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Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase

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Abstract

Isolated lignins from alfalfa deficient in caffeic acid 3-*O*-methyltransferase contained benzodioxanes resulting from the incorporation of the novel monomer, 5-hydroxyconiferyl alcohol. Due to the high level incorporated into the soluble lignin fraction and the use of sensitive NMR instrumentation, unique structural features were revealed. A new type of end-unit, the 5-hydroxyguaiacyl glycerol unit, was identified. It was possible to establish that coniferyl alcohol, sinapyl alcohol, and the novel 5-hydroxyconiferyl alcohol can cross-couple with the 5-hydroxyguaiacyl units that are formed in the lignin, the latter giving rise to extended chains of benzodioxane units. There is also evidence that 5-hydroxyconiferyl alcohol couples with normal (guaiacyl or syringyl) lignin units. Lignin in the alfalfa deficient in caffeoyl CoA 3-*O*-methyltransferase was structurally similar to the control lignin but the transgenic exhibited a dramatic decrease in lignin content (~20%) and modest increase in cellulose (~10%) reflecting a 30% increase in cellulose:lignin ratio. The compositional changes in both transgenics potentially allow enhanced utilization of alfalfa as a major forage crop by increasing the digestibility of its stem fraction.
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Keywords: Alfalfa (*Medicago sativa* L.); Fabaceae; Lignin; *O*-Methyltransferase; Benzodioxane; 5-Hydroxyguaiacyl glycerol; NMR; Transgenic plants

1. Introduction

Alfalfa (*Medicago sativa* L.), an herbaceous perennial, is a leading forage crop. It has a high nutritive value because it is rich in protein, minerals, and vitamins, and if harvested prior to flowering can retain a low fiber and high energy content. The intake and digestibility of forage by dairy animals directly affect their production of meat and milk. A reduction in feeding value results from a lower leaf to stem ratio and the deposition of lignin and polysaccharides in stem cell walls during maturation (Buxton and Hornstein, 1986; Poehlman and Sleper, 1995).

In legumes such as alfalfa, the lignin precursors coniferyl (4-hydroxy-3-methoxycinnamyl) $\mathbf{1_G}$ and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohol $\mathbf{1_S}$ give rise to guaiacyl (G) and syringyl (S) units in the lignin polymer. The formation of coniferyl and sinapyl alcohols requires the activity of *O*-methyltransferases (OMTs), which methylate the 3-hydroxyl of 3,4-dihydroxyphenyl units and the 5-hydroxyl of 5-hydroxyguaiacyl units (Fig. 1). In angiosperms, caffeic acid 3-*O*-methyltransferase (COMT; EC 2.1.1.6) was originally thought to be bifunctional, converting caffeic acid to ferulic acid and 5-hydroxyferulic acid to sinapic acid (Higuchi, 1985; Davin and Lewis, 1992). However, analysis of transgenic tobacco and poplar with suppressed COMT activity has shown that COMT is mainly responsible for the biosynthesis of S monomers (Atanassova et al., 1995; Van Doorsselaere et al., 1995; Tsai et al., 1998;

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Lapierre et al., 1999), and 5-hydroxyconiferyl aldehyde has been demonstrated to be a preferred substrate, at least in vitro (Osakabe et al., 1999). The highlighted pathway (Fig. 1) with hydroxylation and methylation occurring preferentially at the aldehyde level is now considered a major in vivo pathway (Humphreys et al., 1999; Osakabe et al., 1999). Methylation of the caffeate moiety occurs at the level of the CoA thiol ester, catalyzed by caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT; EC 2.1.1.104) (Pakusch et al., 1989; Schmitt et al., 1991). CCoAOMT has been suggested to be involved in a parallel pathway to lignin monomer formation (Ye et al., 1994; Zhong et al., 1998) resulting in both G and S monomers. In wild-type alfalfa, CCoAOMT activity has been reported to be as high as COMT activity throughout stem development, with both enzymes preferentially expressed in the stem vascular tissues (Inoue et al., 1998). Recently, it has been suggested that the 3-*O*-methylation reaction might also take place at the level of caffeoyl aldehyde, a preferred substrate for alfalfa COMT in vitro (Parvathi et al., 2001).

Previous studies have examined the impact of COMT and CCoAOMT on lignin content in angiosperms. Depending on the plant species studied, variable changes resulting from a COMT-deficiency have been reported (Dwivedi et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Boudet et al., 1995; Van Doorsselaere et al., 1995; Sewalt et al., 1997; Lapierre et al., 1999; Jouanin et al., 2000; Guo et al., 2001a; Marita et al., 2001). CCoAOMT-deficient transgenics all display a general reduction in lignin content (Zhong et al., 1998, 2000; Meyermans et al., 2000; Guo et al., 2001a).

Hwang and Sakakibara (1981) first suggested that 5-hydroxyconiferyl alcohol $\mathbf{1_{5H}}$ could be incorporated into lignins after identifying a benzodioxane dimer in hydrogenolysis products from *Fraxinus mandshurica* Rupr. var. *japonica* Max. Later, 5-hydroxyguaiacyl monomers were discovered in thioacidolysis products from COMT-deficient *bm3* maize and transgenic poplar (Lapierre et al., 1988; Ralph et al., 2001a). More diagnostic dimeric benzodioxane products were recently identified in thioacidolyis and DFRC (derivatization followed by reductive cleavage) products (Ralph et al., 2000a; Lapierre et al., 2001a). Thioacidolysis partially cleaves benzodioxanes releasing small amounts of the 5-hydroxyguaiacyl monomers and the benzodioxane dimer. DFRC degradation leaves the benzodioxanes **2** (Fig. 2) intact and therefore does not release any monomers, but smaller amounts of the analogous benzodioxane dimers **3** can be released by cleaving such units that are surrounded by normal β-ether units in the polymer. The dimeric thioacidolysis or DFRC dimers

