NMR characterization of lignins from transgenic poplars with suppressed caffeic acid *O*-methyltransferase activity

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Perturbing the lignin biosynthetic pathway provides a tool for understanding the complex process of lignification. Caffeic acid *O*-methyltransferase (COMT) is required to produce syringyl units in lignins. Down-regulating the expression of its gene in poplar dramatically affects the lignin composition. 2D and 3D NMR investigations detail structural differences between lignins from a control and COMT-deficient poplars obtained by means of two independent transformation techniques. This first application of 3D NMR to natural abundance lignins reveals the full side-chain network and provides diagnostic evidence for the intimate incorporation of 5-hydroxyconiferyl alcohol into the lignins to form novel benzodioxanes as major structures. The flexibility of a plant to utilize novel monomers to produce functional lignins provides opportunities for engineering the structure and affecting the consequent properties of lignins.

Introduction

Lignin is a complex phenylpropanoid polymer found as a substantial component in all terrestrial plants.[1] It is located in the cell walls of conducting and supporting tissues assisting in water transport and mechanical strength. It is also utilized as a defense mechanism by the plant against pathogen infection.[2] Lignin is polymerized from hydroxylated and methoxylated monomers derived from cinnamic acid. In angiosperms such as poplar the major lignin precursors are coniferyl (4-hydroxy-3-methoxycinnamyl) and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl) alcohols which give rise to guaiacyl units (G) and syringyl units (S), respectively (Fig. 1). The incorporation of syringyl units in angiosperm tree species represents an important step in their evolution from gymnosperms. The resulting lignin is less condensed and more easily degraded in wood pulp production.[1,3,4] Methylating enzymes, *O*-methyltransferases (OMTs), are required for the production of syringyl units. As a result, these enzymes have been targeted for biotechnological manipulation.[5–14] The genetic alteration of lignin content and/or structure by perturbing enzymes such as COMT (caffeic acid *O*-methyltransferase) in the lignin biosynthetic pathway provides a novel tool for understanding the complex process of lignification. By manipulating enzymes in the lignin pathway, researchers also hope to achieve various industrial and/or agricultural goals such as enhanced cell wall digestibility in ruminants and reduction of negative environmental impacts of chemical pulping and bleaching necessary in the papermaking process.[15–18]

It has recently been proposed that caffeoyl coenzyme A *O*-methyltransferase (CCoAOMT) participates in the production of guaiacyl and syringyl precursors *via* caffeoyl-coenzyme A (CoA) as an intermediate,[14] whereas COMT primarily contributes to the formation of syringyl precursors from 5-hydroxyconiferyl aldehyde as the major substrate.[19–21] Preliminary evidence from analytical thioacidolysis[13] and NMR[22,23] suggested that 5-hydroxyconiferyl alcohol (Fig. 1) is incorporated into lignin forming novel benzodioxane† structures in COMT down-regulated poplar with no detectable amounts occurring in wild-type poplar. Here we expand and detail that evidence from NMR of lignins of two COMT-deficient poplars from independent transformation techniques and reveal the full structural implications of COMT down-regulation.

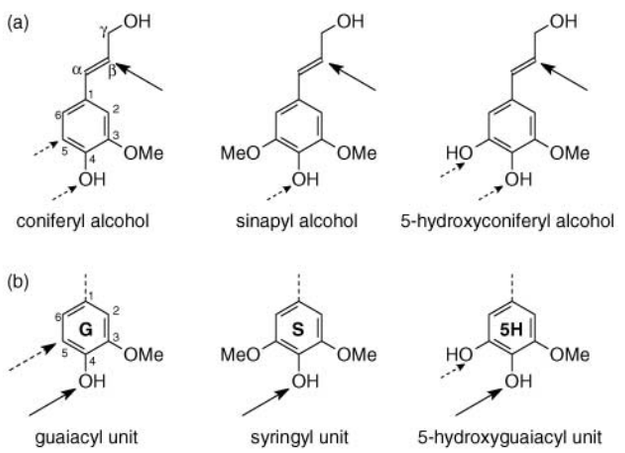

Fig. 1 Lignin precursors and resultant structures in lignin. (a) The two major monolignols, coniferyl and sinapyl alcohols, and the novel monolignol, 5-hydroxyconiferyl alcohol, supplied to the cell wall in COMT-deficient plants. The arrows indicate sites for radical coupling during lignification; for the monolignols, coupling with the lignin polymer is overwhelmingly at the β-position; coupling of monolignols with other monolignols in reactions less prevalent during lignification are at sites with (smaller) dashed arrows. (b) Guaiacyl and syringyl units in lignins, and 5-hydroxyguaiacyl units resulting from 5-hydroxyconiferyl alcohol incorporation. Further radical coupling polymerization reactions are primarily at the O-4-positions, with 5-coupling of guaiacyl units also being substantial.

† The IUPAC name for benzodioxane is 2,3-dihydro-1,4-benzodioxine.

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Results and discussion

HMQC and HMQC-TOCSY spectra

2D short-range ^{13}C–^{1}H correlation spectra, gradient-enhanced HMQC,[24,25] correlate a carbon with its directly attached proton. The HMQC-TOCSY experiment correlates a given carbon with its attached proton and other protons within the same coupling network.[26] The intensities observed depend in part on proton–proton coupling constants so all correlations are not seen and intensities can vary. The extended correlations of the HMQC-TOCSY spectra allow lignin components to be more readily identified,[27] but HMQC spectra are more quantitatively accurate.

