

## SUPPLEMENTARY MATERIAL

### Identification of the Structure and Origin of a Thioacidolysis Marker Compound for Ferulic Acid Incorporation into Angiosperm Lignins (and a *pseudo*-Marker Compound for Cinnamoyl-CoA Reductase Deficiency)

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#### S1. Comment: Tyramine Ferulates in Tobacco Lignins

Tobacco lignins were found to have a substantial component derived from tyramine ferulate. The levels of this component appeared elevated in certain CCR- and CAD-deficient transgenics (Ralph *et al.*, 1998). Although we were careful to note that tyramine ferulate is a well-known wound-response product in tobacco, we remained convinced by the correlation data from detailed NMR experiments that the component was well integrated into the polymeric fraction characterized as lignin. It has, however, been claimed that tyramine ferulates were not part of the polymer fraction at all and were only low molecular-mass impurities (Anterola and Lewis, 2002). The argument, based on noting that the tyramine peaks in 1D <sup>13</sup>C-NMR spectra were much sharper than those from the bulk lignin polymer, is flawed. As is well known, terminal units that are especially rotationally mobile have lower NMR relaxation rates and hence sharper peaks than the bulk polymer (Ralph *et al.*, 1999). Thus the terminal free-phenolic tyramine units attached to ferulates incorporated into the backbone of these isolated polymers, as well as free-phenolic *p*-coumarate units found acylating the  $\gamma$ -methylol of sidechain units in grass lignins (Ralph *et al.*, 1994), and *p*-hydroxybenzoates similarly found acylating lignin units in palms, poplars and willows (Smith, 1955; Nakano *et al.*, 1961; Landucci *et al.*, 1992; Sun *et al.*, 1999; Meyermans *et al.*, 2000; Li and Lundquist, 2001; Lu and Ralph, 2003), all display such sharp peaks.

A simple argument, beyond noting the fact that the lignins were molecular-mass-fractionated, as stated in the original CCR paper (Ralph *et al.*, 1998), would have mollified the criticism—if the tyramine ferulate units were from low molecular mass components, the ferulate moieties would also display observably sharp peaks. However, finding the ferulate moieties, and proving that they were attached to the tyramines, required more sophisticated NMR experiments. The simple reason is that the ferulate moiety cross-couples integrally into the polymer, combinatorially coupling at its 4-O-, 5-, and 8-positions like the monolignols themselves, as has been reviewed (Ralph *et al.*, 2004). Ferulates are consequently involved in such a variety of structures that they become difficult to detect in simple 1D spectra. Whether tyramine ferulates are truly part of lignin can be debated, but the contention that they have

been “unequivocally disproven” (Anterola and Lewis, 2002; Patten *et al.*, 2005) as being incorporated into the polymer by combinatorial radical coupling reactions is not supported by the facts.