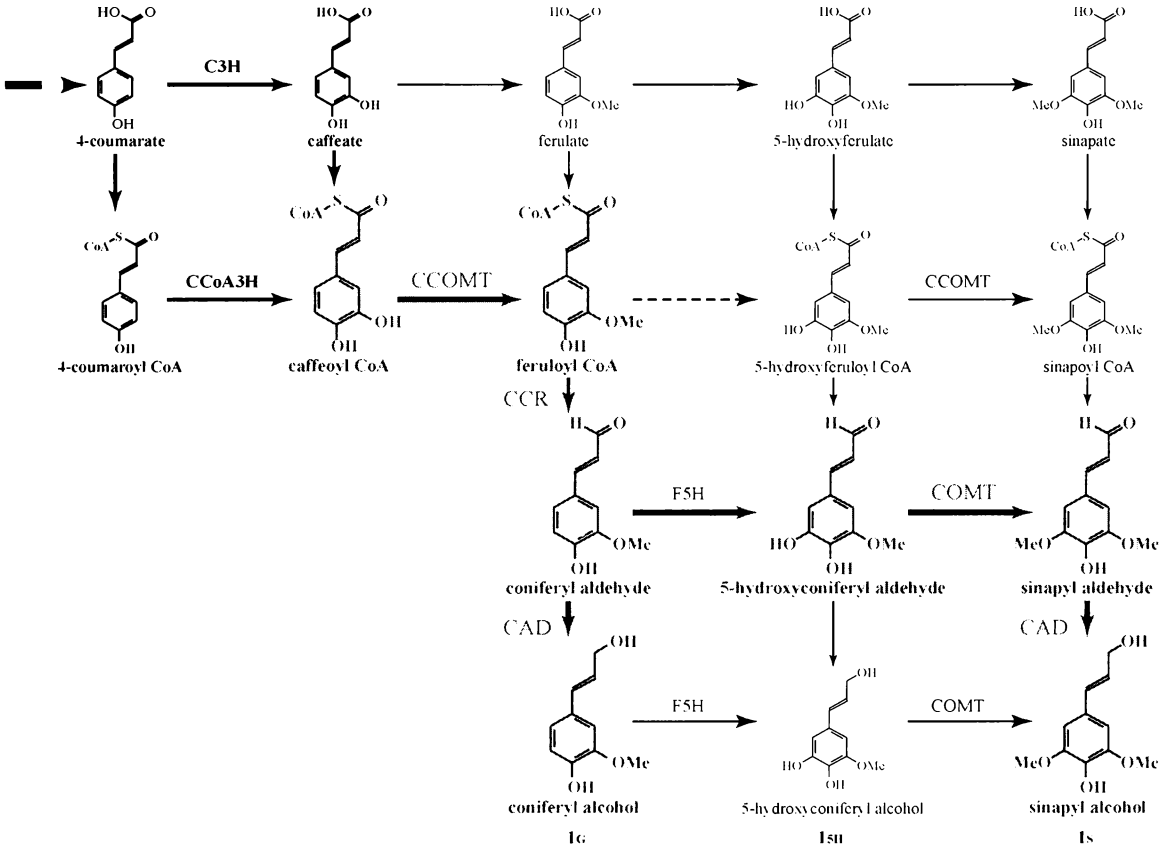

Fig. 1. The monolignol pathway in angiosperms. Bolder structures and arrows (with larger-diameter arrows and bolded structures) represent preferred pathways as recently clarified (Humphreys et al., 1999; Osakabe et al., 1999; Li et al., 2000).

reveal whether it was a guaiacyl or syringyl unit attached to the 5-hydroxyguaiacyl unit within the lignin and therefore whether coniferyl or sinapyl alcohol had added to the new 5-hydroxyguaiacyl end of the growing polymer. Recently, NMR studies have provided further evidence of the incorporation of 5-hydroxyconiferyl alcohol into lignins and revealed the occurrence of benzodioxane structures in the lignins of COMT-deficient poplar at significant levels (Marita et al., 2001; Ralph et al., 2001b, 2001c).

The present study examines alterations to lignin structure and composition resulting from independent down-regulation of COMT and CCoAOMT in alfalfa plants. Previous work has shown genetically modified alfalfa to have altered lignin composition (Guo et al., 2001a) and improved *in situ* digestibility (Guo et al., 2001b). The results of this study reveal new details of the incorporation of novel units in the lignin of COMT-deficient alfalfa including units not previously identified and an increase in the cellulose:lignin ratio in CCoAOMT-deficient alfalfa.

2. Results and discussion

NMR and DFRC methods were used to determine structural and compositional changes that could be attributed to the enzyme deficiencies in lignins from COMT- and CCoAOMT-down-regulated alfalfa. A previously examined COMT down-regulated hardwood incorporated novel benzodioxane units (Marita et al., 2001; Ralph et al., 2001b) into the lignin, but it was not clear whether these novel units would analogously occur in an herbaceous legume. A detailed structural analysis of the lignins and improved compositional data from both transgenics was therefore required.

2.1. COMT-deficient alfalfa

2.1.1. HMQC NMR spectra

2D NMR experiments (gradient-enhanced HMQC; Kay et al., 1992) allowed structural analysis of the major units in isolated lignin from alfalfa. In Fig. 3, the

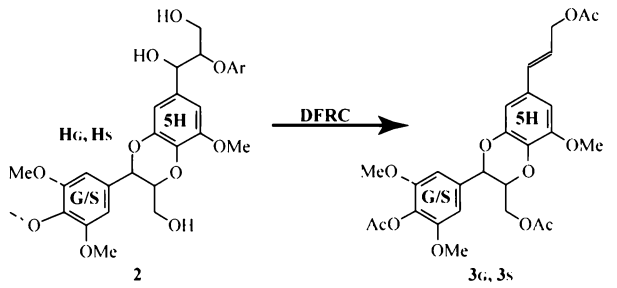

Fig. 2. Benzodioxanes flanked by normal β-ethers (structures of general type **2**) release diagnostic benzodioxane dimers, $\mathbf{3}_G$ and $\mathbf{3}_S$, following DFRC degradation.

HMQC data for the wild-type alfalfa lignin were similar to those for other non-transgenic dicot lignins with structural features being well resolved and readily recognized features (Marita et al., 1999; Ralph et al., 1999). As in previously examined COMT-deficient poplars (Marita et al., 2001; Ralph et al., 2001b), the COMT-deficiency in the alfalfa resulted in a lignin with a striking new feature, the appearance of benzodioxane units **H** which can, therefore, now be considered a signature for COMT-deficiency. Benzodioxanes **H** result from the incorporation of 5-hydroxyconiferyl alcohol into the lignin by typical endwise coupling reactions (Ralph et al., 1999). The relative **H**-unit levels in this spectrum, Fig. 3b, were far higher than in other lignins we have studied. As will be discussed later, the high level is due in part to a preferential partitioning of this structure into the soluble fraction analyzed by NMR. Other features of the spectrum, compared with the control, are a relative decrease in normal β-aryl ether units (β-*O*-4, **A**) and resinol units (β-β, **C**), and an increase in dibenzodioxocins (5–5/β-*O*-4, α-*O*-4, **D**).

Quantification of interunit type structures based on measuring volume integrals in the 2D HMQC spectra (Zhang and Gellerstedt, 2000; Marita et al., 2001; Ralph et al., 2001b) of wild-type and COMT-deficient alfalfa are presented in Table 1. Again it is striking that benzodioxane units **H** (38%) in the COMT-deficient alfalfa approach the level of the normal β-ether units **A** (44%). They are the second most abundant interunit type, far exceeding the levels of traditional units such as β-5 (**B**) and β-β (**C**). In the wild-type alfalfa, no benzodioxane units **H** were detected; the lignin was comprised of mainly β-ether units **A** (81%). The percentage of phenylcoumaran units **B** did not change as a result of COMT down-regulation. There was a decrease in resinol units **C** in the COMT-deficient alfalfa (3%) compared to wild-type (6%) presumably as a result of the depletion in syringyl units. Cinnamyl end-group **X** levels were similar (3–4%). Dibenzodioxocins **D**, which arise from coupling reactions of a monolignol with 5–5-coupled G-units, appeared to be elevated (from 1 to 4%). A higher **D** and a lower **C** unit content is consistent with a lower incorporation of sinapyl alcohol. It suggests that the lignin may be forming something of a block-copolymer with G-rich regions and 5-hydroxyguaiacyl-rich regions as will be discussed later. An elevated **B** level would also normally be expected in G-rich regions (paralleling the **D** content) but this is not seen.