Since wild-type lignins isolated from the same wild-type poplar genotype (*Populus tremula* × *P. alba* cv. 717 1-B4) control plants grown in both cases were similar, only one is presented and discussed. The HMQC data for the control poplar acetylated lignin (Fig. 2b) are typical of normal syringyl-rich syringyl/guaiacyl lignins from a variety of plants.[27,28] The lignin of the antisense COMT down-regulated poplars are comprised of both syringyl and guaiacyl units but exhibit an increase in guaiacyl units and a lower frequency of syringyl units when compared to wild-type,[9,12] as also validated by 1D ^{13}C NMR data (not shown). The sense-suppressed COMT poplars have guaiacyl-rich lignins almost completely lacking in syringyl units,[13] as also evidenced from 1D NMR (not shown).

Important additional structural details are revealed in the 2D HMQC spectra. β-Aryl ether (β-O-4, **A**) units and resinol (β-β, **C**) units are well resolved in the wild-type and both transgenic poplar lignins (Figs. 2b–d). In the wild-type lignin, phenylcoumaran (β-5, **B**) units indicative of the presence of guaiacyl components are present to a lesser degree than **B** units in either transgenic line. Phenylcoumaran units form during lignification by the coupling of either coniferyl or sinapyl alcohol at its β-position with the free phenolic end of a guaiacyl oligomer/polymer at its 5-position (Fig. 1). Since lignin of both COMT transgenic lines contains more guaiacyl units than wild-type (due to the suppression of syringyl-lignin formation),[13] it is logical that the frequency of **B** units should mimic the abundance of guaiacyl units in the lignin. Dibenzodioxocins (5–5/β-O-4, α-O-4, **D**) result from the reaction of 5–5-coupled guaiacyl units with a monolignol,[29,30] and are therefore also correlated with % guaiacyl content. The syringyl-rich wild-type lignin (Fig. 2b) does not appear to have any **D** units. The lignin from the antisense COMT transgenic has increased levels (Fig. 2c, visible more clearly at lower contour levels) resulting from the increased guaiacyl content. Both the α- and β-^{13}C–^{1}H correlations from dibenzodioxocin **D** units are well resolved in the sense-suppressed COMT transgenic (Fig. 2d) characteristic of its guaiacyl-rich lignin polymer. α,β-Diaryl ether **E** units are present in the synthetic lignin (Fig. 2a) and derive mainly from nucleophilic addition of coniferyl alcohol to lignin-intermediate quinomethanes; no detectable amount is seen in any of these poplar lines, a sign that lignin units derive almost entirely from radical coupling.

There is a further striking difference between the wild-type lignin and the COMT transgenic lignins. The two transgenic poplar lignins contain benzodioxanes (**H**) as major components in their lignins. Benzodioxanes are formed by β-O-4-coupling of a monolignol with a 5-hydroxyguaiacyl unit followed by internal trapping of the resultant quinomethane by the phenolic 5-OH.[22] With the down-regulation or gene-silencing of COMT in poplar it is proposed that 5-hydroxyconiferyl alcohol is shipped out into the cell wall where it is incorporated into the growing lignin polymer similarly to the incorporation of the other lignin monomers, coniferyl and sinapyl alcohols. NMR therefore provides diagnostic evidence for 5-hydroxyconiferyl alcohol incorporation into lignin to form novel benzodioxane structures (**H**). The benzodioxanes are readily apparent in the HMQC spectra of acetylated

Table 1 Quantitative subunit ratios derived from volume integrals of contours in the side-chain region of ^{13}C–^{1}H correlation (HMQC) spectra of acetylated lignins

Lignin	Unit type (relative proportion)						
Poplar	**A**	**B**	**C**	**D**	**H**	**X**	Σ(β-ethers)[c]
COMT-silenced[a]	53	13	5	6	18	5	78
COMT-antisense[b]	65	12	5	3	10	4	78
Control	88	3	7	0	0	2	88

[a] Sense-suppressed COMT down-regulated poplar.[13] [b] Antisense COMT down-regulated poplar.[9] [c] Σ(β-ethers) = **A** + **D** + **H**.

COMT-deficient transgenic poplar lignins (Figs. 2c–d): the α-proton at 4.98 ppm correlates with the α-carbon at 76.8 ppm, and the β-proton at 4.39 ppm correlates with the β-carbon at 75.9 ppm. The γ-correlations in 2D NMR overlap with those of other lignin units (structures **A** and **D**) but are revealed in 2D HMQC-TOCSY and 3D TOCSY-HSQC spectra as discussed below. The side-chain correlations agree well with those in a model compound for the *trans*-benzodioxane synthesized by biomimetic cross-coupling reactions between coniferyl alcohol and a 5-hydroxyguaiacyl unit.[22] The inclusion of 5-hydroxyconiferyl alcohol as a monomer in various lignins is becoming well documented;[23,31–34] these NMR spectra provide the proof that the resulting benzodioxanes can be prevalent structures in native lignins.