#### S2. Comment on CAD Markers and Hydroxycinnamaldehyde Incorporation into Angiosperm Lignins

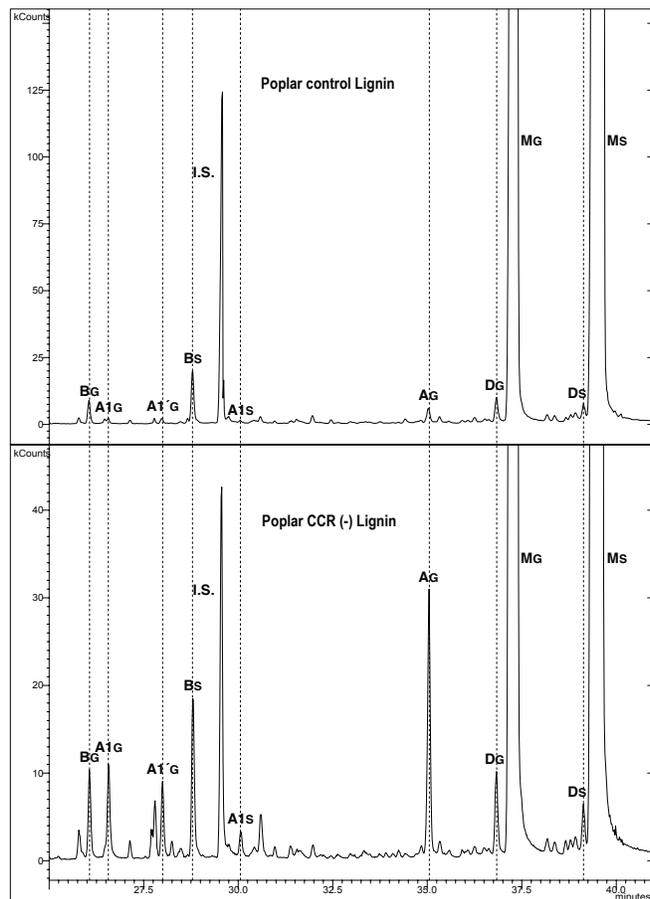
Markers for the incorporation of hydroxycinnamaldehydes into lignins were recently reported by our groups (Kim *et al.*, 2002; Lapierre *et al.*, 2004). The indene markers derive diagnostically from hydroxycinnamaldehydes that have 8-O-4-coupled with the phenolic end of the polymer, providing compelling evidence that hydroxycinnamaldehydes are monomers in lignification. Importantly, the marker levels from thioacidolysis track with the degree of CAD deficiency in angiosperms (Lapierre *et al.*, 2004). A recent report (Laskar *et al.*, 2004) indicated that the red coloration seen in CAD-deficient plants could be extracted out with methanolic HCl and was not related to the lignin. The consequent implication that hydroxycinnamaldehydes were not incorporated into lignins is however erroneous. Simple assessment of the marker levels shows that MeOH/HCl extraction does not remove the source of the markers, hydroxycinnamaldehydes that have 8-O-4-coupled into the lignins. Methanolic-HCl extraction, even after multiple such extractions, indeed removes the red coloration but does not reduce the release of such markers by thioacidolysis. In fact, on a weight basis the marker yield is slightly higher, logically due to the loss of mass from extracted components (including some hemicellulosic components) from the material; the data for a CAD-deficient poplar with 10% residual CAD-activity (Lapierre *et al.*, 2004) are: 7.0  $\mu$ M/g of cell wall for unextracted material and 8.7  $\mu$ M/g for MeOH/HCl-extracted cell walls vs 213 and 247  $\mu$ M/g of normal syringyl and guaiacyl monomers (Lapierre, 2006, reported here for the first time). We find none of this to be surprising—coupling or cross-coupling of hydroxycinnamaldehydes produces an array of combinatorial products that are NOT colored, along with traces of highly colored, but unidentified materials (Kim *et al.*, 2003). Nor, incidentally, do such hydroxycinnamaldehyde 8-O-4-coupling products stain with phloroglucinol-HCl—we have already cautioned against using this stain (for hydroxycinnamaldehyde end-groups

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in lignins) as an indicator of hydroxycinnamaldehyde incorporation into angiosperms or as an assay for CAD deficiency in angiosperms (Kim *et al.*, 2003).

### S3. Supplementary GC-MS spectra from Poplar, Arabidopsis, Tobacco, and a Ferulic Acid Model Compound

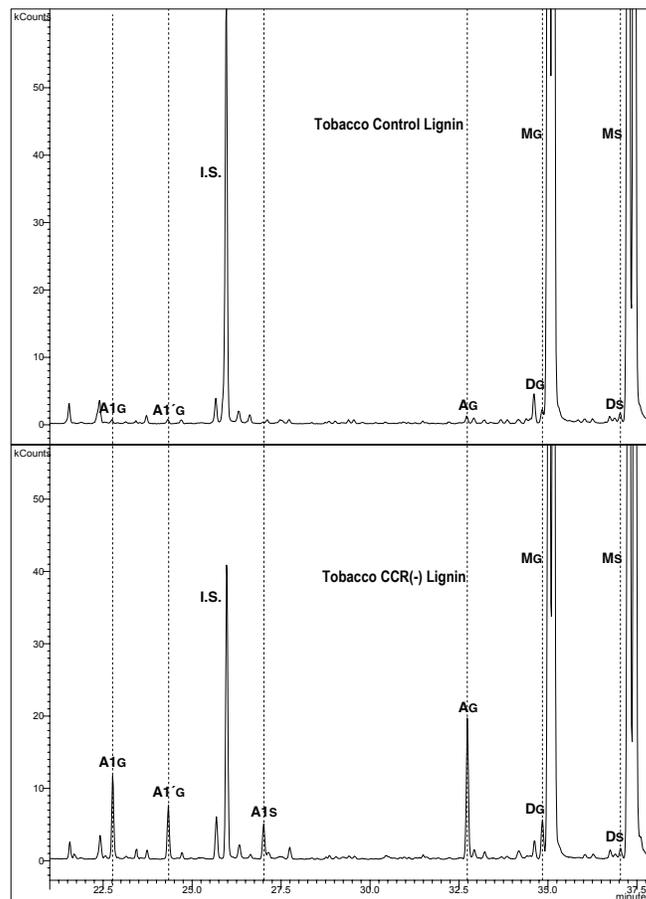
Spectra S1-S3 from other mutants/transgenics illustrate that the observations in the present paper are not restricted to a single line of poplar transgenics. Contrary to a recent report (Laskar *et al.*, 2006), evidence for ferulic acid incorporation into lignins can be readily found (including in *Arabidopsis*), via the thioacidolysis marker **AG** reported in the current paper, and in the etherified ferulic acid products **A1** also liberated. The methylation study, Figure S3, also reveals that the ferulic acid moiety in the *bis*-ether marker precursor **A5** is incorporated integrally into the lignin—the unit is ~80% etherified. Figure S4: thioacidolysis products from ferulate trimer **A5FFF**.