2.1.2. HMBC NMR spectra

Long-range correlation spectra provide valuable insight into which types of units are connected to each other. HMBC data reveal the extent of S/G compositional changes in β-aryl ether units **A** in the extractable isolated lignins by showing α-proton correlations with diagnostic carbons in their aromatic rings. Equivalent

syringyl 2- and 6-carbons, resonating at ~105 ppm, are well separated from their guaiacyl counterparts (for which 2- and 6- carbons are different, at ~113 and ~121 ppm). The gradient-selected 2D HMBC subspectra, Fig. 4, clearly depict that wild-type alfalfa contains normal β-ether S- and G- units. The COMT-deficient transgenic alfalfa has a very low syringyl component; detectable S-unit correlations are seen at lower contour levels approaching the baseplane noise level. The S/G compositional shifts observed by NMR agreed with the results of lignin compositional determination from thioacidolysis (Guo et al., 2001a) and the DFRC and nitrobenzene data that follow.

The HMBC correlation spectrum offers considerably more detail of the transgenic than the wild-type. The detail was such that we reran this experiment at 500 MHz using a cryoprobe system for markedly improved sensitivity. The side-chain region of that HMBC spectrum is shown in Fig. 5. The pink-highlighted region at around 6 ppm in the proton dimension is from the α-proton on α-OAc units, normally in β-aryl ether units **A**. The normal β-ethers are to the left, centered at 6.09, 6.04, and 6.00 ppm. The correlations clearly show that the lignin from the COMT-deficient alfalfa transgenic was significantly syringyl depleted; lower intensity correlations detectable from the α-proton (in β-ether units $\mathbf{A_S}$) to the $S_{2/6}$ carbons at ~105 ppm are now revealed due to the greater signal-to-noise of the spectrum. They remain dwarfed by very strong correlations to the guaiacyl G_2 and G_6 carbons at 112.9 and 120.7 ppm and G_1 at 132.2 ppm (etherified) and 136.6 ppm (free-phenolic ends). {Free-phenolic groups are somewhat over-represented in lignins isolated by ball-milling}. But it is the new α-proton correlations that are more intriguing. In HMBC spectra, correlations up to three bonds are detectable. The correlations to protons at 6.00 and 5.95 ppm (colored red, where they are distinct) are clearly to carbons on a 5-hydroxyguaiacyl ring, a 5H-ring in our nomenclature. Thus, there are correlations from a β-ether α-proton to $5H_2$ (105.3

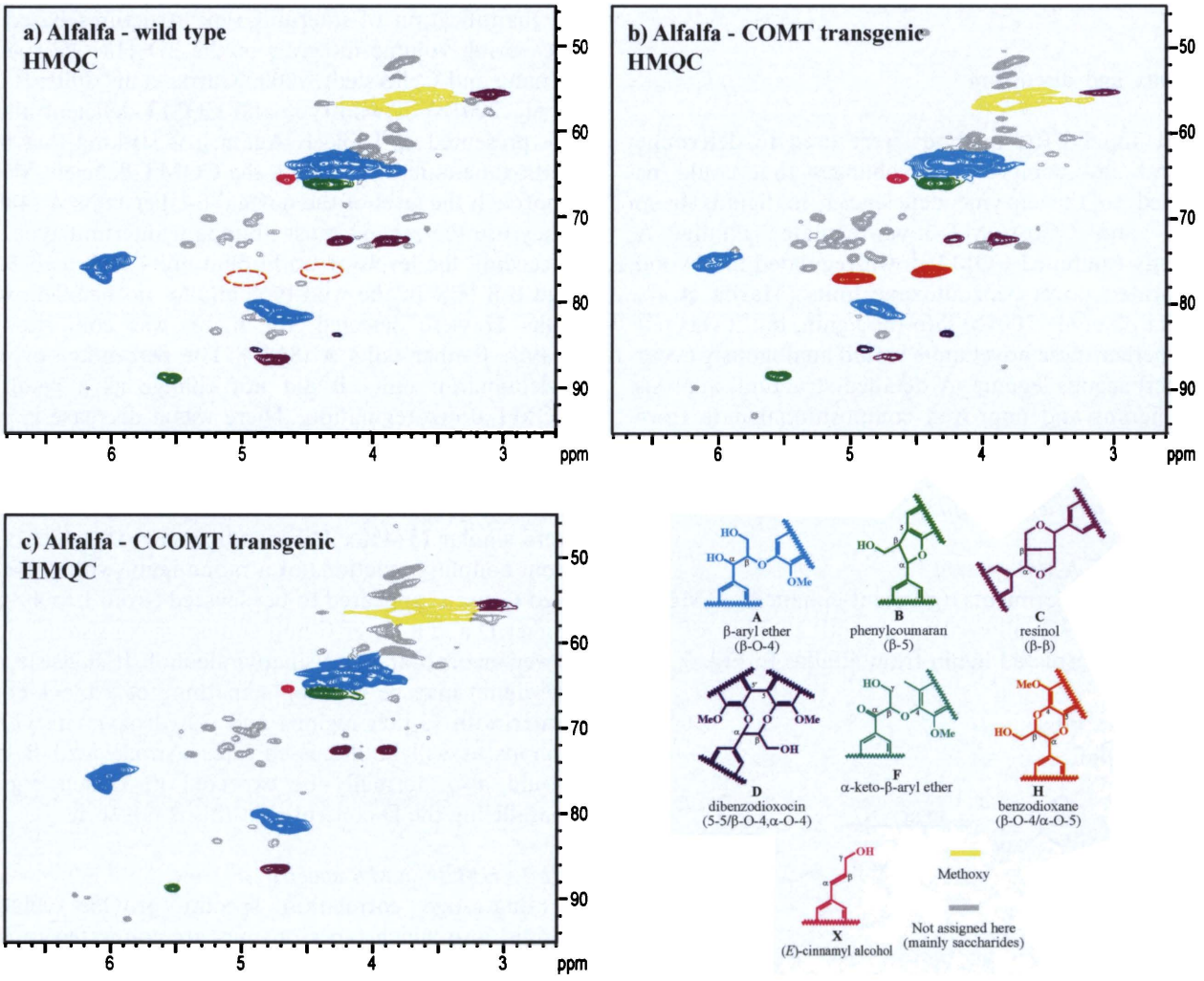

Fig. 3. Partial 2D ^{13}C–^{1}H correlative HMQC NMR spectra of lignins from (a) the wild-type (b) the COMT-deficient and (c) the CCoAOMT-deficient transgenic alfalfa. The major structural units (**A**–**D**, **X** and novel **H**) and color-coded chemical structures are present. Dashed ovals in (a) delineate the areas in which benzodioxane units **H** would correlate (if they were present).

ppm), $5H_6$ (109.5 ppm), and $5H_1$ (130.2 ppm) carbons (as well as to the β- (79.9 ppm) and γ- (63.0 ppm) carbons. This is a clear indication that 5-hydroxyguaiacyl units are involved in normal β-ether structures $\mathbf{A_G}$, Fig. 5—structures arising from the reaction of 5-hydroxyconiferyl alcohol monomers, at their β-positions, with the free-phenolic end of guaiacyl units on the growing polymer (at their 4-*O*-positions). We currently do not have enough model compound data to ascertain whether the 5-hydroxyguaiacyl unit is further etherified, but in preparing radical coupling products in the lab, such 5-hydroxyguaiacyl endgroups are not stable unless further etherified. Furthermore, the chemical shifts are all consistent with etherified 5-hydroxyguaiacyl units in benzodioxane structures (Ralph et al., 2001c). Thus, it is anticipated that all 5-hydroxyguaiacyl groups detected here are etherified (by further coupling reactions during lignification), logically in benzodioxane structures.