A reasonable quantification of all these units is by measuring volume integrals in the 2D HMQC spectra.[35] Since HMQC signal intensities depend largely on the $^{1}J_{C-H}$ coupling constants (which are all similar), and to a lesser degree to off-resonance effects, volume integrals are essentially equal for equal numbers of protons in the side-chains of all of these structures. Zhang and Gellerstedt have detailed how correction factors can be applied,[35] but they represent small corrections that are not required for comparative work. There are some units not included in these percentages since they have no resonances in the aliphatic side-chain region of the NMR spectra, specifically cinnamaldehyde endgroups and β-1 structures, so the data are comparative only and should not be interpreted as absolute percentages in lignins. Table 1 provides quantitative comparisons between the wild-type and both COMT-deficient transgenic lines. The data for all three samples parallel the qualitative observations made above from examining their respective HMQC spectra. The most prevalent β-ether units **A** are lower in COMT-deficient transgenics than in wild-type due to the increased guaiacyl contents (guaiacyl units have an additional major coupling pathway that syringyl units do not—5-β-coupling with monolignols, Fig. 1). These β-ether units **A** in the sense-suppressed COMT transgenic (53%) are at much lower levels than in the antisense COMT transgenic (65%) largely due to the higher level of 5-hydroxyconiferyl alcohol incorporation (into units **H**). **B** units are comparable in lignins from both COMT transgenics (13 and 12%), both reflecting a substantial increase over wild-type lignin (3%). **C** units decrease similarly in both transgenic lignins (5%) compared to wild-type lignin (7%) as a result of the lower syringyl content; although units **C** can derive from coniferyl or sinapyl alcohols, the longer lifetime of sinapyl alcohol radical results in higher **C** contents in high-syringyl lignins; *e.g.* 2% in spruce (a softwood derived predominantly from coniferyl alcohol), *vs.* 5% in beech (a hardwood derived from both coniferyl and sinapyl alcohols).[36] Cinnamyl alcohol endgroups **X** increase a few percentage points for both COMT transgenic poplars over wild-type. No **D** or **H** units are detectable in wild-type lignin but are present in both transgenic lignins. The 5-hydroxyconiferyl alcohol-derived benzodioxane units **H** are the second most abundant interunit type in the COMT transgenic (18%) and the third most abundant interunit type in the antisense COMT transgenic

