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Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and an indicator for cinnamoyl CoA reductase deficiency)

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Summary

A molecular marker compound, derived from lignin by the thioacidolysis degradative method, for structures produced when ferulic acid is incorporated into lignin in angiosperms (poplar, Arabidopsis, tobacco), has been structurally identified as 1,2,2-trithioethyl ethylguaiacol [1-(4-hydroxy-3-methoxyphenyl)-1,2,2-tris(ethylthio)ethane]. Its truncated side chain and distinctive oxidation state suggest that it derives from ferulic acid that has undergone *bis*-8-*O*-4 (cross) coupling during lignification, as validated by model studies. A diagnostic contour for such structures is found in two-dimensional ^{13}C–^{1}H correlated (HSQC) NMR spectra of lignins isolated from cinnamoyl CoA reductase (CCR)-deficient poplar. As low levels of the marker are also released from normal (i.e. non-transgenic) plants in which ferulic acid may be present during lignification, notably in grasses, the marker is only an indicator for CCR deficiency in general, but is a reliable marker in woody angiosperms such as poplar. Its derivation, together with evidence for 4-*O*-etherified ferulic acid, strongly implies that ferulic acid is incorporated into angiosperm lignins. Its endwise radical coupling reactions suggest that ferulic acid should be considered an authentic lignin precursor. Moreover, ferulic acid incorporation provides a new mechanism for producing branch points in the polymer. The findings sharply contradict those reported in a recent study on CCR-deficient Arabidopsis.

Keywords: lignin monomer, downregulation, transgenic, mutant, radical coupling, NMR.

Introduction

Plant cell-wall utilization in various natural and industrial processes is generally inhibited by the lignin component. Such processes include ruminant digestion, chemical pulping, and biomass saccharification for ethanol production. Considerable interest has therefore focused on producing low-lignin plants, but it has also become evident over the last decade that reducing plant cell-wall cross-linking or inducing structural and compositional changes in the lignin polymer produces reactivity and property changes that may be beneficial for many processes (reviewed by Baucher *et al.*, 2003; Boerjan *et al.*, 2003; Ralph *et al.*, 2004b).

Recent analysis has shown that transgenic plants perturbed in monolignol biosynthesis may export products from incomplete biosynthesis to the cell wall, resulting in novel (or more abundant) structures in the polymer. For example, CAD (cinnamyl alcohol dehydrogenase)

downregulation in angiosperms results in increased incorporation of hydroxycinnamaldehydes (precursors to the monolignols) during the lignification process (Kim *et al.*, 2003; Lapierre *et al.*, 2004). Hydroxycinnamaldehydes incorporated via 8-*O*-4 coupling with the growing polymer give rise to novel unsaturated styryl ether structures in the lignin that release diagnostic thioacidolysis markers (Kim *et al.*, 2002; Lapierre *et al.*, 2004). Such molecular marker compounds allow elucidation of the impact of gene downregulation on the lignification process.

CCR (cinnamoyl CoA reductase) is the key enzyme that reduces pathway intermediates from the cinnamic acid oxidation level to the cinnamaldehyde level in the pathway to the final cinnamyl alcohols, the monolignols **M1** (Figure 1; Boerjan *et al.*, 2003). Polymeric lignins derive primarily from coniferyl alcohol (**M1G**) and sinapyl alcohol (**M1S**). In previous studies, downregulation of CCR in tobacco suggested that structures at the ferulic acid oxidation level accumulated (Dauwe *et al.*, 2007; Piquemal *et al.*, 1998; Ralph *et al.*, 1998). Hydroxycinnamic acid amides have been repeatedly implicated in the inducible defense of some plant species, particularly in Solanaceae. In the case of tobacco, potato or tomato, the main representative of such compounds is tyramine ferulate (Negrel and Javelle, 1997). Evidence is