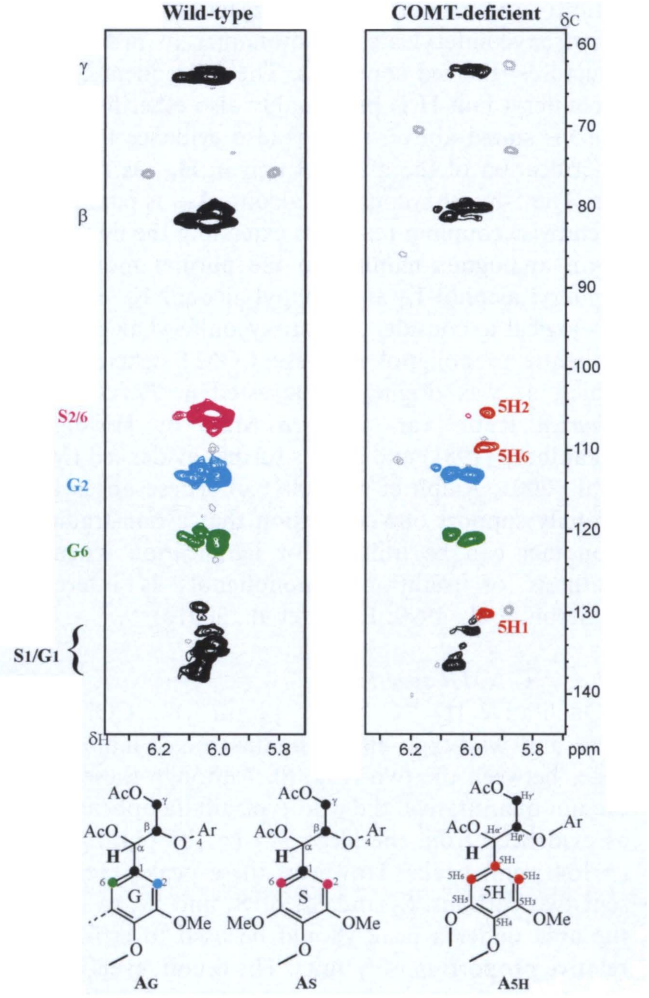

Fig. 4. Gradient-selected 2D HMBC sub-spectra showing α-proton correlations in β-aryl ether units **A** (specifically with $S_{2,6}$, G_2, and G_6 carbons). In the wild-type alfalfa lignin, guaiacyl ($\mathbf{A_G}$) and syringyl ($\mathbf{A_S}$) β-ethers are evident. In the COMT-deficient alfalfa lignin, there are only trace syringyl ($\mathbf{A_S}$) correlations, but significant new correlations from 5-hydroxyguaiacyl ($\mathbf{A_{5H}}$) β-ethers (red where distinct).

The correlations at 5.86 ppm (green) in the proton domain were a mystery. The proton corresponds to an α-proton on a carbon bearing an acetate; the acetate carbonyl correlation is evident at 170.0 ppm. It is also attached to a 5-hydroxyguaiacyl nucleus with strong correlations to $5H_2$, $5H_6$, and $5H_1$ carbons similar to the β-ether units above (in red; Fig. 5). However, the β-carbon is uniquely different at 72.9 ppm. HMQC–TOCSY (not shown) and HMQC spectra suggest that the carbon assignments are: ~74 ppm (α), 72.9 ppm (β), and 62.1 ppm (γ); other than the α-proton shift at 5.86 ppm from the HMQC and HMBC, the other protons are not clearly revealed due to overlap. The data suggest that the structure has an acetylated glycerol sidechain, and is therefore a 5-hydroxyguaiacyl-glycerol unit $\mathbf{G_{5H}}$. Glycerol structures **G** are typically rare in lignins. Data for such units appear in the "NMR Database of Lignin and Cell Wall Model Compounds" (Ralph et al., 2001d); for example, a phenylcoumaran-guaiacyl glycerol dimer (database compound number 262) has carbons/protons at 74.6/5.92 (α), 73.3/5.38 (β), and 63.0/4.17,3.82 (γ). Whether their origin here is from hydrolysis of β-aryl ether units in the lignin polymer, from actual endgroups formed somehow during the polymerization (still possibly via the β-ether) or have arisen during ball-milling is unknown, but they are clearly unique to the 5H-units. Unfortunately, no current degradative method will give unique products from this structure to allow its detection from the native lignin; all methods will give the same products as from a normal β-ether unit. Some insight may be gained from further model experiments.

In the benzodioxane α-proton correlation region (yellow shading), new details regarding the course of coupling reactions involving 5-hydroxyconiferyl alcohol are evident. It is revealed that the **H**-unit benzodioxane rings themselves are the result of adding either coniferyl or 5-hydroxyconiferyl alcohol to the 5-hydroxyguaiacyl end of the evolving lignin polymer; thus, the correlations

Table 1
Subunit ratios derived from volume integrals of contours in the sidechain region of ^{13}C-^{1}H correlation spectra of alfalfa lignin

Alfalfa lignin	Unit type, relative proportion						
	A	**B**	**C**	**D**	**H**	**X**	Σ(β-ethers)[a]
Wild-type	81	8	6	1	0	3	83
COMT-deficient[b]	44	8	3	4	38	4	85
CCoAOMT-deficient[b]	88	5	6	0	<1	2	88

Percentages are not traditional interunit linkage percentages since no account is taken of units that do not have specific contours in the sidechain region of their HMQC spectra (e.g. 5–5, 4-O-5, β-1 units). These percentages are ratios of the volume integrals of contours arising from units **A–D**, **H**, and **X**.

[a] Σ(β-ethers) = **A** + **D** + **H**.

