995). 4-Hydroxycinnamaldehyde was obtained by reduction of cinnamic acid ester or chloride followed by oxidation of the alcohol by manganese dioxide. These protocols involve many reaction steps and separations, leading to poor yields and difficulties with purification. Daubresse et al. (1994) developed a mild protocol to synthesize coumaryl, coniferyl, and sinapyl aldehydes and alcohols, performed under phase-transfer conditions.

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We tried to use this procedure for synthesis of caffeoyl aldehyde and 5-hydroxyconiferaldehyde but without success. Nevertheless, the idea of using a Wittig reaction (McMurry, 2000) with (1,3 - dioxolan - 2 - yl) - methyltriphenylphosphonium bromide inspired us to develop a new procedure for synthesis of these compounds. We have found that 18-crown-6 can effectively catalyze the Wittig reaction and give good yields for synthesis of monolignol precursors. We here describe the synthetic procedures, and the use of caffeoyl and 5-hydroxyconiferyl aldehydes and alcohols to determine substrate preferences for OMTs in extracts from several plant species.

2. Results and discussion

2.1. New synthetic approaches for hydroxycinnamyl aldehydes and alcohols

To investigate fully the monolignol biosynthetic pathway, it is a prerequisite to obtain all potential substrates in the pathway. Caffeoyl aldehyde, caffeoyl alcohol, 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol are all potential substrates for monolignol *O*-methyltransferase but are not commercially available at this time. In recent reports, 5-hydroxyconiferaldehyde was prepared from 5-hydroxyvanillin (Osakabe et al., 1999). This protocol demands several steps of reaction and purification (Fig. 1), and gives only moderate overall yield (reported to be around 25%). Daubresse et al. (1994) have described mild syntheses of cinnamyl aldehydes and alcohols from 4-acetoxybenzaldehyde. The key step for this procedure is a Wittig reaction with (1,3-dioxolan-2-yl-methyl)-triphenylphosphonium bromide catalyzed by tris-[2-(2-methoxyethoxy)-ethyl]amine (TDA-1). We therefore attempted to prepare caffeoyl aldehyde and 5-hydroxyconiferaldehyde using this procedure. However, in our hands, the yields of dioxolanes obtained from 3,4-diacetoxybenzaldehyde and 3,4-diacetoxy-5 methoxybenzaldehyde were too low and the predominant products of the reaction were the corresponding piperonals even if the phenolic groups were already acetylated. To exploit the convenience of synthesis of cinnamyl aldehydes and alcohols with (1,3-dioxolan-2-yl-methyl)-triphenylphosphonium bromide, we tried two alternative catalysts for Wittig reactions, 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) and tetrabutylammonium bromide, and found that 18-crown-6 most efficiently catalyzed the reactions with 3,4-diacetoxy-5-methoxybenzaldehyde and 3,4-diacetoxybenzaldehyde. The new procedure (Fig. 2) described here requires fewer steps than previous methods, can be