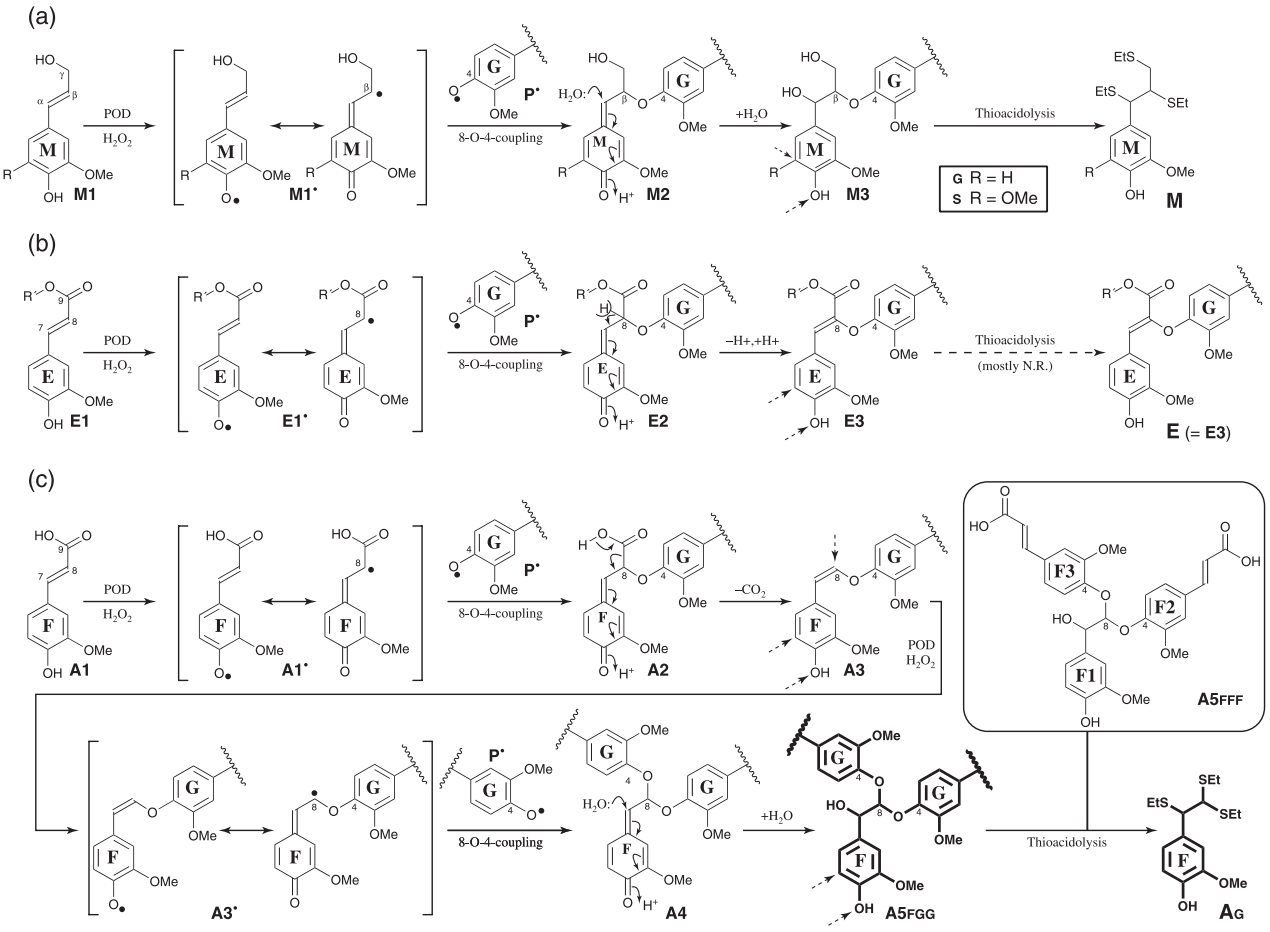


Figure 1. Reaction schemes for incorporating various monomers into lignins via 8-*O*-4 coupling.
(a) Monolignol **M1** radicals **M1**· (for which two of the resonance forms are shown) cross-couple with guaiacyl and syringyl radicals **P**· (illustrated with only guaiacyl radicals **PG**· for simplicity) in the growing polymer, via β-*O*-4 coupling (= 8-*O*-4 coupling; by convention, α, β and γ are used for the side chain positions of hydroxycinnamyl alcohols, but are labeled 7, 8, and 9 in hydroxycinnamates and hydroxycinnamaldehydes). The intermediate quinone methide **M2** is aromatized via nucleophilic water addition to produce the β-ether unit **M3** in lignin. Thioacidolysis of units **M3**, even if they further incorporate into the polymer by 4-*O*-β coupling, produces the diagnostic guaiacyl monomers **M** (as pairs of diastereomers) (Figure 2).
(b) Analogous 8-*O*-4 coupling of ferulate ester **E1** produces an intermediate quinone methide **E2** which preferentially re-aromatizes by elimination of the acidic 8-proton, forming products **E3**. Thioacidolysis essentially leaves such structures intact (i.e. uncleaved).
(c) Analogous 8-*O*-4 coupling of ferulic acid **A1** produces an intermediate quinone methide **A2** that will efficiently re-aromatize via CO_2 loss. The resulting truncated side chain product **A3** is now uniquely capable of radical coupling at its 8-position (again) as well as on the ring. The second 8-*O*-4 coupling reaction produces the *bis*-8-*O*-4-ether **A5** via the usual quinone methide **A4**. As a result of the double-oxidative coupling reaction, the 8-carbon in **A5** is at the oxidation level of an aldehyde. Inset structure **A5FFF** is the dehydrotrimer produced from ferulic acid. Thioacidolysis of compounds **A5** (or their 4-*O*-ethers) yields the marker product **AG**. Dashed arrows on compounds **M3**, **E3**, **A3** and **A5** indicate sites for further combinatorial radical coupling during lignification. N.R. indicates no reaction. The small **G** descriptor indicates guaiacyl, **S** indicates syringyl in all cases, e.g. **M1G** is coniferyl alcohol, **M1S** is sinapyl alcohol.

quite strong that tyramine ferulate co-polymerizes into tobacco lignins (Ralph *et al.*, 1998). A comment on a opposing view is given in Appendix S1.

Here we report identification of the structure of a valuable marker compound that is present in lignins from several CCR-deficient plant species, and demonstrate its derivation from novel structures produced when ferulic acid is incorporated into lignins. As will be shown, this marker has two rather profound new implications regarding lignification – first, ferulic acid itself is apparently an authentic lignin monomer, and, second, a new mechanism for chain branching in lignins is evident from a unique aspect of ferulic acid's incorporation mechanism.

Results and discussion

Discovery of the thioacidolysis marker compound

The INRA group in Thiverval-Grignon has a long history in the development and application of the analytical thioacidolysis method to define lignin composition and aspects of its structure (Lapierre, 1993). The method selectively cleaves β-aryl ether units in lignin polymers, releasing low-molecular-mass thio-ethylated compounds. For example, the guaiacyl and syringyl monomers (**M**, Figure 1, together with the *p*-hydroxyphenyl monomer, not shown) are routinely used to determine the ratio of such units involved in β-ether units and to draw inferences regarding the composition of lignins from their monomers, coniferyl and sinapyl alcohols (**M1G** and **M1S**, respectively).

We have routinely applied thioacidolysis methods to lignin biosynthetic pathway mutants and transgenics to delineate the effects of perturbing various steps in the pathway. Thioacidolysis was found to produce diagnostic marker compounds from hydroxycinnamaldehydes that are incorporated endwise into lignins by 8-*O*-4 coupling, particularly in CAD-deficient angiosperm mutants and transgenics (Kim *et al.*, 2002; Lapierre *et al.*, 2004). In a similar way, we have noted over many years that CCR deficiency produces a single 'marker' compound (**AG**) during total-ion chromatography (Figure 2b). As precise measurement of CCR activity in plant extracts is a difficult task (Baltas *et al.*, 2005; Chabannes *et al.*, 2001; Goujon *et al.*, 2003; Kawasaki *et al.*, 2006), we cannot, as in the case of CAD deficiency, correlate enzyme levels with the marker level. From a limited number of analyses, however, the level of this compound appears to track the degree of CCR deficiency in poplar, tobacco and Arabidopsis (C.L., unpublished results). We describe this marker as only an indicator for CCR deficiency here because there is a background level from wild-type control plants, and because other plants in which the incorporation of ferulic acid (and ferulate esters) has been widely implicated, notably grasses, release more elevated levels of this compound.