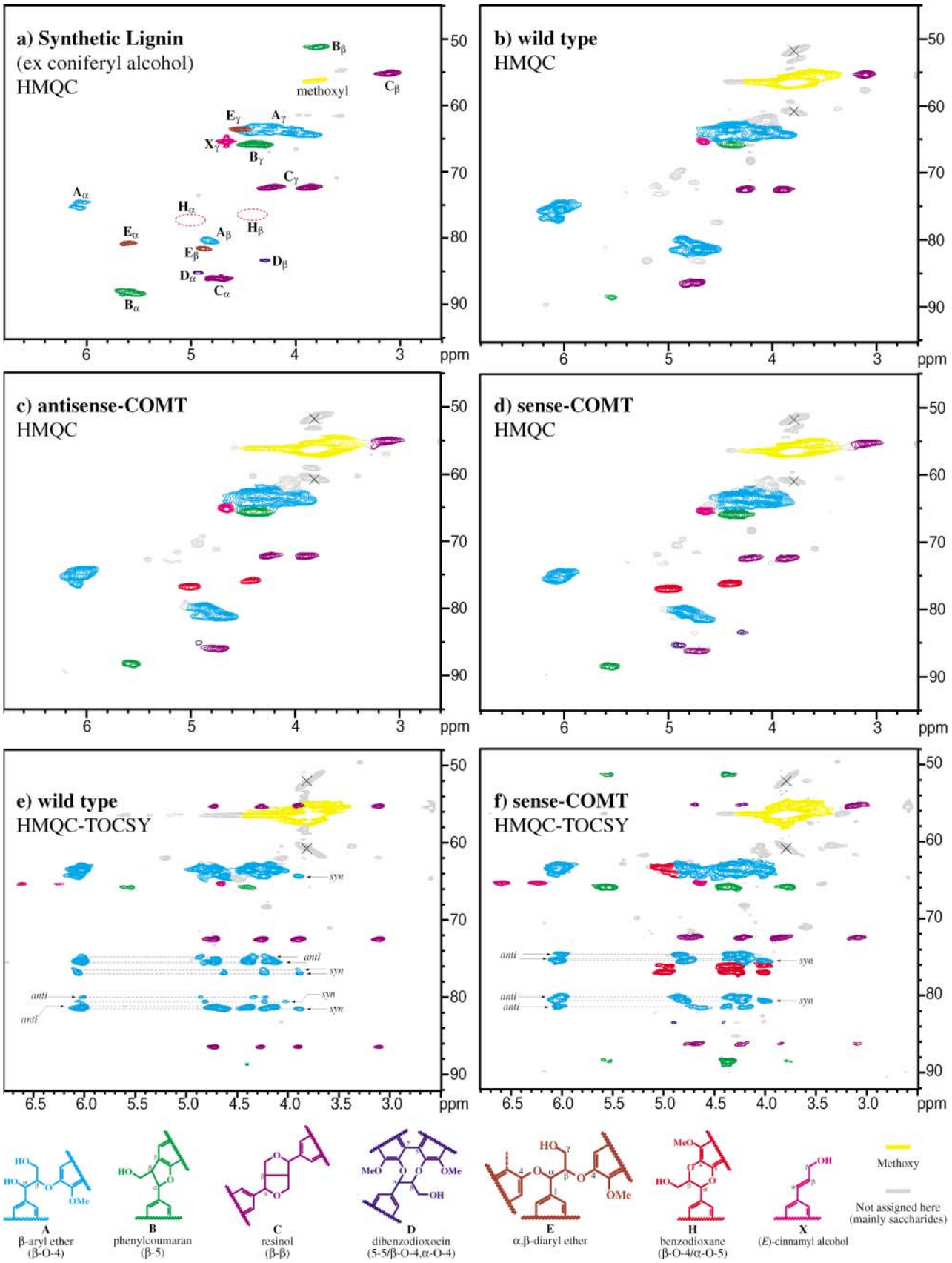

Fig. 2 2D ^{13}C–^{1}H correlative NMR spectra (360 MHz) of acetylated lignins. [(a)–(d)] Gradient-selected 2D HMQC spectra of acetylated lignins from (a) a synthetic lignin derived from coniferyl alcohol; (b) the wild-type poplar; (c) the antisense COMT transgenic poplar; (d) the sense-suppressed COMT transgenic poplar. [(e) and (f)] 2D HMQC-TOCSY (125 ms TOCSY mixing time) spectra of lignins from (e) the wild-type poplar; (f) the sense-suppressed COMT transgenic poplar. For β-ether **A** units, *syn*- (= *threo*-) and *anti*- (= *erythro*-) isomers[47] are indicated; *syn*-isomers are characterized by one high-field (<4 ppm) γ-proton. × = symmetrical artifacts from an intense methoxy signal.

(10%) following phenylcoumaran **B** units (12%). Since the acetone-soluble lignins analyzed by NMR represent about 80% of the total lignin in the sense-suppressed COMT transgenic and 64% in the antisense COMT transgenic, it seems logical that the benzodioxanes would remain major components of the lignins even if preferentially partitioned by the isolation

process. Interestingly despite the two COMT transgenic lignins compositional differences, their total β-ether frequencies (normal β-ether **A** plus dibenzodioxocins **D** plus benzodioxanes **H**) are around 78% compared to 88% for wild-type.

An interesting example of COMT-deficiency recently came to light. In a transgenic arabidopsis up-regulated for ferulate 5-hydroxylase (F5H; the hydroxylase necessary to generate syringyl units), benzodioxane units **H** can also be detected in HMQC spectra.[23] Apparently the COMT in these plants is not able to keep pace with the up-regulated F5H, again resulting in incorporation of 5-hydroxyconiferyl alcohol into the lignin. Using the same 2D quantification the **H**-unit abundance was determined at 10% in the isolated lignin for that transgenic. As noted recently, F5H and COMT appear to be a coactive pair of enzymes required for sinapyl alcohol production.[21]

The HMQC-TOCSY data (Figs. 2e, f) complement the HMQC data and help authenticate assignments of structures. In these experiments a carbon correlates with directly attached protons and others within the same proton-coupling network. Generally, all side-chain protons are within the same coupling network for any lignin structural unit. Additional data revealed by the HMQC-TOCSY spectra concern the stereochemistry of β-ether units and the identity of the γ-carbon/proton pairs in benzodioxane **H** units. Lignin-intermediate β-O-4-guaiacyl ether quinomethanes add water to give equal ratios of *syn*- and *anti*-isomers. By contrast analogous syringyl ether quinomethanes favor *anti*-isomers by about 80 : 20.[37] Consequently, the wild-type poplar lignin has predominantly *anti*-β-ethers (Fig. 2e), whereas the lignin from the sense-suppressed COMT transgenic, with a more guaiacyl-rich lignin, like softwoods and other guaiacyl-rich transgenics,[28] has more equal isomer distributions (Fig. 2f). More detailed assignments of *syn*- and *anti*-isomers for syringyl *vs*. guaiacyl and etherified *vs*. free phenolic units appear possible from the detail observed but will require the use of much more extensive model compound data than are currently available. The new benzodioxane **H** units, which are also strictly β-O-4 ethers (from initial β-O-4-coupling of 5-hydroxyconiferyl alcohol with a guaiacyl or syringyl unit) are not reflected in the **A** peaks, which come only from "normal" syringyl- and guaiacyl-β-O-4 units.

For the benzodioxane units **H**, the HMQC-TOCSY (with a 125 ms TOCSY mixing period) shows beautiful correlations between the α- and β-carbons and the full side-chain proton coupling network (Fig. 2f). The α-carbon correlates with its own α-proton (4.96 ppm), as well as the β-proton (4.36 ppm), and both γ-protons (4.23, 3.98 ppm). Seen just above these correlations are the β-carbon correlations to the α-proton, its own β-proton, and both γ-protons. As was discussed previously with the HMQC data, the benzodioxane unit **H** γ-carbon correlations are masked by β-ether unit **A** and dibenzodioxocin unit **D** γ-carbon correlations and the crucial C_γ–H_γ data for benzodioxane units **H** were therefore not available from the HMQC experiment. The benzodioxane correlations discernible in the HMQC-TOCSY spectrum reveal the γ-proton chemical shifts, which compare well with model compound data,[22] and therefore provide further diagnostic evidence for the presence of benzodioxanes in the COMT transgenic lignins.