[b] Down-regulated COMT and CCoAOMT alfalfa (Guo et al., 2001a).

at 5.05 ppm (blue) are to guaiacyl ring G_2, G_6, and G_1 carbons in structures $\mathbf{H_G}$. Most of these correlations are to free-phenolic guaiacyl units indicating that lignification had not proceeded further than one unit past the incorporated 5-hydroxyconiferyl alcohol, or that this was where the lignin chain was broken by ball-milling. {It must also be remembered that we are analyzing only the fraction that extracted into dioxane:water.} Smaller amounts of etherified guaiacyl units in these structures are seen in Fig. 5 (yellow shading). A large fraction of the **H**-units are however attached to subsequent **H**-units in structures $\mathbf{H_{5H}}$. The evidence for this is the contours (red), which correlate the benzodioxane α-proton with carbons $5H_2$, $5H_6$ and $5H_1$ in the next ring down, also a

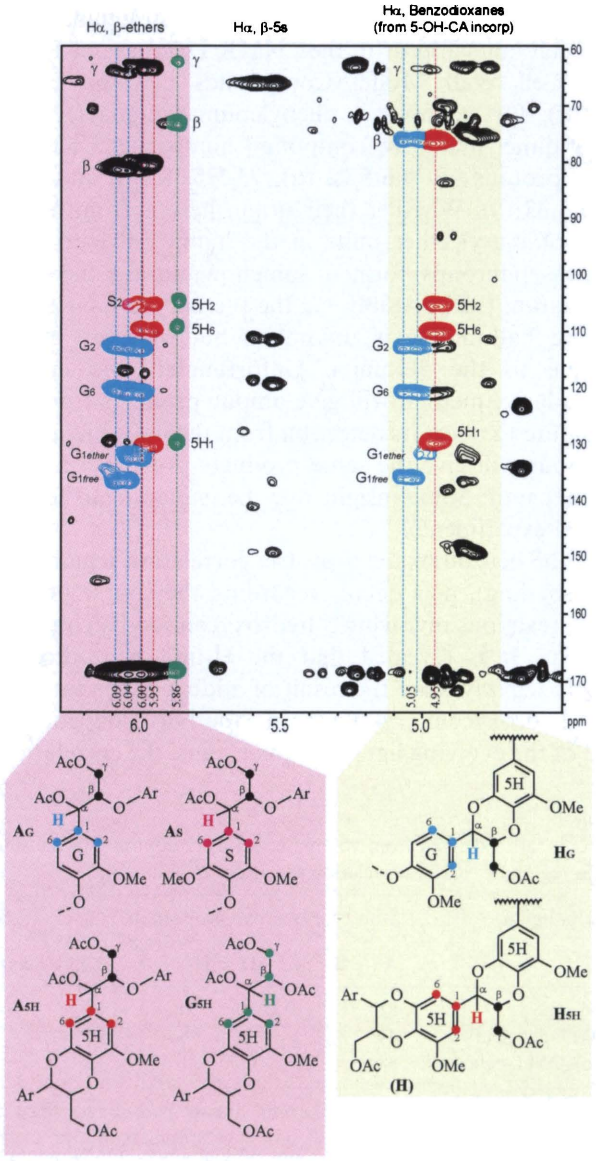

Fig. 5. Partial 2D HMBC of lignin isolated from the COMT-deficient alfalfa transgenic, run on a 500 MHz cryoprobe instrument revealing the low level syringyl β-ether ($\mathbf{A_S}$) component, new 5-hydroxyguaiacyl glycerol structures **G**, and revealing new details on benzodioxane units **H**. See text for details.

5-hydroxyguaiacyl ring. Correlations to S-units are either very minor or unresolved; as shown later, there is DFRC evidence for $\mathbf{H_S}$ structures.

HMBC experiments, therefore, provide compelling evidence that 5-hydroxyconiferyl alcohol is incorporated intimately into the polymerization process. 5-Hydroxyconiferyl alcohol cross-couples with guaiacyl units in the growing polymer as evidenced by the red contour peaks showing that 5-hydroxyguaiacyl rings are involved in normal β-ethers in structures of type $\mathbf{A_{5H}}$. Further lignification reactions occur at the new 5-hydroxyguaiacyl end of the polymer, producing benzodioxane structures. These structures are prevalent, as shown by the HMQC spectra, Fig. 3b, and the quantification in Table 1. In the examined isolated lignin, the HMBC spectrum (Fig. 5) shows that about half of the 5-hydroxyguaiacyl units are etherified (into benzodioxane structures of type $\mathbf{H_G}$) by reacting with coniferyl alcohol monomers (the blue contours in the yellow region) and about half by reacting with another 5-hydroxyconiferyl alcohol monomer to produce $\mathbf{H_{5H}}$ structures (the red contours). The subsequent 5-hydroxyconiferyl unit **H** is presumably also etherified for the reasons stated above; there is also evidence for further etherification of the guaiacyl unit in $\mathbf{H_G}$, as noted earlier. The 5-hydroxyconiferyl alcohol $\mathbf{1_{5H}}$ is participating in endwise coupling reactions extending the lignin chain in an analogous manner to the normal monolignols, coniferyl alcohol $\mathbf{1_G}$ and sinapyl alcohol $\mathbf{1_S}$. Therefore, it is logical to consider 5-hydroxyconiferyl alcohol as an authentic monolignol in these COMT-deficient transgenics, as was originally suggested in *Fraxinus mandshurica* Rupr. var. *japonica* Max. by Hwang and Sakakibara (1981) and as was further evidenced (Marita et al., 2001; Ralph et al., 2001b,c). These observations strongly support our contention that a non-traditional monomer can be utilized for lignification when biosynthesis of traditional monolignols is interrupted (Sederoff et al., 1999; Ralph et al., 2001b).

2.1.3. ^{13}C NMR spectra

Qualitative 1D ^{13}C NMR spectra from COMT-deficient and wild-type alfalfa lignins reveal major differences between the two (Fig. 6). Although these spectra are not quantitative, the wild-type alfalfa appears S-rich as evidenced from the large $S_{2/6}$ (~105 ppm) and $S_{3/5}$ (~154 ppm) peaks. However, these peaks each represent two carbons, S_2 and S_6, or S_3 and S_5, so one half the area under a peak should be used to estimate the relative proportion of S units. The S unit levels are then comparable to those of the more disperse G units. In the COMT-deficient alfalfa, negligible amounts of $S_{2/6}$ and $S_{3/5}$ resonances are present; the indicated peaks do not exhibit normal correlations at a significant level. The G component dominates, and 5-hydroxyguaiacyl (5H) carbons in benzodioxane structures **H** are readily

identifiable by comparison with ^{13}C NMR data from a benzodioxane model compound (Lu, 2002; unpublished).

2.1.4. DFRC analysis

DFRC degradation products from cell walls and dioxane:water soluble and insoluble fractions of COMT-deficient and wild-type alfalfa were examined. The DFRC S:G ratios for the cell walls of wild-type and COMT-deficient alfalfa were 0.64 and 0.08 (Table 2), values that corresponded with previously published thioacidolysis results (Vallet et al., 1998; Guo et al., 2001a). Both DFRC and thioacidolysis methods release S and G monomers attached as normal β-ethers in lignins. Nitrobenzene oxidation also confirmed a significant decrease in syringaldehyde:vanillin ratios from 0.91 in wild-type to 0.11 μmol g^{-1} lignin in COMT-deficient alfalfa cell walls (Table 3). As noted earlier,