Structural identification of the marker

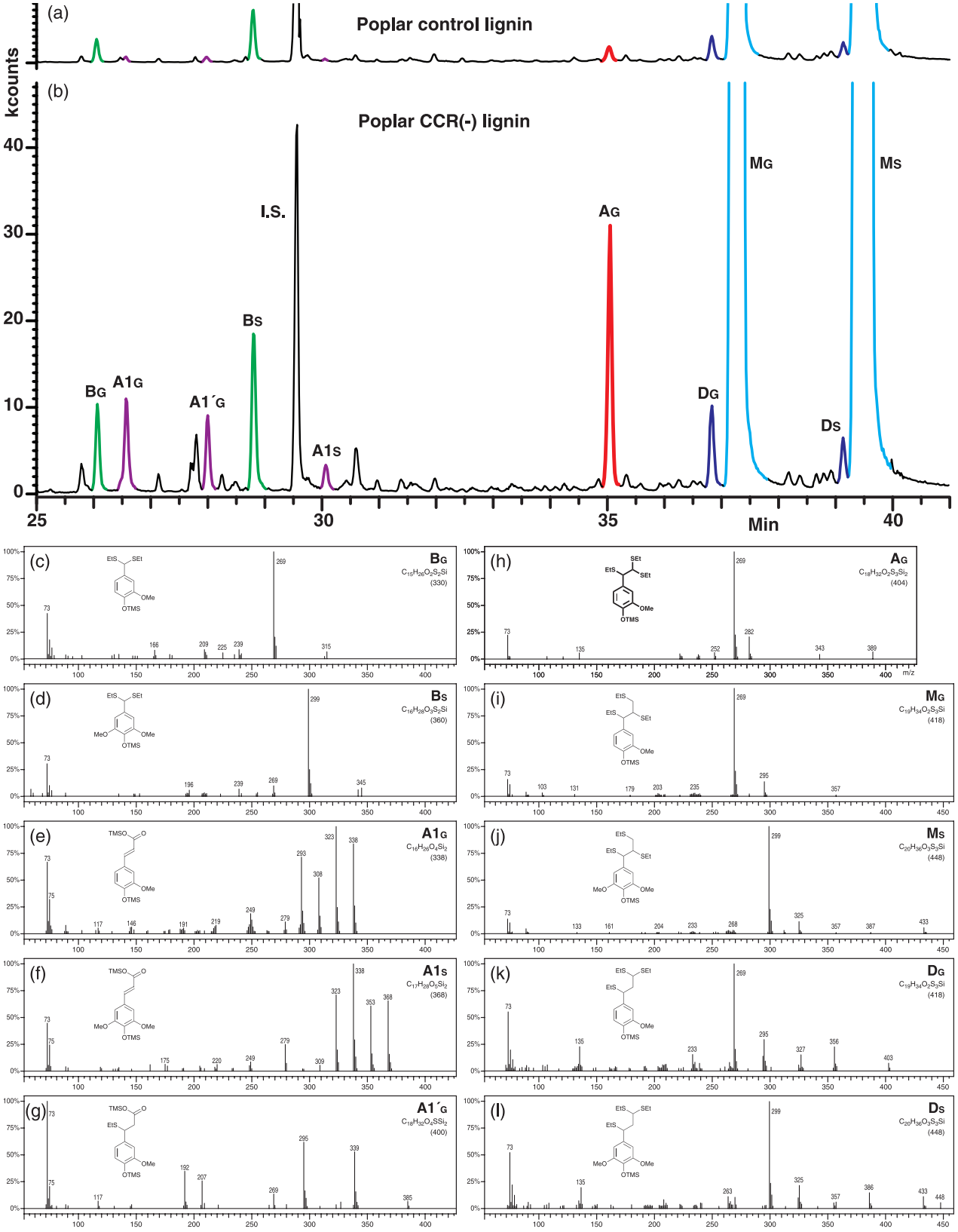
Mass spectrometry (Figure 2h) suggested that the marker compound **AG** was a tri-thioethyl derivative of ethylguaiacol. Raney–Ni desulfurization of the thioacidolysis product produces simple ethylguaiacol from this component. The ethyl side chain is somewhat unusual as lignin units typically have propyl sidechains, although minor side chain degradation can occur during thioacidolysis of some types of structural units (Rolando *et al.*, 1992). Mass spectrometry also indicated the regiochemistry of the thioethyl groups, suggesting that the structure was the 1,2,2-trithioethyl ethylguaiacol **AG** (Figures 1 and 2); the primary fragment (and base peak) at *m*/*z* 269 (Figure 2h) is from the benzylic cation resulting from cleaving the C7–C8 bond; the other benzyl cation produced by splitting off EtS· is found at 343, a more minor peak. The oxidation state of the 8- or β-carbon, at the level of an aldehyde, was a puzzling but important clue to identification of the source of such structures.

Identifying the in planta *source of the marker*

Mechanistic insight comes from identifying the source of this thioacidolysis marker. It seemed logical that products at the oxidation level of the acid (including the CoA thioester itself) might accumulate when CCR is downregulated. As noted above, there were indications that tyramine ferulate levels were elevated in tobacco as a possible result of CCR downregulation (Piquemal *et al.*, 1998). We had some indications that the marker detected here in CCR-downregulated poplar, Arabidopsis and tobacco related to the incorporation of ferulic acid; increased levels of cell wall-linked ferulic acid have already been reported in CCR-deficient *A. thaliana* (Goujon *et al.*, 2003) and tobacco (Chabannes *et al.*, 2001) lines.

The truncated side chain and the oxidation state of the marker **AG** were revealing. We are familiar with the incorporation of ferulate esters (**E1**) into lignins from studying such processes in grasses and in model systems, as

Figure 2. GC–MS data for trimethylsilylated thioacidolysis products from control and CCR-deficient poplar lignins.
(a) Total-ion chromatogram for products from control poplar lignin.
(b) Total-ion chromatogram for thioacidolysis products from CCR-deficient poplar lignin, showing the CCR marker **AG** together with the traditional guaiacyl and syringyl thioacidolysis monomers **MG** and **MS**, as well as other products of interest, including structures **D** from cinnamaldehyde end groups, structures **B** from benzaldehyde endgroups, and structures **A1** from 4-*O*-etherified ferulic (**A1G** and **A1′G**) and sinapic acids (**A1S**). Chromatograms (a) and (b) are scaled the same relative to the internal standard (I.S.).
(c–l) Mass spectra of the peaks identified in the total-ion chromatogram from CCR-deficient poplar lignin (b).



reviewed by Ralph *et al.* (2004a). None of these pathways produced products that could release thioacidolysis products with the truncated side chain and the observed oxidation state. However, on examination of the anticipated pathway by which free ferulic acid (**A1**) itself may enter into the radical (cross) coupling reactions of lignification, a pathway explaining both of the puzzling features of the marker became evident. As shown in (Figure 1c), 8-*O*-4 cross-coupling of ferulic acid with the phenolic end of a lignin unit produces an intermediate quinone methide **A2** in a way that is analogous to the production of **M2** by β-*O*-4 cross-coupling of the monolignol **M1** (Figure 1a). The difference is that the quinone methide **M2** in the case of coniferyl alcohol requires re-aromatization by water addition. In the case of ferulate ester (**E1**) coupling (Figure 1b), 8-proton elimination from the quinone methide **E2** competes with such water addition, giving a cinnamate-8-*O*-4-ether (**E3**), which is not cleaved by thioacidolysis (see Experimental procedures). For ferulic acid (**A1**), however, decarboxylation of quinone methide **A2** is favored over both processes, producing the styryl ether structure **A3**, as inferred from ferulic acid radical coupling products (see below; Münzenberger *et al.*, 2003; Ward *et al.*, 2001). This explains the truncated side chain, but does not explain how the oxidation state of the thioacidolysis product arises; **A3** produces only the 1,2-dithioethyl guaiacol upon thioacidolysis.