3D TOCSY-HSQC NMR spectra

Three-dimensional (and/or higher-dimensional) NMR experiments are routinely applied to labeled proteins. Some success has come from applying the 3D HMQC-TOCSY (with one ^{13}C and two ^{1}H axes) to synthetic lignins,[27] and to uniformly ^{13}C-enriched lignins,[38,39] but these papers suggest that such experiments are difficult with real isolated unlabeled lignins. Fig. 3 shows the first 3D gradient-selected TOCSY-HSQC spectrum of a natural ^{13}C-abundance lignin. It is the acetylated lignin from the sense-suppressed COMT transgenic taken on a high magnetic field 750 MHz instrument. In less than 24 hours, the 3D experiment provided ample sensitivity to authenticate all of the major units in the natural ^{13}C-abundance sense-suppressed COMT transgenic lignin. Analogous experiments run on a 360 MHz instrument over 60 h were similarly successful. In the 3D TOCSY-HSQC experiment, spectra are acquired with three orthogonal dimensions, labeled F_1, F_2, and F_3. The acquired dimension is F_3 (proton). F_2 is carbon and F_1 is proton. A slice in the F_2–F_3 plane is basically a 2D ^{13}C–^{1}H HSQC spectrum at a given proton chemical shift (defined by the position along the proton F_1 axis). 2D F_2–F_3 projections/slices for the prominent structures in the lignin are shown in Figs. 3d–g. The slices show: a) the 3D contour map; b) a 2D HMQC for comparison with c) the first 2D slice in the F_2–F_3 plane (which is essentially a 2D composite spectrum of all the units on all the other planes); d–g) F_2–F_3 slices at various proton frequencies (in F_1) showing almost perfect isolation of the major structural units in HSQC-type sub-spectra.

When a proton frequency is unique to a given structure, a "pure" F_2–F_3 slice and HSQC of only that structure can be obtained. F_2–F_3 slices for both the β-aryl ether **A** units and phenylcoumaran **B** units at their respective α-proton frequency along the proton F_1 axis show this phenomenon nicely (Figs 3d, e). Only ^{13}C–^{1}H correlations for the given unit are seen in each slice. Fig. 3d is a "pure" slice of β-aryl ether **A** units at the α-proton frequency of 6.05 ppm. This slice shows both *syn*- and *anti*-β-ether isomers. Slices either side of this slice (not shown) resolve *syn*- from *anti*-isomers. Fig. 3e is a "pure" slice of phenylcoumaran **B** units at the α-proton frequency of 5.62 ppm. However, a "pure" F_2–F_3 slice of the resinol **C** units is not obtainable at any of its proton frequencies. The best slice represents resinol **C** units at its β-proton frequency of 3.09 ppm (Fig. 3f). At this particular frequency some saccharide/methoxy peaks are also detected but the slice uniquely isolates all of the resinol **C** units. The F_2–F_3 slice of the new benzodioxane structure is spectacular with its α-, β-, and γ-correlations fully resolved (Fig. 3g). In the 3D experiment, the γ-correlations of benzodioxane in the F_2–F_3 plane are nicely resolved and isolated from the plane at its α-proton frequency (5.01 ppm) as well as at the β-proton frequency (~4.47 ppm; not shown) along the F_1 axis. By obtaining a "pure" slice, there is no ambiguity between correlations of different structures. For example, the γ_1- and γ_2-correlations of the benzodioxane units that are unresolved from the γ_1- and γ_2-correlations of the β-aryl ether **A** units and the dibenzodioxocin **D** units in 2D spectra are unique to their respective 3D slices. This is the first reported identification of a new lignin component using 3D NMR experiments at natural abundance. The data in this slice agree with those from a benzodioxane model compound.[22]

Another detail regarding benzodioxane units **H**, the degree of etherification, is revealed in the 3D and 2D spectra. Unfortunately, the ball-milling step in the lignin isolation process produces extra phenolic groups, so isolated lignins have a higher phenolic content than natural lignins.[40] In acetylated lignins, units that were free phenolic in lignin become phenol-acetylated, whereas those that were originally etherified remain so. Phenol acetylation causes H_α in **H** units to move to a lower field (higher ppm). Thus, unetherified units have H_α at 5.04 ppm, whereas etherified units are at 4.96 ppm. The 2D slice for the 3D experiment shown in Fig. 3g has only a trace of correlations for the acetylated component. Other slices reveal slightly more. However, it appears that the benzodioxane units **H** are substantially etherified and therefore have been fully integrated into the polymer by further monolignol coupling reactions during lignification. Monomer substitution (5-hydroxyconiferyl alcohol for sinapyl alcohol) therefore appears to have been successfully accommodated in these transgenics, refuting the recent claim that "the free interchange of monomeric units in any biopolymer assembly" is unprecedented.[41] The substitution of L-fucose