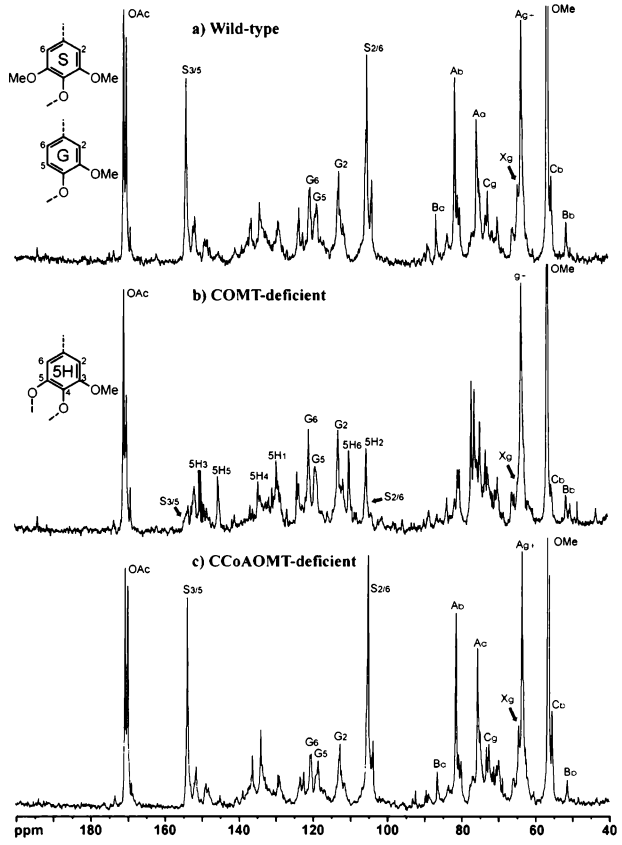

Fig. 6. 1D ^{13}C NMR spectra of lignins from the (a) wild-type (b) COMT-deficient and (c) CCoAOMT-deficient transgenic alfalfa. 1D ^{13}C NMR assignments are very complex (Nimz et al., 1981). Only prominent peaks from major units are labeled; peak labeling in the aromatic region is only for uncondensed β-ether units (so not all guaiacyl 5-carbons are in the G_5 peak, for example). Syringyl (S) units appear to dominate in both wild-type and the CCoAOMT-deficient alfalfa but because S_2 and S_6 or S_3 and S_5 carbons in normal β-ethers resonate identically these peak areas need to be halved in order to make S:G comparisons. Guaiacyl (G) and 5-hydroxyguaiacyl (5H) units are prevalent in the S-depleted lignin of the COMT-deficient alfalfa. The S:G ratio is higher in the CCoAOMT-deficient alfalfa.

dioxane:water extractions caused partitioning of the lignin into soluble and insoluble fractions. Therefore it was not surprising that the DFRC S and G data (wild-type and COMT-deficient) for the dioxane:water soluble lignin fractions differed substantially from their corresponding cell wall values. The soluble fraction of both wild-type and COMT-deficient alfalfa contained a greater proportion of S-units as evidenced by the larger S:G ratio in the soluble fraction versus the insoluble fraction. The release of S-monomers from this soluble fraction is consistent with the NMR observations of normal S β-ether units $\mathbf{A_S}$ (Fig. 5).

The DFRC method has been recently extended to examine benzodioxanes releasable by cleaving (normal) β-ethers in lignins (Lapierre et al., 2001; Ralph et al., 2001a). The benzodioxane ring remains completely intact during DFRC so no 5-hydroxyguaiacyl monomers are released. The yields are low because, to release a benzodioxane dimer **3**, such a unit in lignin must be surrounded on both sides by normal β-ether units in structures such as **2** (Fig. 2). For this reason syringyl products $\mathbf{3_S}$ are also over-represented since a syringyl unit is most likely to be connected via a β-ether linkage to the next unit. As has been seen in the NMR data presented above, benzodioxane units are frequently linked to a further benzodioxane unit and therefore cannot be released as dimeric fragments. The COMT-deficient alfalfa released more $\mathbf{3_G}$ (4.5 μmol g^{-1} lignin) than $\mathbf{3_S}$ (1.2 μmol g^{-1} lignin) as anticipated from the S-depletion in this transgenic.

2.1.5. Cell wall composition

Klason lignin, total neutral sugars and total uronic acids were determined for both alfalfa samples (mg g^{-1} CW; Table 3). Measurable cell wall components were

Table 2
Monomeric composition (μmol g^{-1} lignin) of cell wall, soluble, and insoluble lignin fractions as measured by DFRC[a]

	Guaiacyl	Syringyl	S:G
Wild-type			
Cell wall	370	238	0.64
Soluble fraction	336	328	0.98
Insoluble fraction	178	93	0.52
COMT-deficient[b]			
Cell wall	315	24	0.08
Soluble fraction	336	46	0.14
Insoluble fraction	108	4	0.04
CCoAOMT-deficient[b]			
Cell wall	185	134	0.72
Soluble fraction	182	240	1.31
Insoluble fraction	135	114	0.85

[a] Duplication of runs gave reproducibility.
[b] Down-regulated COMT and CCoAOMT alfalfa (Guo et al., 2001a).

based on two replicates. Unlike reported increases in lignin content for COMT-deficient hardwoods (Marita et al., 2001; Ralph et al., 2001b), COMT-down-regulation in these alfalfa samples resulted in a 10% decrease in Klason lignin content between wild-type and transgenic. This decrease in lignin content, similar to values recently reported (Guo et al., 2001a), was complimented by a (relative) increase (~10%) in cellulose (as measured by glucose) and an increase (~6%) in xylans (as measured by xylose). Uronic acid levels did not drop significantly (<1%) overall. Such changes could result in an increase in the digestibility of alfalfa stems. Typically, alfalfa leaves provide the most nutrition to the animal, but stems represent a limiting component. Any increase in availability of nutrients tied up in the stem should positively affect the overall benefit of the forage to the animal. Guo et al.'s examination of rumen digestibility of alfalfa forage in fistulated steers revealed just that—improved digestibility of forage from COMT down-regulated alfalfa plants (Guo et al., 2001b).

2.2. CCoAOMT-deficient alfalfa

Isolated lignin from CCoAOMT down-regulated alfalfa was similarly analyzed by NMR to determine what structural changes have occurred and whether these changes are analogous to changes observed in CCoAOMT-deficient hardwoods.

2.2.1. HMQC spectra, Fig. 3

All major structural units were still present in the lignin of the CCoAOMT-deficient alfalfa at similar intensities to wild-type levels (Fig. 3) and as in a previous study on CCoAOMT-deficient poplar (Meyermans et al., 2000; Marita, 2001). The only structural and compositional differences were detected near baseplane levels where **F** (α-keto-β-aryl ether) and **H** (benzodioxane) units became discernable.

Quantification of all the interunit type structures based on measuring volume integrals in the 2D HMQC spectra (Zhang and Gellerstedt, 2000) of wild-type and CCoAOMT-deficient alfalfa are presented in Table 1. Quantification of individual interunit types parallels the qualitative observations from their respective NMR spectra. Most noticeably, normal β-ether units **A** predominate in both with a slight elevation in CCoAOMT-deficient (88%) over wild-type (81%) alfalfa. Dibenzodioxocins (**D**) were present at low levels in the wild-type but not CCoAOMT-deficient transgenic alfalfa. Conversely, benzodioxanes (**H**) were present at low levels in CCoAOMT-deficient transgenic but not in the wild-type alfalfa. The difficulty and time constraints associated with doing these NMR studies does not allow us to determine whether these changes are statistically significant.