The key difference with ferulic acid incorporation lies in what happens in the subsequent coupling step. Obviously, the radical from this new free-phenolic end group is capable of further radical coupling with other lignin monomers or with other phenolic radicals on the polymer. The unsaturated and truncated side chain in **A3** allows a pathway that has no analog in the products with full 3-carbon side chains, namely the possibility of a second coupling to the 8-position (which may even be favored). A second 8-*O*-4 (cross) coupling reaction now produces a *bis*-8-*O*-4-ether **A5** (via quinone methide **A4**), a product that has the correct oxidation state to generate the observed thioacidolysis product. That such a reaction occurs is already well demonstrated; dehydrogenation (radical coupling) of ferulic acid produces compound **A5FFF** as a major trimer (Münzenberger *et al.*, 2003; Ward *et al.*, 2001), as described in Experimental procedures.

Do such *bis*-8-*O*-4-coupled compounds produce the correct thioacidolysis product? The thioacidolysis marker is the major product from trimer **A5FFF** (see Figure S4). Also released were the products **A1G** (ferulic acid itself) and **A1′G** (the ferulic acid EtSH addition product). Such ferulic acid-derived monomers are also readily detected in thioacidolysis chromatograms from poplar (Figure 2 and Table 1) as well as Arabidopsis (not shown) and tobacco (Figure S2). The marker **AG**, together with the ferulic acid products **A1G** and **A1′G** are also efficiently released from a synthetic lignin incorporating ferulic acid, a lignin in which the *bis*-8-*O*-4-coupled unit can be readily validated by NMR (see below, and Figure 3d). These observations are sufficient to strongly suggest that the thioacidolysis marker results from ferulic acid incorporation into lignin.

Table 1 Thioacidolysis yield for minor monomers (μmol g^{-1} of washed and dried wood or of DHP)

Sample	Major thioacidolysis monomers (**MG** and **MS**) Total yield	Molar ratio (**MS**/**MG**)	CCR marker (**AG**)	Ferulic acid and its addition product (**A1G** and **A1′G**)
Poplar WT	470 ± 18	2.34 ± 0.04	0.24 ± 0.02	Trace
Poplar FS40 sense	332 ± 21	2.14 ± 0.04	1.35 ± 0.15[a]	0.27 ± 0.02
Poplar FAS13 antisense	229 ± 11	1.96 ± 0.04	8.23 ± 0.30[b]	3.78 ± 0.27
DHP	637 ± 6	1.12 ± 0.03	29.1 ± 0.9	8.06 ± 0.83

Values are means (± standard error between duplicates).
AG, G-CHR-CHR_2; **A1G**, G-CH = CH-COOH; **A1′G**, G-CHR-CH_2-COOH (R = SEt).
[a]1.28% of G-monomers.
[b]10.6% of G-monomers.

Supporting evidence comes from the following experiments. A correlation peak indicating that the ferulic acid had indeed undergone *bis*-8-*O*-4 (cross) coupling was readily observed in two-dimensional HSQC NMR spectra (Figure 3d) from a synthetic lignin prepared from coniferyl and sinapyl alcohols (50:50) and 6% [8-^{13}C]ferulic acid. The ^{13}C–^{1}H correlation peak at approximately 104/6.0 ppm is diagnostic of the 8-carbon/proton in structures **A5**. Incidentally, a previous paper describing the ferulic acid trimer (Ward *et al.*, 2001) incorrectly assigned these ^{13}C and ^{1}H peaks to the 7-carbon/proton. Long-range ^{13}C–^{1}H correlation experiments (not shown) indicated that coupling had occurred primarily with guaiacyl units, as might be expected for steric and electronic reasons. Thioacidolysis of this synthetic co-polymer produced the characteristic marker compound **AG**. Interestingly, as shown in the inset to (Figure 3d), the synthetic co-polymer has the same orange coloration as the xylem from CCR-deficient zones in various plants. Similar coloration as synthetic co-polymers was found previously when ferulic acid was administered to fresh stem sections of control plants (Piquemal *et al.*, 1998). The orange coloration need not result directly from the incorporation of ferulic acid into the lignin, but seems to be associated with the presence of ferulic acid during coupling. In the same manner, the red coloration in CAD-deficient plants, and the similar color of synthetic lignins produced from monolignols and coniferaldehyde, is attributed to the presence of coniferaldehyde but not to its actual incorporation into the polymer (see Appendix S2).