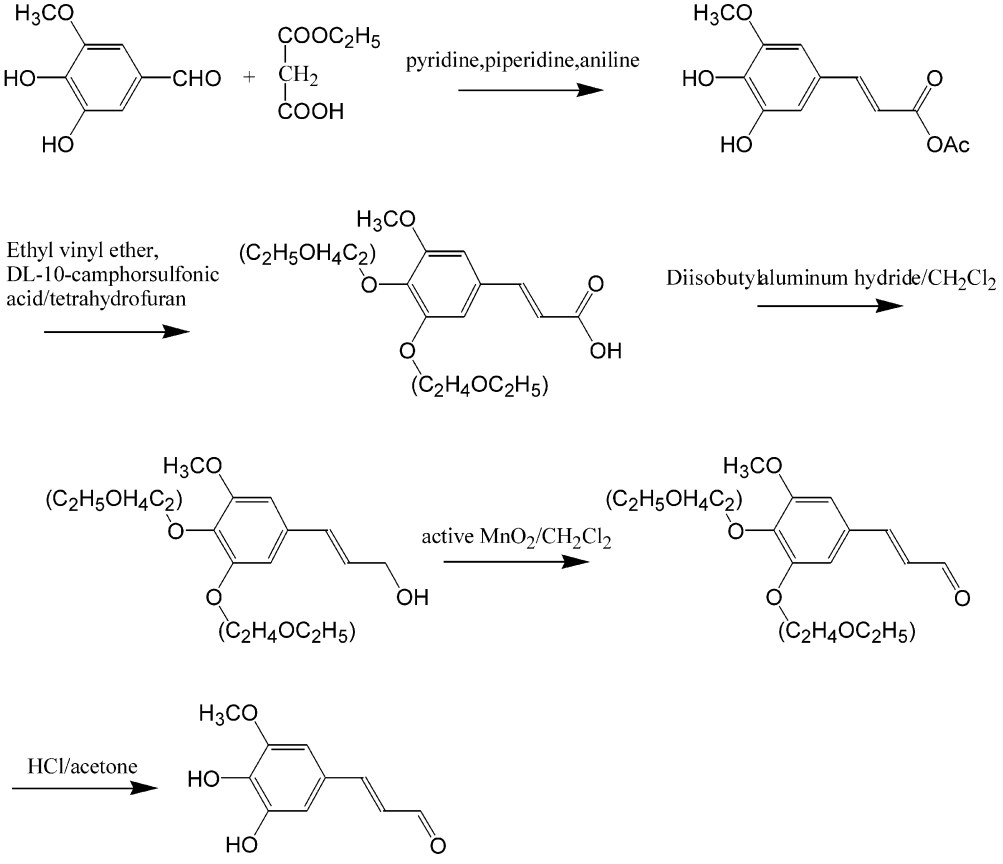

Fig. 1. Synthesis of 5-hydroxyconiferaldehyde from 5-hydroxyvanillin and monoethyl malonate.

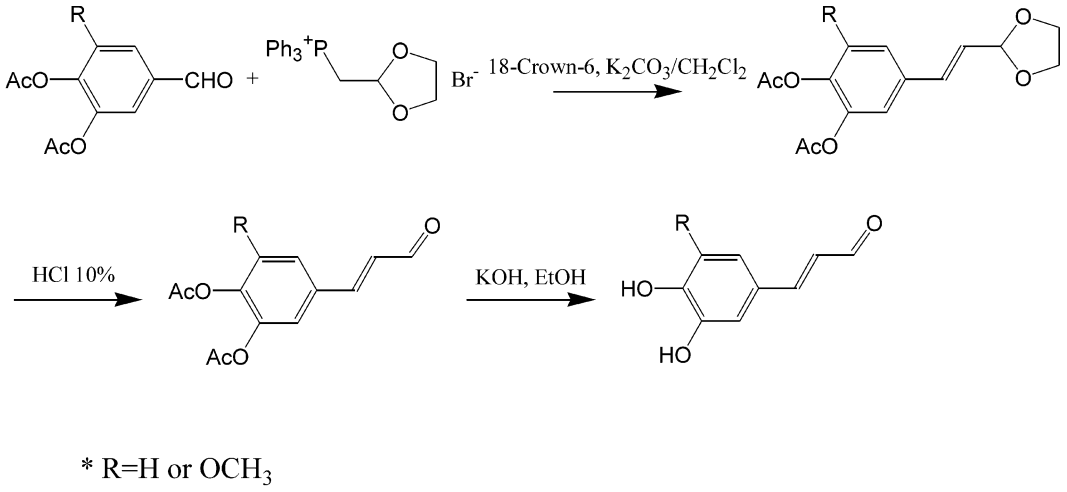

Fig. 2. Synthesis of caffeoyl and 5-hydroxyconiferyl aldehydes by a Wittig reaction with the corresponding hydroxybenzaldehydes and (1,3-dioxolan-2-yl-methyl)- triphenylphosphonium bromide.

performed in ambient atmosphere, and gives higher yields than previous methods (50–60% being routine in our hands).