2.2.2. ^{13}C NMR spectra

1D ^{13}C NMR spectra of CCoAOMT-deficient and wild-type alfalfa reveal a modest increase in the S:G ratio for CCoAOMT-deficient transgenic compared to the wild-type alfalfa as revealed by a reduction in G_2, G_5, and G_6 peaks relative to the $S_{2/6}$ peaks in the CCoAOMT-deficient alfalfa relative to wild-type, Fig. 6. This was substantiated by the decrease in phenylcoumaran units **B** observed by HMQC, by an increase in syringaldehyde:vanillin ratio from 0.91 (wild-type) to 1.16 (CCoAOMT-deficient transgenic) by nitrobenzene oxidation (Table 3) and by changes in molar ratios from

Table 3
Klason lignins (acid insoluble residues), nitrobenzene determinations (syringaldehyde:vanillin), total neutral sugars, and uronic acids as percentages of isolated alfalfa cell walls (mg g^{-1} CW ± standard deviation) based on two replicates

	Wild-type	COMT-deficient[a]	CCoAOMT-deficient[a]
Klason lignins	164±5	147±7	129±5
Nitrobenzene S:V[b]	0.91±0.02	0.11±0.02	1.16±0.10
Neutral sugars			
Fucose	1.7±0.0	1.8±0.1	1.5±0.1
Arabinose	23.0±0.3	24.2±0.3	22.7±0.6
Rhamnose	4.8±0.3	5.3±0.5	5.2±0.8
Galactose	15.8±0.5	16.8±0.4	17.3±0.7
Glucose	376.4±21.3	413.1±19.6	411.1±21.1
Xylose	142.0±0.7	150.7±3.2	159.6±2.3
Mannose	19.1±1.0	19.4±0.6	18.6±1.0
Totals	**583**	**631**	**636**
Uronic acids	77[c]	70±3	69±4

[a] Down-regulated COMT and CCoAOMT alfalfa (Guo et al., 2001a).
[b] Syringaldehyde:vanillin ratio.
[c] Based on one replicate.

DFRC presented below. Similar results have been reported in previous studies involving other plant species (Zhong et al., 1998; Meyermans et al., 2000).

2.2.3. DFRC analysis

DFRC degradation products were examined from cell walls and dioxane:water soluble and insoluble fractions of CCoAOMT-deficient alfalfa. The S:G ratios, for the cell wall components corresponded with previously published thioacidolysis results (Vallet et al., 1998; Guo et al., 2001a; Table 2). A discrepancy between releasable monomers from the cell wall and dioxane soluble lignin samples was observed implying lignin partitioning. Compared to wild-type, the S:G ratio was greater for both cell wall and dioxane soluble lignin samples but with an overall decrease in releasable S- and G-monomers compared to wild-type (Table 2). The decrease in releasable lignin monomers following DFRC corroborates the general down-regulation of lignin monomers typically seen in CCoAOMT-deficient plants (Meyermans et al., 2000; Guo et al., 2001a). There is a debate as to whether CCoAOMT is part of an independent pathway specific to G-monomer synthesis (Ye et al., 1994; Van Doorsselaere et al., 1995; Dixon et al., 2001) or is part of a dependent pathway that contributes to the synthesis of both G- and S-units (Humphreys et al., 1999; Osakabe et al., 1999; Meyermans et al., 2000). Although there is a greater decrease in releasable G-monomers compared to S-monomers following DFRC, the decrease in the incorporation of S-units in the CCoAOMT-deficient alfalfa lignin tends to support a dependent pathway.

DFRC of cell walls from CCoAOMT-deficient alfalfa did not reveal benzodioxane lignin degradation products even though at baseplane levels in the HMQC NMR, benzodioxanes were observed suggesting that they may be beyond the detectable limit of the DFRC analysis run under these conditions. A larger sample size may be necessary for detection of the dimer products by DFRC.

2.2.4. Cell wall composition

Klason lignin, total neutral sugars and total uronic acids were determined for alfalfa samples (mg g^{-1} CW; Table 3). The CCoAOMT down-regulation in alfalfa resulted in a 21% decrease in lignin content between wild-type and transgenic, higher than recently reported values by Guo et al. (2001a). This decrease in lignin content is comparable to the 18.5 to 20% reduction in lignin content observed for sense-suppressed CCoAOMT transgenic poplar (Jouanin et al., 2000; Marita et al., 2001; Ralph et al., 2001b). Unlike the COMT-deficient alfalfa, the complimentary (relative) increase (~11%) in cellulose (as measured by glucose) and increase (~9%) in xylans (as measured by xylose) were not equivalent to the reduction in lignin content. This corresponds to a 39% increase in cellulose:lignin ratio compared to wild-type. Uronic acid levels did not drop significantly (<1%) overall. It is likely that the decrease in in situ digestibility reported by Guo et al. (2001b) of CCoAOMT-deficient alfalfa compared to COMT-deficient alfalfa is a consequence of these CCoAOMT-deficient alfalfa cell wall attributes, particularly the polysaccharide:lignin ratio.

2.3. Partitioning of lignin

Previously, additional ball-milling has increased the dioxane:H_2O soluble lignin fraction recovered during the lignin isolation process. Examination by NMR of the re-extracted material indicated that the signature COMT-deficient **H**-units (benzodioxanes) had been preferentially partitioned into the first extract by the lignin isolation process.

DFRC degradation products of cell wall and isolated fractions (dioxane:H_2O soluble and insoluble fractions) also revealed considerable lignin partitioning (Table 2). The trend across alfalfa samples, wild-type and both transgenics, was a substantial increase in S:G from soluble lignin fractions compared to the cell walls with the greatest increase from 0.72 to 1.31 (S:G) for the CCoAOMT-deficient alfalfa. Less dramatic changes between S:G ratios of insoluble fractions and cell wall fractions were observed. The total S or G monomers (μmol g^{-1} lignin) recovered from soluble lignin fractions were greater than the monomers recovered from the insoluble fractions for all alfalfa samples. The earlier observations suggest that β-ether-linkages were separated preferentially into the soluble fraction since DFRC only cleaves the β-ether linkages, and more of the β-ether linkages involved S-units rather than G-units. The higher yields seen in the soluble fraction confirm this hypothesis (Table 2).

Releasable benzodioxane dimers **3** (Fig. 2) from cell wall, soluble and insoluble fractions of alfalfa samples (wild-type and both transgenics) were also examined by DFRC. The COMT-deficient soluble lignin fraction had the only measurable benzodioxane dimer levels by GC. This confirms the partitioning of benzodioxane units observed by NMR.

Considering the stratification of lignification across an alfalfa stem section and between stem internodes (Vallet et al., 1998) and prior demonstration that "milled wood lignin" (dioxane:water soluble lignin) in wood comes preferentially from secondary cell walls in softwoods (Goring, 1989), differences in S:G ratios between whole cell wall and dioxane soluble lignin fractions is reasonable. It remains evident that units such as benzodioxanes are a major component of the lignins, despite the preferential (temporal and spatial) partitioning in the polymer, because of their abundance in initial extracts.