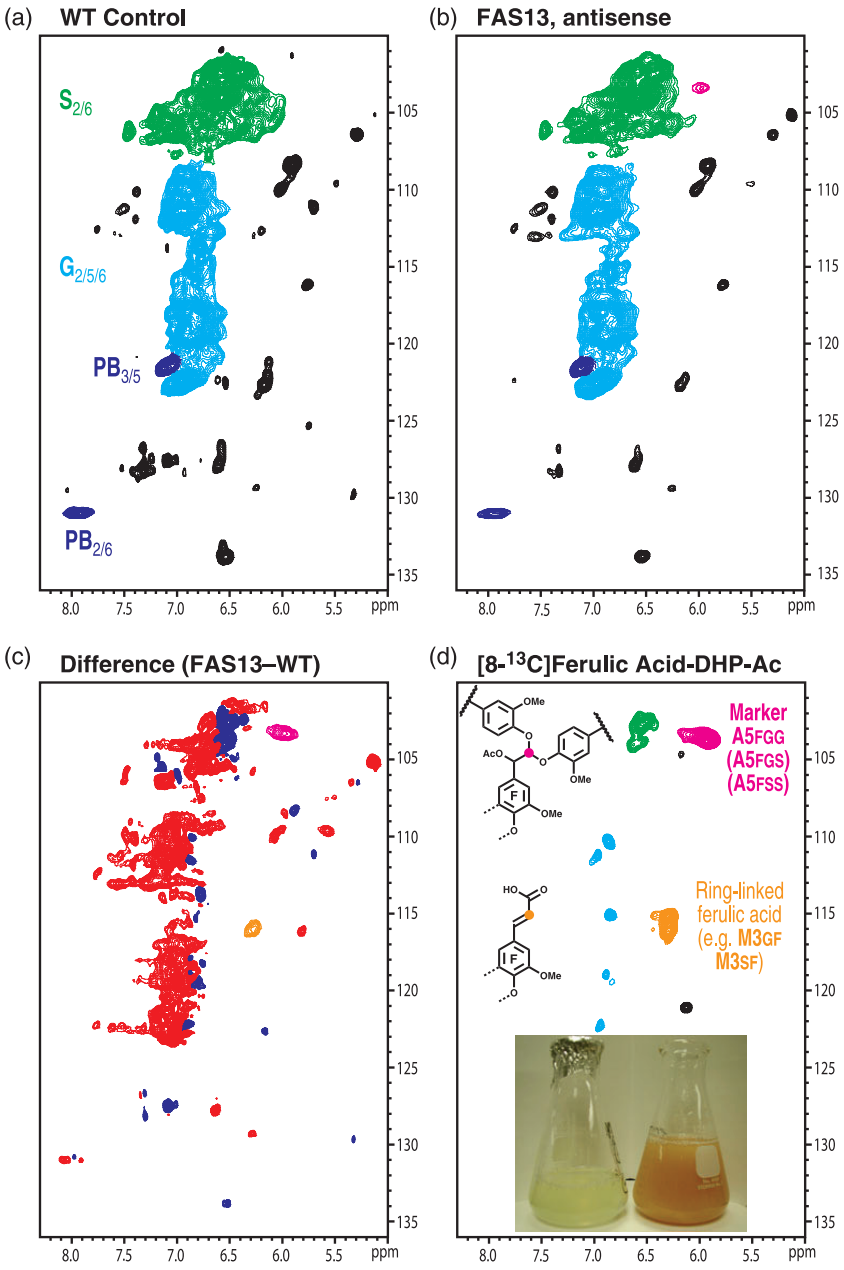


Figure 3. Partial HSQC NMR spectra (aromatic regions) from control, antisense CCR and synthetic lignin (DHP) samples.
(a) Wild-type control poplar acetylated cellulolytic enzyme lignin.
(b) 'FAS13' antisense CCR acetylated cellulolytic enzyme lignin. The magenta contour is consistent with the 8-C/H of marker **A5**.
(c) Difference spectrum: the red peaks (mainly guaiacyl) are higher in the transgenic sample, the blue peaks are lower (mainly syringyl, and more pronounced than they appear – the blue contours are very high); the higher ferulic acid-derived peaks are colored magenta and orange.
(d) Same region from a synthetic (50:50 guaiacyl:syringyl) lignin incorporating 6% [8-^{13}C]ferulic acid. Due to the label, the 8-C/H correlations from the marker **A5** (magenta) and from ring-linked ferulic acid end groups (e.g. **M3**, orange) show up clearly. Both correlations match those in the antisense spectra, as shown most clearly in the difference spectrum of (c). The inset shows flasks of control DHP (left, derived from coniferyl alcohol and sinapyl alcohol only) and the orange coloration that results when ferulic acid is a component of the DHP precursors (right). The xylem tissue in many CCR-deficient tissues has a similar orange coloration.

A ^{13}C–^{1}H correlation diagnostic for structure **A5**, the marker-originating moiety, can also be detected in NMR spectra of CCR-downregulated poplar (Figure 3b,c) and tobacco. The level of the component is low; uncorrected volume integration indicates a marker contour level at only approximately 1% of the syringyl volume, although the volumes underestimate the actual relative amounts of marker versus syringyl moieties as the $^{1}J_{C-H}$ coupling constant (anticipated to be about 160 Hz for an acetal) is considerably different from the value chosen in the experiment (145 Hz, a compromise between normal aliphatic and aromatic coupling constants). The synthetic lignin (Figure 3d), the poplar difference spectrum (Figure 3c) and CCR-deficient tobacco spectra (not shown) also display evidence for the ring-attached ferulic acid (116/6.3 ppm, the 8-C/H correlation), e.g. **M3GF** (Figure 4), resulting from radical coupling of a monolignol (at its β-position) with ferulic acid (at its 4-*O*-, or perhaps 5-position). All these observations provide strong support for the involvement of free ferulic acid **A1G** in lignification in CCR-deficient plants.

Further supporting evidence for the origin of the marker comes from noting other plants in which this thioacidolysis marker compound arises. Over many years, marker **AG** has also been observed at low levels in thioacidolysates from grasses. Grasses are well-known to contain ferulate esters, but we consider such esters an unlikely source of this

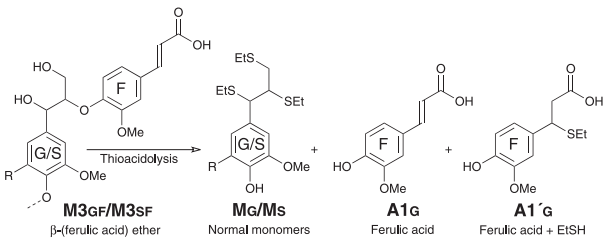


Figure 4. Thioacidolysis products from 4-*O*-linked ferulic acid in lignins. Coupling of ferulic acid (at its 4-position) with a monolignol (at its β-position) can create heterodimers that become further incorporated into the lignin polymer. Together with the traditional thioacidolysis monomer **MG**, the resulting ethers release ferulic acid **A1G** and an ethanethiol addition product **A1′G** during thioacidolysis. Both compounds can be found at elevated levels in chromatograms from CCR-deficient poplar (Figure 1b and Table 1), as well as Arabidopsis (not shown) and tobacco (see Figure S2).

particular marker. It presumably derives from the small amounts of ferulic acid that are also present and incorporated during lignification. Incidentally, using sensitive detection methods (and particularly looking at selected-ion chromatograms in mass spectral data), traces of the marker **AG** (and the other products **A1G** and **A1′G**) can usually be detected in the control plants (see, for example, Figure 2a for poplar). We assume that this indicates that small amounts of ferulic acid are naturally exported to the wall to enter the lignification process even under 'normal' circumstances. We have found previously that pathway-derived monomers, e.g. 5-hydroxyconiferyl alcohol, that appeared to be present only in caffeic acid *O*-methyltransferase (COMT)-downregulated transgenics can almost always be found using sufficiently sensitive methods in non-transgenic plants (Li *et al.*, 2003).