3,4 - Dihydroxybenzaldehyde and 3,4 - dihydroxy - 5 - methoxybenzaldehyde can be acetylated at both phenolic hydroxyls using acetic anhydride under alkaline conditions. The acetylated products were then mixed with (1,3-dioxolan-2-yl-methyl)- triphenylphosphonium bromide, 18-crown-6 and solid K_2CO_3 in dry dichloromethane to produce the corresponding dioxolanes. The process can easily be monitored by GC/MS in one-hour intervals to ensure the maximum yield. Dioxolanes are obtained as isomers with a Z/E ratio of about 60/40, as described by Daubresse et al. (1994). After removal of the solid phase by filtration, the mixture can be hydrolyzed without further purification. Acid hydrolysis of the dioxolanes leads to nearly pure (E)-acetylated aldehydes as revealed by GC/MS. After purification of the 3,4-diacetoxyconiferyl aldehyde by column chromatography, the acetyl groups can be removed by transesterification in absolute EtOH. Fig. 3 shows the mass spectra of the resulting caffeoyl aldehyde and 5-hydroxyconiferaldehyde after trimethylsilylation. Methoxime derivatization of the products was also conducted to confirm the presence of the aldehyde groups. Fig. 4 shows the mass spectra of caffeoyl aldehyde and 5-hydroxyconiferaldehyde after methoximation and trimethylsilylation.

Caffeoyl alcohol and 5-hydroxyconiferyl alcohol can be obtained by condensation of 3,4-dihydroxybenzaldehyde or 5-hydroxyvanillin, respectively, with monoethyl malonate according to the method of Umezawa et al. (1991). However, the yield for these compounds (around 20%) is much lower than that reported for the synthesis of coniferyl alcohol (around 50%). With the procedure described by Daubresse et al. (1994), we were able to synthesize caffeoyl alcohol and 5-hydroxyconiferyl alcohol from 3,4-diacetoxycaffeoyl aldehyde and 3,4-diacetoxyconiferaldehyde with a yield of approximately 70%. Fig. 5 shows the mass spectra of the obtained products. The results reported here show that a Wittig reaction with (1,3-dioxolan-2-yl-methyl)-triphenylphosphonium bromide is a relatively straightforward method for synthesis of caffeoyl and 5-hydroxyconiferyl aldehydes and alcohols in good yield. The method can be performed with limited demands on synthetic expertise.

2.2. Substrate preferences of O-methyltransferases in dicots and monocots

To determine if the various hydroxycinnamyl aldehydes and alcohols were effective substrates for alfalfa COMT, the alfalfa COMT cDNA (Inoue et al., 1998) was cloned into the pET15b vector and expressed in *E. coli*. The His-tagged recombinant COMT enzyme was purified to homogeneity from the soluble protein using nickel affinity chromatography. Enzyme assays were conducted with the usual substrates, caffeic acid, 5-hydroxyferulic acid, and caffeoyl and 5-hydroxy-coniferyl aldehydes and alcohols. HPLC/diode-array analyses with parallel radio-detection showed that all these compounds acted as substrates for recombinant alfalfa COMT and gave the predicted products (Parvathi et al., 2001). The relative activites are summarized in Table 1. These results provide in vitro evidence for a potential alternative pathway to monolignols involving methylation of hydroxycinnamyl aldehydes and alcohols.

To determine whether OMT activity against all the hydroxycinnamyl aldehydes and alcohols was also found in other plant species, we assayed crude extracts from alfalfa, *Medicago truncatula* (a close relative of alfalfa), tobacco, wheat and tall fescue. The results (Table 1) indicate that, in each case, all substrates were effectively methylated, and, in each case, caffeic acid was