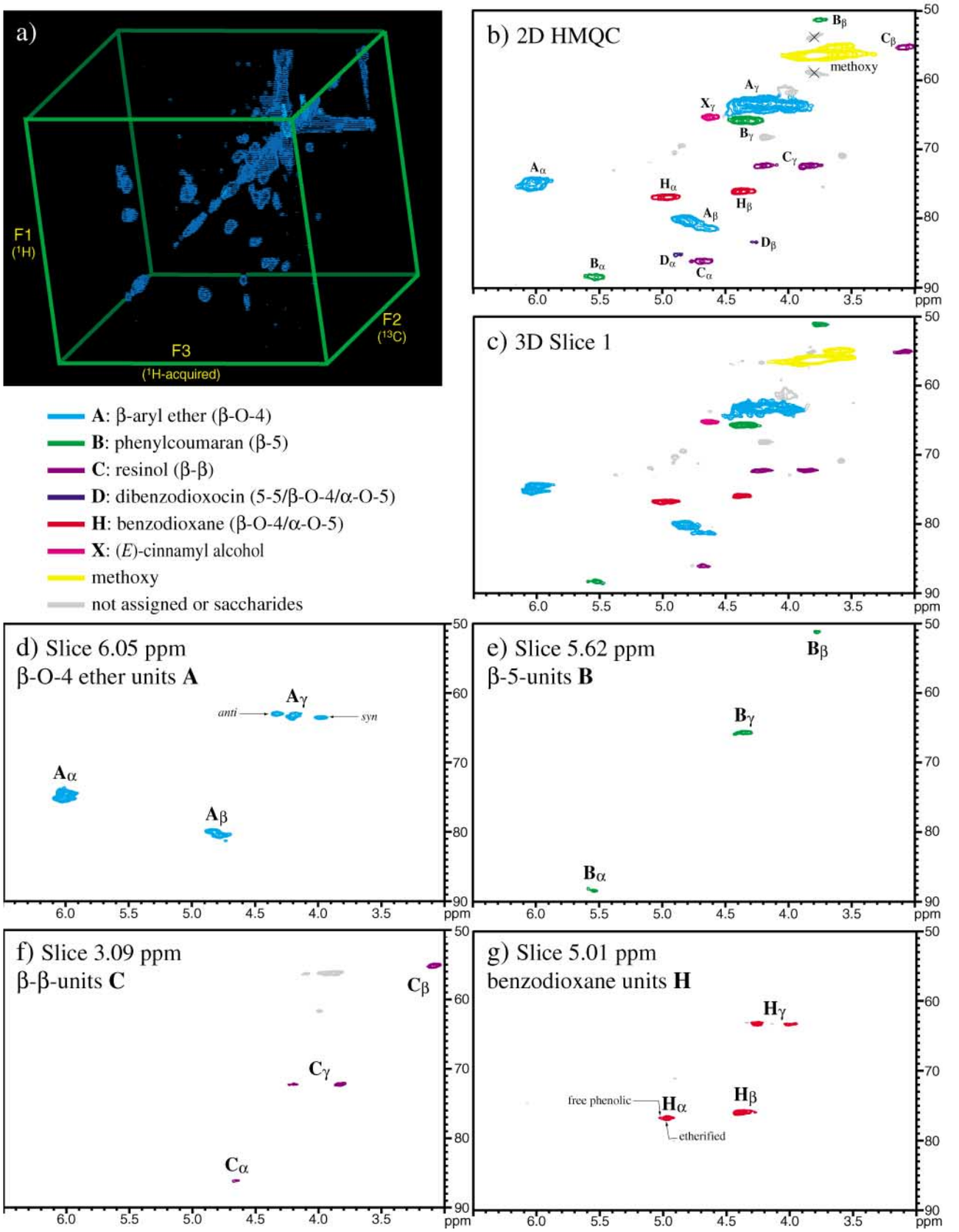

Fig. 3 3D NMR (750 MHz) "isolation" of the major units in COMT-deficient transgenic poplar lignins. (a) A 3D gradient-selected TOCSY-HSQC spectrum (70 ms TOCSY mixing time) of a natural ^{13}C-abundance lignin (acetylated) from the sense-suppressed COMT transgenic; (b) 2D gradient-selected HMQC spectrum; (c) the first F_2–F_3 plane which is essentially a 2D ^{13}C–^{1}H HSQC spectrum; (d)–(g) 2D F_2–F_3 slices for the major structural units (**A**, **B**, **C**, and novel **H**).

with L-galactose in fucose-deficient *mur1* mutants of arabidopsis is a previously documented monomer substitution occurring in polysaccharides,[42] where the polymer biosynthesis is more highly structurally controlled than in lignification.[43]

Conclusions

Many recent studies have shown that altering the expression of genes specific to the lignin biosynthetic pathway produces profound alterations in plant lignins. Our results demonstrate that

the manipulation of the monolignol supply for the purposes of changing lignin composition has been successful in poplar. Specifically, the down-regulation of caffeic acid *O*-methyltransferase (COMT) through either sense-suppression or antisense results in changes in the syringyl and guaiacyl components, and in the incorporation of 5-hydroxyconiferyl alcohol *as a monolignol* into the poplar lignin polymer producing lignins with novel units (benzodioxanes) in substantial quantities. The presence of these novel units in isolated lignins, as revealed by diagnostic 2D and 3D NMR experiments, indicates that the plant is capable of sending intermediate monolignols such as 5-hydroxyconiferyl alcohol out into the cell wall for incorporation. The resulting plants are phenotypically normal and healthy indicating no detrimental impact of such compositional shifts on the water transport and mechanical strengthening roles of lignin. The realization that novel units such as benzodioxanes can be tolerated in lignins should encourage further research into bioengineering plants with broad compositional changes in their lignins in order to achieve enhanced cell wall digestibility and/or reduction of negative environmental impacts of chemical pulping and bleaching related to papermaking.