Apparent conflicts in observations regarding CCR downregulation

The findings here are in stark contrast to those reported in a recent study on CCR-deficient Arabidopsis (Laskar *et al.*, 2006). We consider that together with earlier papers (Anterola and Lewis, 2002; Davin and Lewis, 2005), this paper (Laskar *et al.*, 2006) mischaracterizes the findings in Ralph *et al.* (1998) and asserts that tyramine ferulates are proposed there as general signatures of CCR deficiency. No such claim is made in Ralph *et al.* (1998), either explicitly or implied, in the references cited or elsewhere. It is also not suggested in the study on the initial identification of the *irx4* Arabidopsis mutant (Jones *et al.*, 2001) or mentioned in the study of downregulation of a *CCR* gene in Arabidopsis by Goujon *et al.* (2003). Tyramine ferulates have only been described in solanaceous plants, and are unknown or insignificant in Arabidopsis and most dicots in general; finding none in CCR-downregulated Arabidopsis is therefore unsurprising. Also, whereas the authors were careful in Ralph *et al.* (1998) not to imply that CCR deficiency caused the apparent increase in tyramine ferulate levels in tobacco, they did suggest that the product was a logical result, in tobacco, of impeding the pathway taking substrates from the acid level (the cinnamoyl CoA thioesters) to the aldehyde, i.e. the step dependent on CCR. A possible alternative explanation is that biosynthesis of the tyramine ferulate and ferulic acid is actively induced by a stress response to the altered cell walls, and is only an indirect result of CCR deficiency. Thioacidolysis and NMR data are not presented in sufficient detail by Laskar *et al.* (2006), or in the correct regions of the spectra, to support the authors' claims for the absence of evidence for the incorporation of acids into the lignin. As in the poplar spectra shown here (Figure 2), we readily found analogous thioacidolysis evidence in Arabidopsis (Table 2 and Figure S3) and tobacco (Figure S2) via the marker **AG** and the diagnostic products **A1G** and **A1′G** from 4-*O*-etherified ferulic acid. We also find evidence for etherified ferulic acid, and the *bis*-ether **A5** that is the likely source of the marker, in NMR spectra from tobacco (not shown); we have not examined CCR-deficient Arabidopsis material by NMR.

We therefore conclude that the thioacidolysis monomer **AG** identified here is a general marker for incorporation of ferulic acid into the lignification process, and is an indicator that can be used judiciously for CCR downregulation in a variety of plants. Thioacidolysis products **A1G** and **A1′G** derived from 4-*O*-8-etherified ferulic acid (via further radical coupling reactions with monolignols) provide additional validation. With NMR evidence for the important marker precursor, the *bis*-(8-*O*-4)-ether **A5**, in the lignin polymer, the case is sufficiently compelling that one of the effects of CCR downregulation is indeed an increased incorporation of ferulic acid (monomer) into lignins.

Implications of ferulic acid in lignin

Ferulic acid is a lignin monomer. Although it often goes without mention in texts and reviews, it is evident that there are phenolic 'monomers' other than the three traditional monolignols that polymerize into lignins (Boerjan *et al.*, 2003; Ralph *et al.*, 2004b, 2007, 2008). Over a decade ago, studies began to suggest that higher levels of available phenolics might become incorporated when mutant or transgenic plants respond to their inability to produce the

Table 2 Thioacidolysis following methylation to determine etherification of units in Arabidopsis control and CCR-deficient mutants

	% OMe/OTMS	
Monomer	Control	Transgenic
M1G	12.52 ± 0.06	15.06 ± 0.35
M1S	1.04 ± 0.04	1.44 ± 0.10
AG	nd	19.9 ± 0.75

Values are means from three independent controls and four independent mutant lines (see Figure S1).
nd, not determined/detected.

normal complement of monolignols (Ralph, 1997). This was first apparent with CAD deficiency, in which the logical hydroxycinnamaldehyde precursors to the monolignols accumulate. Evidence is now indisputable, from diagnostic NMR data and from thioacidolysis markers, that hydroxycinnamaldehydes do indeed incorporate into lignins, by endwise radical coupling mechanisms, particularly in CAD-deficient angiosperms (Kim *et al.*, 2002, 2003; Lapierre *et al.*, 2004; Ralph *et al.*, 1998, 2001). Similarly, as again revealed by thioacidolysis and NMR, in COMT-deficient angiosperms, the novel monomer 5-hydroxyconiferyl alcohol (partially) substitutes for the sinapyl alcohol whose production is thwarted, producing novel benzodioxane structures in the lignins (Jouanin *et al.*, 2000; Marita *et al.*, 2001, 2003; Morreel *et al.*, 2004; Ralph *et al.*, 2001). Contrary to claims that incorporation of non-monolignol monomers has been 'unequivocally disproven' (Anterola and Lewis, 2002; Lewis, 1999; Patten *et al.*, 2005), monomer substitution is well authenticated in lignification, and evidence continues to mount that natural and mutant/transgenic lignins may derive from quite an array of phenolic precursors (Boerjan *et al.*, 2003; Boudet, 1998; Lu *et al.*, 2004; Ralph, 2006; Ralph *et al.*, 2004b, 2007, 2008; Sederoff *et al.*, 1999).

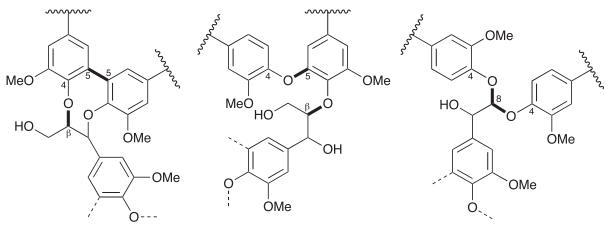
The evidence presented here suggests that ferulic acid itself is a previously unrecognized monomer in lignification, being incorporated at low levels in various types of 'normal' plants, and at elevated levels in various CCR-deficient transgenics. The presence of the thioacidolysis marker **A**G and the implicated (and NMR-evidenced) precursor in lignins, the *bis*-8-*O*-4-ethers **A5**, is difficult to rationalize in any other way. Ferulic acid therefore needs to join the growing list of compellingly authenticated monomers.

Ferulic acid incorporation provides a new chain-branching mechanism in lignification. Lignins are comprised primarily of linear chains of resinol, β-ether, phenylcoumaran and occasional spirodienone units, produced via monomer coupling reactions producing β-β-, β-*O*-4-, β-5- and β-1 bonds, respectively. All arise from coupling of a monolignol at its β-position with another monolignol (at its β-, 4-*O*- or 5-position), or, more commonly in so-called endwise polymerization, with the phenolic end of the growing polymer chain (at its 4-*O*- or 5-position); 5-coupling is obviously not possible in syringyl moieties where the 5-position is methoxylated. Resinol units, particularly the predominant syringaresinol units in angiosperm lignins, arise only from the coupling of two monolignols. As a syringaresinol unit can only initiate a chain and can never be formed in an already growing chain (which has no possibility of 8-position coupling), there can only be a single resinol unit in any linear (endwise-derived) chain.

Although lignin is often referred to as a branched 3-dimensional network polymer, lignins are largely linear. Branch points in the chain occur by only two known coupling mechanisms. Coupling of the phenolic end units of two preformed oligomers can occur via 4-*O*-5 or 5–5 coupling, the latter only being possible with guaiacyl units and the former requiring at least one guaiacyl unit (Ralph *et al.*, 2004b). In the case of the 5–5 unit, further coupling with a monolignol (at its β-position) creates novel dibenzodioxocin structures BR1 (Figure 5; Karhunen *et al.*, 1995). In the 4-*O*-5-coupled end unit, the chain is simply extended by normal etherification, producing structures BR2. Both cases represent branch points as the lignin polymer may extend in three directions from either branch unit BR1 or BR2 (Figure 5).