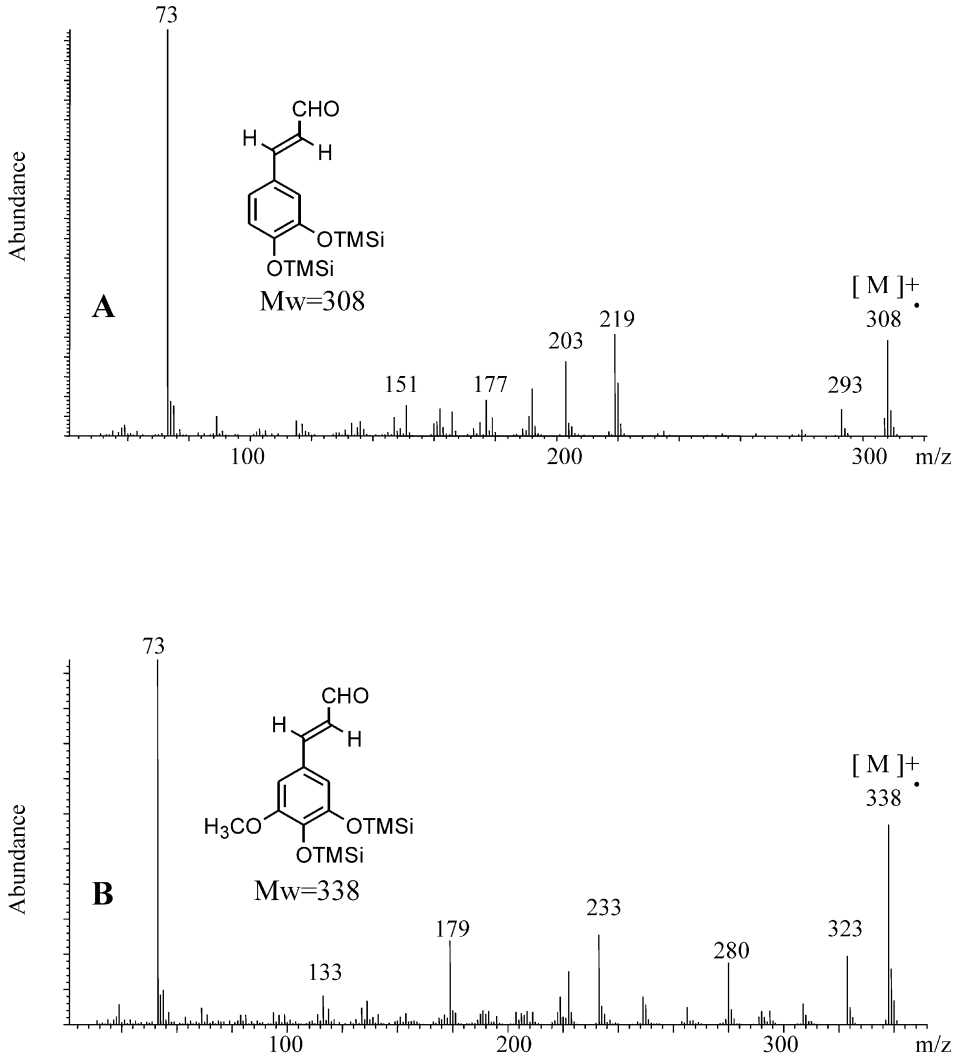

Fig. 3. Mass spectra of caffeoyl aldehyde (**A**) and 5-hydroxyconiferaldehyde (**B**) after TMSi derivatization.

the poorest substrate. In alfalfa, *M. truncatula*, tobacco and fescue, 5-hydroxyferulic acid, caffeoyl aldehyde, caffeoyl alcohol and 5-hydroxyconiferyl alcohol were methylated much more effectively than caffeic acid. In contrast, 5-hydroxyconiferaldehyde was methylated with about the same efficiency as caffeic acid in extracts from alfalfa and *M. truncatula*, whereas this compound was as good a substrate as the other aldehydes and alcohols in extracts from wheat. There were no obvious differences in substrate preferences between monocots and dicots in this experiment (compare alfalfa and tall fescue).

Recently, some OMTs have been found to have an unexpectedly broad range of substrates and even to exhibit functional overlap between different biosynthetic pathways. For example, a series of 38 potential substrates have been tested with four recombinant OMTs of *Thalictrum tuberosum* (Frick and Kutchan, 1999; Frick et al., 2001). Some of the enzymes showed similar catalytic activity with catechol, caffeic acid and the alkaloid norcoclaurine. It is not clear whether these enzymes are also active with caffeoyl aldehyde or 5-hydroxyconiferaldehyde, although this would not appear unlikely. To fully address OMT substrate preferences in vivo, or the potential activities of the large number of OMTs now appearing in plant gene EST databases or being identified from proteomic analyses (Van der Mijnsbrugge et al., 2000; Bell et al., 2001), even broader ranges of substrates will be needed. The relatively facile synthetic procedure reported here will make such substrates more widely available.