Experimental

General

All reagents were purchased from Aldrich. The 2D (two-dimensional) 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). The conditions for all samples were ~100 mg of acetylated lignin in 0.4 ml of acetone-d_6, with the central solvent peak as internal reference (δ_H 2.04, δ_C 29.80). Experiments used were 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. The TOCSY spin lock period was 125 ms; the HMBC experiments used a 100 ms long-range coupling delay. The 3D (three-dimensional) NMR experiment was acquired on a Bruker DMX-750 instrument fitted with a 5 mm triple-resonance (^{1}H, ^{13}C, ^{15}N) gradient inverse probe. The 3D gradient-selected TOCSY-HSQC experiment was trivially modified to a two-channel version from "mlevietf3gs3d" (a 3 channel experiment). The TOCSY spin lock period was 70 ms in this case; 2D TOCSY experiments indicated that 70 ms also provided suitable TOCSY transfer especially for new benzodioxane structures. Other conditions were kept the same as those stated above. Carbon/proton designations are based on conventional lignin numbering (see structures in Fig. 1). Lignin sub-structures are labeled by the convention established in a recent book chapter.[27]

The synthetic lignin used in Fig. 2a was prepared by the action of peroxidase and hydrogen peroxide on coniferyl alcohol in which the methoxy was specifically ^{13}C-depleted and trideuterated (from a previous study).[44]

Plant materials and lignin isolation

Two independent sets of COMT-deficient poplar (*Populus tremula* × *P. alba*) samples were examined. Wild-type controls from both sets of samples were similar. The two COMT-down-regulated transgenic poplars resulted from different down-regulation mechanisms: (a) a *Populus tremula* × *P. alba* cv. 717 1-B4 transformed *via Agrobacterium tumefaciens* containing an antisense COMT cassette resulting in a line (ASB10B) with suppressed COMT activity,[9,12] and (b) an attempted sense overexpressed transgenic line (70SOMT-3) with a double 35S promoter where COMT activity was negligible due to a gene-silencing phenomenon.[13]

Lignin isolation from the control and antisense-COMT poplars was essentially as previously described.[28,45] Debarked stems were collected from 6-month-old poplars, ground, and extracted 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 isolated lignins represented 65 and 64% of the total lignin in the control and antisense-COMT poplars. A portion (200 mg) of the dioxane soluble lignin was acetylated overnight and washed with water–EDTA to remove trace metal contaminants.[45]

Isolated lignins from sense-suppressed COMT poplar previously described[12,46] were also used for NMR analysis. A portion (150 mg) of the isolated lignin was acetylated (as above) and partitioned into acetone-soluble and insoluble fractions. The acetone-soluble fraction (used for NMR) represented 82% of the original isolated lignin.

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References

1 K. V. Sarkanen and C. H. Ludwig, *Lignins: Occurrence, Formation, Structure and Reactions*, Wiley-Interscience, New York, 1971.
2 C. P. Vance, T. K. Kirk and R. T. Sherwood, *Annu. Rev. Phytopathol.*, 1980, **18**, 259.
3 H. M. Chang and K. V. Sarkanen, *Tappi*, 1973, **56**, 132.
4 V. L. Chiang and M. Funaoka, *Holzforschung*, 1990, **44**, 140.
5 U. N. Dwivedi, W. H. Campbell, J. Yu, R. S. S. Datla, R. C. Bugos, V. L. Chiang and G. K. Podila, *Plant Mol. Biol.*, 1994, **26**, 61.
6 W. Ni, N. L. Paiva and R. A. Dixon, *Transgenic Res.*, 1994, **3**, 120.
7 R. Atanassova, N. Favet, F. Martz, B. Chabbert, M.-T. Tollier, B. Monties, B. Fritig and M. Legrand, *Plant J.*, 1995, **8**, 465.
8 A. M. Boudet, C. Lapierre and J. Grima-Pettenati, *New Phytol.*, 1995, **129**, 203.
9 J. Van Doorsselaere, M. Baucher, E. Chognot, B. Chabbert, M.-T. Tollier, M. Petit-Conil, J.-C. Leplé, G. Pilate, D. Cornu, B. Monties, M. Van Montagu, D. Inzé, W. Boerjan and L. Jouanin, *Plant J.*, 1995, **8**, 855.
10 K. Inoue, V. J. H. Sewalt, G. M. Ballance, W. Ni, C. Sturzer and R. A. Dixon, *Plant Physiol.*, 1998, **117**, 761.
11 R. Zhong, W. H. Morrison III, J. Negrel and Z.-H. Ye, *Plant Cell*, 1998, **10**, 2033.
12 C. Lapierre, B. Pollet, M. Petit-Conil, G. Toval, J. Romero, G. Pilate, J. C. Leple, W. Boerjan, V. Ferret, V. De Nadai and L. Jouanin, *Plant Physiol.*, 1999, **119**, 153.
13 L. Jouanin, T. Goujon, V. de Nadaï, M.-T. Martin, I. Mila, C. Vallet, B. Pollet, A. Yoshinaga, B. Chabbert, M. Petit-Conil and C. Lapierre, *Plant Physiol.*, 2000, **123**, 1363.
14 H. Meyermans, K. Morreel, C. Lapierre, B. Pollet, A. De Bruyn, R. Busson, P. Herdewijn, B. Devreese, J. Van Beeumen, J. M. Marita, J. Ralph, C. Chen, B. Burggraeve, M. Van Montagu, E. Messens and W. Boerjan, *J. Biol. Chem.*, 2000, **275**, 36899.
15 M. Baucher, B. Monties, M. Van Montagu and W. Boerjan, *Crit. Rev. Plant Sci.*, 1998, **17**, 125.
16 A.-M. Boudet, *Trends Plant Sci.*, 1998, **3**, 67.
17 R. W. Whetten, J. J. MacKay and R. R. Sederoff, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 1998, **49**, 585.
18 C. Y. Chen, M. Baucher, J. H. Christensen and W. Boerjan, *Euphytica*, 2001, **118**, 185.
19 T. Higuchi, in *Biosynthesis and Biodegradation of Wood Components*, ed. T. Higuchi, Academic Press, Orlando, FL, 1985, p. 557.
20 J. Van Doorsselaere, B. Dumas, M. Baucher, B. Fritig, M. Legrand, M. Van Montagu and D. Inzé, *Gene*, 1993, **133**, 213.
21 L. Li, J. L. Popko, T. Umezawa and V. L. Chiang, *J. Biol. Chem.*, 2000, **275**, 6537.
22 J. Ralph, C. Lapierre, F. Lu, J. M. Marita, G. Pilate, J. Van Doorsselaere, W. Boerjan and L. Jouanin, *J. Agric. Food Chem.*, 2001, **49**, 86.