3. Conclusions

Down-regulating COMT resulted in the incorporation of novel units undetected in the normal wild-type, because the plant apparently utilizes monomers from incomplete monolignol synthesis to augment the production of lignin. It appears that COMT-down-regulation in a legume such as alfalfa leads to similar compositional changes as COMT-down-regulation in a hardwood such as poplar. The extent of these changes varies but the signature effects are clearly evident. For the first time, we have provided evidence that the next reaction in the lignification sequence following incorporation of 5-hydroxyconiferyl alcohol can be the coupling with either coniferyl alcohol (or sinapyl alcohol) or another 5-hydroxyconiferyl alcohol monomer. These compositional changes evident in the CCoAOMT-deficient alfalfa potentially enhance the utilization of alfalfa as a major forage crop by increasing the digestibility of its normally poorly digestible stem fraction (Galyean and Goetsch, 1993; Guo et al., 2001b). The data also further support the bi-functional role of CCoAOMT in the lignin pathway and the possible interactions between the two *O*-methyltransferases, CCoAOMT and COMT.

4. Experimental

4.1. Plant materials and lignin isolation

Stems from wild-type, COMT-deficient and CCoAOMT-deficient alfalfa (*Medicago sativa* cv Regen SY) were grown in the greenhouse under standard conditions (Guo et al., 2001a). All transformations were performed with clonally propagated material of one selected highly regeneratable line. Transformation with a full length COMT cDNA sequence in the sense orientation under control of the bean *PAL2* promoter resulted in a silenced-COMT line (SC5). The down-regulated CCoAOMT line (ACC305) resulted from transformation with a full length CCoAOMT cDNA sequence in the antisense orientation under control of the same promoter (Guo et al., 2001a).

Lignin isolation from the alfalfa was essentially as previously described (Marita et al., 1999). Alfalfa stems (internodes 6–9) were ground and extensively extracted sequentially with water, methanol, acetone, and chloroform. The isolated cell walls were ball-milled, digested with crude cellulases, and extracted into 96:4 dioxane:H_2O. The dioxane:water soluble lignin was ultra-filtered (YM10–43 mm; Amicon-Millipore Corp., Bedford, MA) to remove water-soluble components (mainly low molecular weight sugars), acetylated overnight, and water/EDTA washed to remove trace metal contaminants. The acetylated lignin (~100 mg) was dissolved in ~400 μl acetone-d_6 for NMR analysis. Soluble lignins were difficult to extract from alfalfa and the yields were: wild-type, 11.5%; COMT-deficient, 17.0%; CCoAOMT-deficient, 18.4% of the total (Klason) lignin.

4.2. Cell wall composition

Klason lignin was determined as an acid-insoluble residue remaining after total hydrolysis of cell wall polysaccharides by a modified Theander and Westerlund (1986) method. Alfalfa acid-insoluble residues can contain a small percentage of protein material (Hatfield et al., 1994). Total uronic acids were estimated colorimetrically using galacturonic acid as the calibration standard (Blumenkrantz and Asboe-Hansen, 1973; Ahmed and Labavitch, 1977). Neutral sugar residues were determined by high-pressure liquid chromatography (HPAE–PAD: high performance anion exchange chromatography–pulse amperometric detection, DX500; Dionex, Sunnyvale, CA) after acid hydrolysis of total cell wall polysaccharides (Jung and Russelle, 1991; Hatfield and Weimer, 1995). Cell wall compositions (summarized Table 3) were determined for the wild-type, sense-COMT and antisense-CCoAOMT alfalfa cell wall samples (~75 mg).

4.3. NMR (nuclear magnetic resonance)

The 1D (^{1}H and ^{13}C) and 2D (HMQC, HMQC–TOCSY and HMBC) NMR spectra were taken on a Bruker DRX-360 instrument fitted with a 5-mm ^{1}H/broadband gradient probe with inverse geometry (proton coils closest to the sample). A 2D HMBC NMR spectrum was taken on a Bruker DMX-500 fitted with a 5-mm TXI GRASP II cryogenic probe (Cryoprobe) with increased sensitivity. The conditions for all samples were acetylated lignin (~100 mg) in acetone-d_6 (0.4 ml) with the central solvent peak as internal reference (δ_H 2.04, δ_C 29.80). Standard Bruker implementations of gradient-selected versions of inverse (^{1}H-detected) heteronuclear multiple quantum coherence (HMQC), HMQC-total correlation spectroscopy (HMQC-TOCSY), and heteronuclear multiple bond correlation (HMBC) experiments were used. The TOCSY spin lock period was 125 ms; the HMBC experiments used an 80 ms long-range coupling delay. Carbon/proton designations are based on conventional lignin numbering (Fig. 3). Lignin sub-structures are labeled by the convention used in a recent review (Ralph et al., 1999); the new glycerol structures are assigned as structures **G** here (e.g. Fig. 5).

4.4. DFRC (derivatization followed by reductive cleavage)

DFRC monomers (H, G, and S acetates) were determined as described (Lu and Ralph, 1997) using

4,4-ethylidenebisphenol as internal reference (response factors were H:1.42, G:1.38, and S:1.45). DFRC degradation products from cell wall (~20 mg), dioxane:H_2O soluble (~2 mg; isolated lignin) and insoluble (~15 mg) fractions were quantified by gas liquid chromatography (GLC). Samples (1 μl injection, 50:1 split ratio) were separated on a 30 m×0.2 mm, 0.2 μm film SPB-5 (Supelco) column fitted in an HP5890 GLC (Hewlett-Packard; Atlanta, GA) using He gas as carrier, 0.4 ml/min. For monomers determination, the column temperature was held at 140 °C for 1 min ramped to 250 °C at 3 °C/min then ramped 10 °C/min to 280 °C and held 1 min before the final ramp of 20 °C/min to 300 °C and held 20 min to give a total run time of 62 min. Monomers of interest eluted between 20 and 40 min.

Release and determination of benzodioxane dimers by the DFRC method has been recently described (Ralph et al., 2001a). The column temperature was held at 200 °C for 1 min ramped to 310 °C at 5 °C/min and held constant for another 25 min. Compounds of interest eluted between 30 and 40 min. Authentic guaiacyl and syringyl DFRC benzodioxane dimers **3**, Fig. 2, were synthesized by Dr. Lu and will be described elsewhere.

4.5. Nitrobenzene oxidation

Nitrobenzene oxidation reactions followed a modified Iiyama and Lam (1990) procedure. Nitrobenzene oxidation products from alfalfa cell walls (~25 mg) were silylated with *N,O*-bis(trimethylsilyl) trifluoroacetamide (40 μl) and tetrahydrofuran (10 μl) at 60 °C for 30 min. Derivatized components were separated by GLC (HP6890-Hewlett-Packard; Atlanta, GA) using a 50 m×0.22 mm ID, BP-1 capillary column (SGE, Victoria, Australia). The GLC conditions were: injection temperature 250 °C; oven temperature 160 °C for 5 min, ramp 4 °C min^{-1} to 200 °C, ramp 10 °C min^{-1} to 240 °C and hold for 20 min at 250 °C; He gas carrier, 0.4 ml/min; injector split ratio 50:1.

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