3. Experimental

3.1. Plant material, enzymes and chemicals

All plants used in this study were grown under standard greenhouse conditions. Stem tissues were collected and ground under liquid N_2. The powdered tissues were

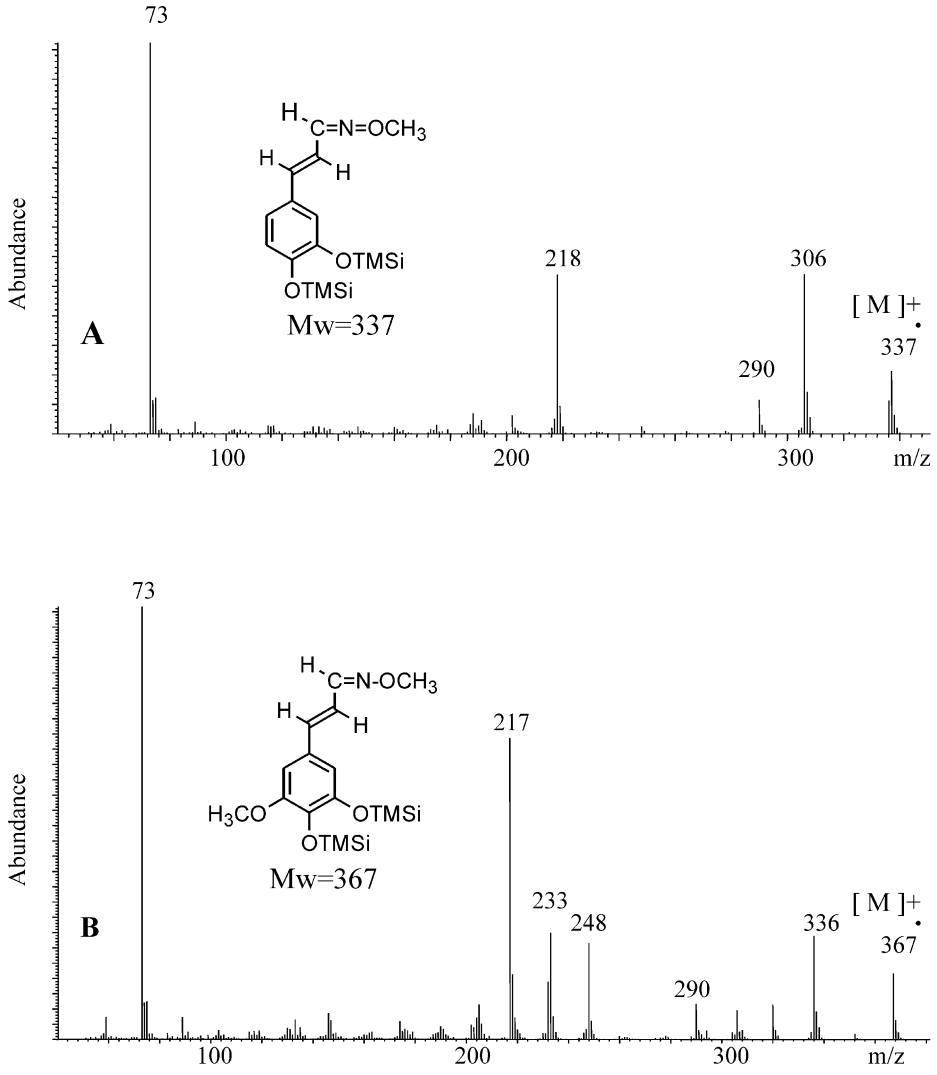

Fig. 4. Mass spectra of caffeoyl aldehyde (**A**) and 5-hydroxyconiferaldehyde (**B**) after methoxime derivatization and TMSi derivatization.

extracted and the soluble proteins were used for enzyme assay (Parvathi et al., 2001). Expression of alfalfa COMT in *E. coli* was performed as described previously (Parvathi et al., 2001). The enzyme was extracted from the *E. coli* cells by sonication and purified from the soluble fraction using His.bind resin (Novagen, Madison, WI, USA) according to the manufacturer's protocol. 3,4-Dihydroxybenzaldehyde, 5-hydroxy vanillin, (1,3-dixoxolan-2-yl-methyl)-triphenylphosphosphonium bromide, 18-crown-6, acetic anhydride, and ethyl acetate were purchased from Aldrich-Sigma Co (St. Louis, MO, USA) and used without further purification.