23 J. Ralph, C. Lapierre, J. Marita, H. Kim, F. Lu, R. D. Hatfield, S. A. Ralph, C. Chapple, R. Franke, M. R. Hemm, J. Van Doorsselaere, R. R. Sederoff, D. M. O'Malley, J. T. Scott, J. J. MacKay, N. Yahiaoui, A.-M. Boudet, M. Pean, G. Pilate, L. Jouanin and W. Boerjan, *Phytochemistry*, 2001, **57**, 993.
24 A. G. Palmer, J. Cavanagh, P. E. Wright and M. Rance, *J. Magn. Reson., Ser. A*, 1991, **93**, 151.
25 L. E. Kay, P. Keifer and T. Saarinen, *J. Am. Chem. Soc.*, 1992, **114**, 10663.
26 L. Lerner and A. Bax, *J. Magn. Reson.*, 1986, **69**, 375.
27 J. Ralph, J. M. Marita, S. A. Ralph, R. D. Hatfield, F. Lu, R. M. Ede, J. Peng, S. Quideau, R. F. Helm, J. H. Grabber, H. Kim, G. Jimenez-Monteon, Y. Zhang, H.-J. G. Jung, L. L. Landucci, J. J. MacKay, R. R. Sederoff, C. Chapple and A. M. Boudet, in *Advances in Lignocellulosics Characterization*, eds. D. S. Argyropoulos and T. Rials, TAPPI Press, Atlanta, GA, 1999, p. 55.
28 J. Marita, J. Ralph, R. D. Hatfield and C. Chapple, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 12328.
29 P. Karhunen, P. Rummakko, J. Sipilä, G. Brunow and I. Kilpeläinen, *Tetrahedron Lett.*, 1995, **36**, 4501.
30 P. Karhunen, P. Rummakko, J. Sipilä, G. Brunow and I. Kilpeläinen, *Tetrahedron Lett.*, 1995, **36**, 169.
31 B. H. Hwang and A. Sakakibara, *Holzforschung*, 1981, **35**, 297.
32 C. Lapierre, M. T. Tollier and B. Monties, *C. R. Acad. Sci., Ser. 3*, 1988, **307**, 723.
33 G. Jacquet, *Structure et réactivité des lignines de graminées et des acides phénoliques associés: développement des méthodologies d'investigation*, PhD thesis, Institut National Agronomique, 1997.
34 S. Suzuki, T. B. T. Lam and K. Iiyama, *Phytochemistry*, 1997, **46**, 695.
35 L. Zhang and G. Gellerstedt, *Achieving quantitative assignment of lignin structure by combining* ^{13}C and HSQC NMR techniques, 6th European Workshop on Lignocellulosics and Pulp Bordeaux, France, 2000, p. 7.
36 E. Adler, *Wood Sci. Technol.*, 1977, **11**, 169.
37 G. Brunow, O. Karlsson, K. Lundquist and J. Sipilä, *Wood Sci. Technol.*, 1993, **27**, 281.
38 I. Kilpeläinen, E. Ämmälahti, G. Brunow and D. Robert, *Tetrahedron Lett.*, 1994, **35**, 9267.
39 E. Ämmälahti, G. Brunow, M. Bardet, D. Robert and I. Kilpeläinen, *J. Agric. Food Chem.*, 1998, **46**, 5113.
40 H.-M. Chang, E. B. Cowling, W. Brown, E. Adler and G. Miksche, *Holzforschung*, 1975, **29**, 153.
41 N. G. Lewis, *Curr. Opin. Plant Biol.*, 1999, **2**, 153.
42 E. Zablackis, W. S. York, M. Pauly, S. Hantus, W. D. Reiter, C. C. S. Chapple, P. Albersheim and A. Darvill, *Science*, 1996, **272**, 1808.
43 R. R. Sederoff, J. J. MacKay, J. Ralph and R. D. Hatfield, *Curr. Opin. Plant Biol.*, 1999, **2**, 145.
44 J. Ralph, Y. Zhang and R. M. Ede, *J. Chem. Soc., Perkin Trans. 1*, 1998, 2609.
45 J. Ralph, R. D. Hatfield, S. Quideau, R. F. Helm, J. H. Grabber and H.-J. G. Jung, *J. Am. Chem. Soc.*, 1994, **116**, 9448.
46 C. Lapierre, B. Monties and C. Rolando, *Holzforschung*, 1986, **40**, 113.
47 J. Ralph, R. F. Helm, R. C. Fort and T. J. Elder, *J. Chem. Soc., Perkin Trans. 1*, 1994, 2117.