The incorporation of ferulic acid into lignin, as in (Figure 1c), provides a new branching pathway. As ferulic acid can couple at its 8-position with two phenolic end units, and the product **A5** (Figure 1c) is capable of further 4-*O* coupling, the resulting structure in the polymer (BR3 from **A5**, Figure 5) clearly represents another branch point. That such units are etherified and therefore are real branch points can be elegantly demonstrated by thioacidolysis following exhaustive cell-wall methylation; thioacidolysis monomers that are methylated were originally free-phenolic, whereas those that are not methylated must have been 4-*O*-etherified during lignification (Table 2). Spectra are shown in Figure S3. In CCR-downregulated Arabidopsis transgenics, the methylated marker **A**G**-Me** accounts for approximately 20% of the total marker (Table 2); most is released as unmethylated **A**G and therefore derives from etherified structures **A5** (Figure 5).

Introduction of ferulic acid into the lignification scheme therefore provides a new mechanism by which branching can occur in the polymer. Although units **A5** (Figure 5) are cleavable ether units, the increased branching may be expected to result in a lignin that appears more cross-linked. Such issues may confound the interpretation of some analytical data, and may provide further insight into some of the conclusions regarding lignin condensation drawn from antibody studies in a companion paper (Leplé *et al.*, 2007). Introducing branching structures **A5** into lignins may also provide a new mechanism for creating more readily cleavable lignins, allowing less energy-intensive biomass processing conditions – the acetals cleave readily under mild acidolytic conditions, depolymerizing the lignins.



BR1 5-5/4-O-β **BR2** 4-O-5/4-O-β **BR3** (from **A5**) 4-O-8/8-O-4

Figure 5. Traditional branch points BR1 and BR2 in lignins and the new branchpoint BR3 resulting from ferulic acid incorporation into lignin.

Implications regarding the theory of lignification. The currently accepted theory for lignification, based on combinatorial radical coupling of phenolic monomers, and, more importantly, cross-coupling of such monomers with the growing polymer, independent of direct protein/enzyme control in the coupling reactions, readily accommodates the observations described above. Basically, any phenolic compound in the lignifying zone can enter into the polymerization, subject to simple chemical constraints such as its ability to oxidize to its phenolic radical and the propensity of that radical to cross-couple with other radicals. Elevated incorporation of various non-monolignols into lignins in transgenic plants already attests to this contention. Sufficient examples are now known that it has even been possible to deduce 'What makes a good monolignol substitute?' (Ralph, 2006).

A challenge hypothesis suggests that lignins must be synthesized with exquisite structural control, putatively on proteins harboring arrays of dirigent sites, and then replicated by template replication (Davin and Lewis, 2005). Among the many difficulties inherent in this notion (as reviewed by Ralph *et al.*, 2004b, 2007) is the contention that other phenolic monomers are not tolerated in the assembly process. The evidence that novel monomers such as the hydroxycinnamaldehydes and 5-hydroxyconiferyl alcohol are incorporated into lignins is compelling if not unequivocal. Ferulates have also been established as being integrally incorporated into lignins in grasses (Ralph *et al.*, 2004a), and probably also in gymnosperms (Carnachan and Harris, 2000). The data on incorporation of hydroxycinnamaldehydes, 5-hydroxyconiferyl alcohol and ferulate esters into lignins have not been refuted. Even the more contentious 'abnormal monomers' such as dihydroconiferyl alcohol and tyramine ferulate continue to garner support for their incorporation into lignins via the accepted lignification mechanisms (Ralph *et al.*, 2004b, 2007).

Evidence has been presented here that ferulic acid should join the list of authentic lignin monomers. It is logical to conclude that lignification is therefore particularly tolerant of novel phenolic monomers, incorporating them into the polymer when they are present during lignification. Such observations are fully compatible with the existing combinatorial coupling mechanism, but are problematic for the dirigent hypothesis that is based on absolute proteinaceous control of polymer assembly.

Conclusions

Evidence has been presented to establish that compound **A**G, 1,2,2-trithioethyl ethylguaiacol, is a marker compound for ferulic acid incorporation into lignins. As such, it may be used judiciously as a marker or indicator to gauge CCR deficiency as long as the background levels in control materials are measured. Certainly, the 34-fold increase (Table 1) detected here in an antisense line of CCR-downregulated poplar transgenics is fully consistent with its being a marker for CCR deficiency. The finding that ferulic acid is incorporated into lignins, at low levels in a range of plants and at elevated levels in various CCR-downregulated transgenics, provides yet another example of non-monolignol monomers that are incorporated into the polymer by radical coupling mechanisms. Such findings are fully consistent with the currently accepted combinatorial theory of lignification. Significantly, ferulic acid incorporation also provides a new mechanism by which branch points can occur in the lignin polymer.

Experimental procedures

General

Solvents used were AR grade and supplied by Fisher (http://www.fishersci.com). Reagents were obtained from Aldrich (http://www.sigmaaldrich.com/). NMR spectra were acquired on a 500 MHz Bruker (http://www.bruker-biospin.com) DRX–500 instrument equipped with an inverse-gradient $^{1}H/^{13}C/^{15}N$ cryoprobe for higher sensitivity. The central chloroform solvent peak was used as internal reference (δ_C 77.0, δ_H 7.27 ppm). The standard Bruker implementation (hsqcetgpsi) for the gradient-selected sensitivity-improved inverse (^{1}H-detected) HSQC experiment (Willker *et al.*, 1992) was used for the spectra in Figure 3; the standard (inv4gslplrnd) gradient-selected inverse-detected HMBC experiment with an 80 msec long-range coupling delay was used for acquiring long-range $^{13}C–^{1}H$ spectra (not shown). These experiments and their applications to lignins have been described previously (Lu and Ralph, 2003; Ralph *et al.*, 1999).

Plant materials

Poplar. The control and CCR-downregulated poplar samples were those used in a companion paper (Leplé *et al.*, 2007). Briefly, lignins were prepared by ball-milling solvent-extracted cell walls, digesting away most of the polysaccharides with crude cellulases, and dissolving and acetylating the so-called 'cellulolytic enzyme lignin' (Hu *et al.*, 2006) using our cell-wall dissolution method (Lu and Ralph, 2003). Acetylated lignins (approximately 60 mg) were dissolved in $CDCl_3$ for NMR.

Tobacco. The tobacco spectra discussed here and presented in Figure S2 were from material used in our previous study of CCR-deficient tobacco (Ralph *et al.*, 1998).