3.2. Synthesis procedures

3,4-Diacetoxy-3-methoxybenzaldehyde (5 mmol) and (1,3-dioxolan-2-yl-methyl)-triphenylphosphosphonium bromide (5 mmol) were dissolved in CH_2Cl_2 (80 ml) with vigorous stirring. Solid K_2CO_3 (5 mmol) and 18-crown-6 (0.05 mmol) were added. The reaction mixture was kept at room temperature for 8 h. The organic phase was separated from the solid phase by filtration. Aqueous HCl (10%, 50 ml) was added to the organic portion and the mixture stirred at room temperature for a further 6 h. During this period, a small amount of organic phase was taken and subjected to GC/MS analysis. After 2 h, 3,4-diacetoxyconiferaldehyde could be detected (MS *m*/*z*: 278, 236, 194, 177, 166, 151). At the end of the reaction, the mixture was diluted with 50 ml H_2O and extracted three times with 100 ml CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ and saturated aqueous NaCl solution successively, dried over $MgSO_4$ and evaporated under vacuum. The residue was dissolved in a small amount of CH_2Cl_2 and passed through a silica gel column (eluted with CH_2Cl_2: EtOAc, 90:10, v/v). The eluate was monitored by TLC and the portions containing 3,4-diacetoxyconiferaldehyde were pooled. After evaporation of

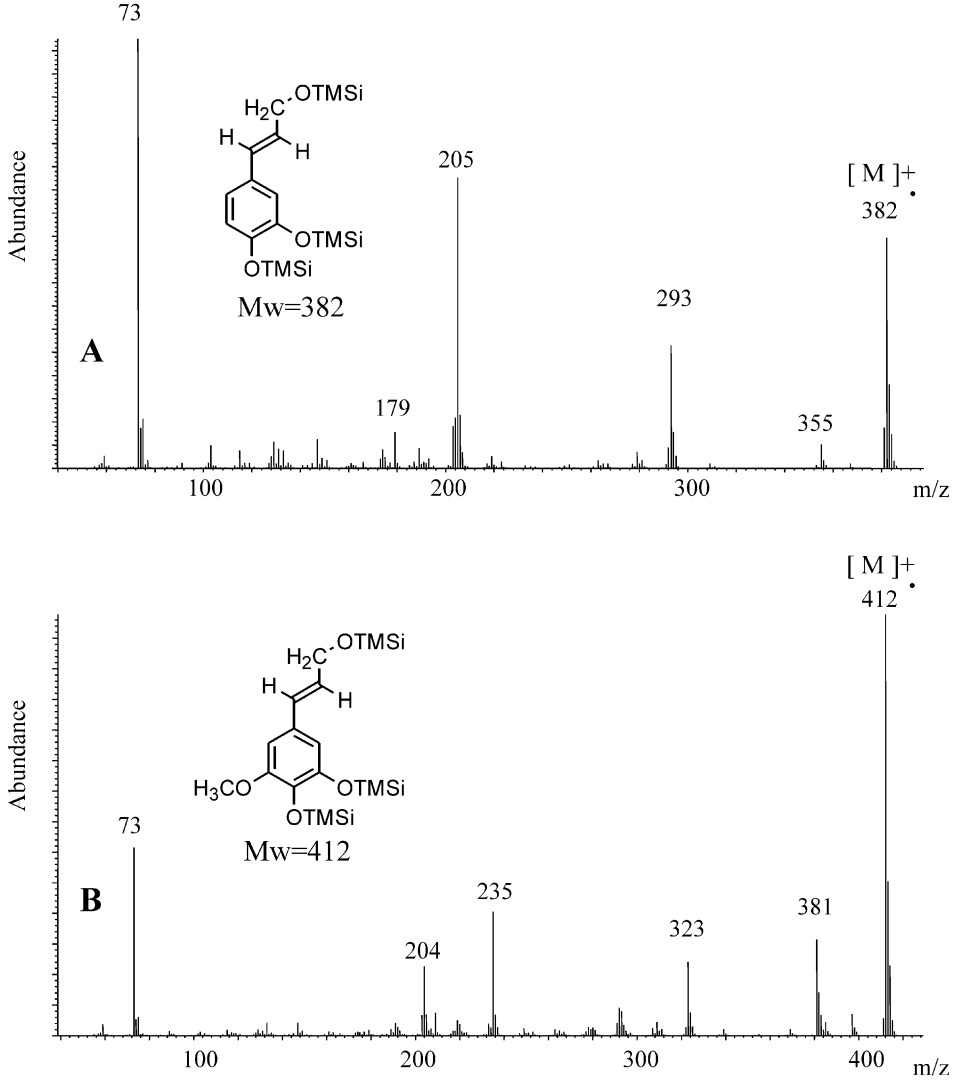

Fig. 5. Mass spectra of caffeoyl alcohol (**A**) and 5-hydroxyconiferyl alcohol (**B**) after TMSi derivatization.

Table 1
Methylation of a range of potential monolignol precursors by purified recombinant alfalfa COMT and crude preparations from alfalfa and four other species[a]

Substrate (50 μM)	OMT activity (pkat mg^{-1} protein)					
	Recombinant alfalfa COMT	Alfalfa	*M. truncatula*	Tobacco	Wheat	Tall fescue
Caffeic acid	2989±105	4.9±0.3	6.4±0.1	1.6±0.1	0.7±0.04	2.6±0.3
5-OH Ferulic acid	9235±110	15.0±0.9	14.7 ±1.5	20.3±1.3	5.8±0.3	31.3±1.3
Caffeoyl aldehyde	11408±725	14.4±0.9	13.3±0.7	20.1±0.4	5.9±0.1	24.0±0.5
Caffeoyl alcohol	17286±364	24.1±0.4	19.1±0.6	23.8±1.7	4.6±0.3	28.4±1.1
5-OH Coniferaldehyde	1655±138	5.2±0.3	6.9±0.5	5.9±0.1	5.7±0.1	9.4±0.9
5-OH Coniferyl alcohol	5965±624	13.0±0.8	16.0±0.7	7.6±0.2	4.2±0.1	26.3±2.0

Data represent the mean and spread of values for two replicate assays.

[a] Crude enzyme extracts from alfalfa, *M. truncatula* and tobacco were prepared from the 6–9th internodes, the extract from wheat was from the pseudo stem and leaf sheath, and the extract from tall fescue was from the 1st–4th stem internodes.

the solvent, 3,4-diacetoxyconiferaldehyde was obtained as a white solid (1.05 g, 72% yield). The 3,4-diacetoxyconiferaldehyde (0.56 g) was dissolved in 100 ml of 0.2 M KOH in 95% EtOH, and stirred for 8 h under N_2. The solvent was removed under vacuum, and the mixture diluted with 50 ml H_2O and extracted with ethyl

acetate (50 ml ×3). The combined organic layers were dried over Na_2SO_4 and evaporated to give 5-OH coniferaldehyde (0.29 g).

5-Hydroxyconiferyl alcohol was synthesized from 3,4-diacetoxyconiferaldehyde (70% yield) according to the procedure described by Daubresse et al. (1994). Caffeoyl aldehyde and alcohol were synthesized using the same procedure starting from 3,4-diacetoxybenzaldehyde with overall yield of 49–53%.

3.3. Derivatization for GC/MS analysis

Methoxime derivatization of the carbonyl groups of caffeoyl aldehyde and 5-hydroxyconiferaldehyde was performed using 0.5 ml MOX reagent (Pierce, Rockford, IL, USA) at 60 °C for 3 h, followed by evaporation to dryness with N_2. TMSi derivatization of the synthesized products and methoxime derivatives was performed using 250 μl MSTFA reagent (Pierce, Rockford, IL, USA) at 30 °C for 2 h.

3.4. Gas chromatography/ mass spectrometry

The synthesized products were identified by GC/MS. The GC/MS was performed on a Hewlett 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). The mass spectra were obtained using electron ionization (70 eV) with a 60 to 650 m/z scanning range.

3.5. Enzyme extraction and assay

Stem internodes from alfalfa were collected and ground under liquid N_2. The powdered tissue was extracted for 1 h at 4 °C in extraction buffer (100 mM Tris–HCl, pH 7.5, 0.2 mM $MgCl_2$, 2mM DTT and 10% glycerol). The samples were then centrifuged at 12,000 g for 10 min at 4 °C and the extracts desalted on PD-10 columns (Pharmacia). The soluble protein fractions were used for COMT enzyme assay. The protein concentration was measured using Bradford's reagent (Bio-Rad) with BSA as standard (Bradford, 1976). COMT activity was assayed as described previously (Gowri et al., 1991, Inoue et al., 1998).

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