Arabidopsis. The CCR data described are derived from Arabidopsis materials used in a previous study (Goujon *et al.*, 2003).

Thioacidolysis

Analytical thioacidolysis was performed as previously described (Lapierre *et al.*, 1995, 1999). Lignin-derived compounds were identified by GC–MS of their TMS derivatives. Quantitative evaluation of marker **A**G and the main **M**G and **M**S monomers was performed on ion chromatograms reconstructed from their prominent benzylic ions at *m*/*z* 269 or 299. Calculations used the monomer response factors relative to the C22 internal standard.

*Thioacidolysis of **E3***

Although free ferulic and *p*-coumaric acids, together with their EtSH addition products, are liberated via thioacidolysis from ferulate and *p*-coumarate units acylating grass cell walls (Rolando *et al.*, 1992), the fate of ethers **E3** was not obvious. Such styryl aryl ethers do not cleave significantly even in a high-temperature base (Grabber *et al.*, 1995), and their acidolytic cleavage has not, as far as we are aware, been examined. When the 4-*O*-methylated dimer (analog of **E3**) was subjected to thioacidolysis, the compound was isolated fully intact (as determined by ^{1}H-NMR). This experiment provides the crucial evidence to infer that the marker compound **A**G does not arise from ferulate esters that have incorporated into lignins.

*Synthesis of ferulic acid dehydrotrimer **A5**FFF*

Compound **A5**FFF (Figure 1) was synthesized essentially as described previously (Ward *et al.*, 2001) in approximately 5% isolated yield (following column and preparative thin-layer chromatography); the major product was the dilactone from 8–8-coupling. As the previously reported (Ward *et al.*, 2001) NMR data were from spectra recorded in CD_3OD (and assignments were incorrectly made), data for the acetate (relevant to assignment of this structure in the acetylated lignins in Figure 3) are reported here. Molecular modeling validates the shielding of one of the methoxyls in F1 or F2. Assignment of which ferulate moieties belonged together was based on COSY, TOCSY and HMBC experiments. ^{13}C data are from HSQC and HMBC experiments. ^{13}C chemical shifts are reported to two decimal places to distinguish close resonances. NMR, $CDCl_3$, δ_C/δ_H (number of protons, multiplicity, coupling constant(s) in Hz, assignment) F1: 74.6/6.26 (1H, d, J = 5.5 Hz, 7), 103.2/6.04 (1H, d, J = 5.5 Hz, 8), 134.0/– (1), 112.9/7.18 (1H, d, J = 1.9 Hz, 2), 150.8/– (3), 139.8/– (4), 122.5/7.02 (1H, d, J = 8.2 Hz, 5), 120.4/7.11 (1H, dd, J = 8.2, 1.9 Hz, 6), 55.88/3.86 (OMe); F2: 146.4/7.63 (1H, d, J = 15.8 Hz, 7), 116.13/6.28 (1H, d, J = 15.8 Hz, 8), 171.6/– (9), 130.3/– (1), 111.3/6.94 (1H, m, 2), 151.0/– (3), 147.3/– (4), 120.4/6.85 (1H, d, J = 8.2 Hz, 5), 122.1/6.94 (1H, m, 6), 55.73/3.64 (OMe); F3: 146.4/7.66 (1H, d, J = 15.9 Hz, 7), 116.07/6.30 (1H, d, J = 15.9 Hz, 8), 171.6/– (9), 129.9/– (1), 111.2/7.00 (1H, bs, 2), 150.8/– (3), 148.1/– (4), 119.7/6.91 (1H, d, J = 8.1 Hz, 5), 122.1/6.97 (1H, dd, J = 8.1, 2.0 Hz, 6), 55.78/3.78 (OMe).

Synthesis of [8-^{13}C]ferulic acid

Ferulic acid, essentially 100% 8-^{13}C-labeled, was prepared from vanillin and triethylphosphonoacetate-2-^{13}C (Aldrich) as previously described for the 9-^{13}C-labeled analog via triethylphosponoacetate-1-^{13}C (Ralph *et al.*, 1992).

Preparation of a synthetic lignin containing 6% ferulic acid The synthetic lignin (DHP) was prepared using the traditional Zutropf method (Freudenberg, 1956). Basically, coniferyl alcohol (91 mg, 0.5 mmol, 0.5 eq), sinapyl alcohol (106 mg, 0.5 mmol, 0.5 eq) and [8-^{13}C]ferulic acid (12 mg, 0.06 mmol, 0.06 eq) were dissolved in dioxane (1 ml) and mixed with water (40 ml). In another flask, H_2O_2 (30%, 100 μl, 1 eq) was added to water (40 ml). These two solutions were added at 2 ml h^{-1} over 20 h to a solution containing horseradish peroxidase [type II, Sigma (http://www.sigmaaldrich.com/), 3 mg, approximately 500 U] in homoPIPES buffer (50 mM, pH 4.5, 20 ml) and $CaCl_2$ (100 mM, 1 ml). After checking for residual peroxidase activity (guaiacol and H_2O_2), a further aliquot of H_2O_2 (11 μl) was added to water (4 ml) and pumped into the enzyme solution at the same rate, providing a total of approximately 1.1 eq of H_2O_2. The DHP was allowed to settle in the refrigerator at 4°C, and the solid isolated by centrifuging (10 min, 20 000 ***g***) and washing three times with distilled water. Freeze-drying yielded approximately 200 mg of DHP. NMR (360 MHz, DMSO-d_6), δ: 6.08/105.1 (8, **A**G); 6.39/116.4 (8, ring-attached ferulic acid, e.g. **M3**GF, Figure 4).

Acetylation (acetic anhydride:pyridine, 1:1, 12 h) of this DHP produced the acetylated DHP used for the spectrum in (Figure 3d). NMR (500 MHz, $CDCl_3$), δ: 5.93/103.8 (8, acetylated **A**G); 6.29/115.2 and 116.1 (8, ring-attached ferulic acid, e.g. **M3**GF, Figure 4).

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Supplementary Material

The following supplementary material is available for this article online:

Appendix S1. Comment on tyramine ferulates in tobacco lignins.

Appendix S2. Comment on CAD markers and hydroxycinnamaldehyde incorporation into angiosperm lignins.

Figure S1. GC–MS spectra for thioacidolysis monomers from poplar control and CCR-deficient lignins.

Figure S2. GC–MS spectra showing thioacidolysis monomers from tobacco.

Figure S3. GC–MS spectra for thioacidolysis products from a previous Arabidopsis methylation study (Goujon *et al.*, 2003).

Figure S4. GC–MS spectra for thioacidolyis of a dehydrotrimer **A5** of ferulic acid (**A5**FFF).

This material is available as part of the online article from http://www.blackwell-synergy.com.

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