**Figure S1.** Thioacidolysis monomers from Poplar control and CCR-deficient lignins. These are the original files used to generate Figure 2 in the main paper, for which the caption should be consulted. Peaks are labeled as in the paper.

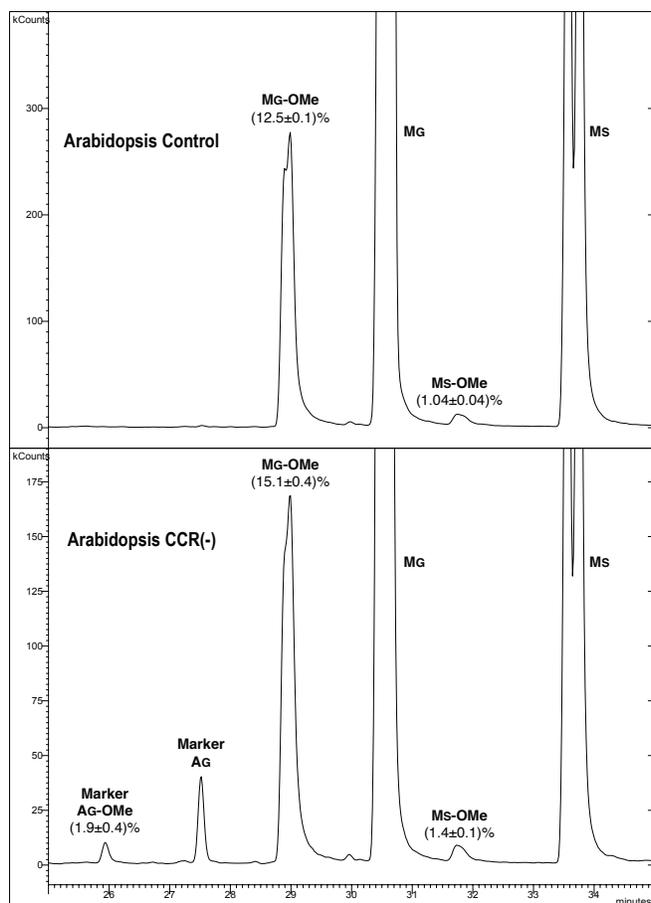
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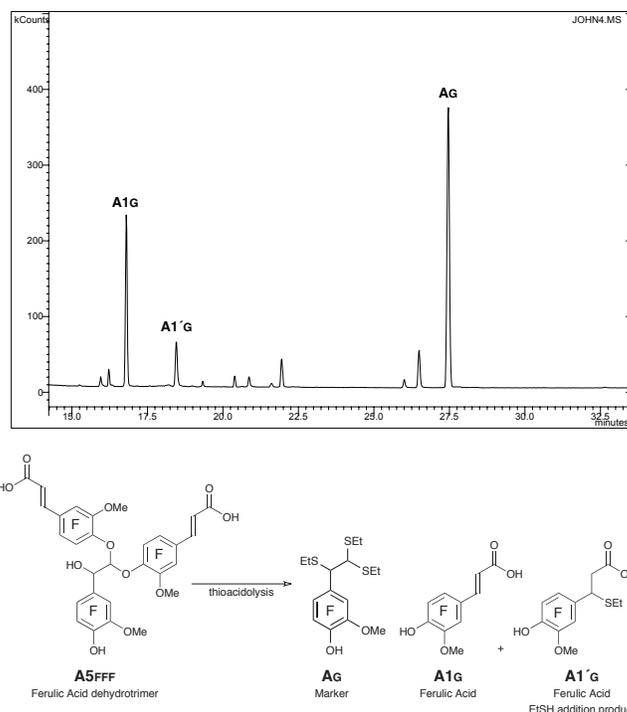
**Figure S2.** Plots showing thioacidolysis monomers from Tobacco. The same marker compound **AG** is evidenced at higher levels in the CCR-deficient tobacco (than in poplar), as are the monomers released from etherified hydroxycinnamic acids (**A1** and **A1'**). Note, however, that there are low levels of these products from the control tobacco line also.

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**Figure S3.** Thioacidolysis products from the *Arabidopsis* methylation study noted in the text of the main paper, that data for which were shown in Table 2. The control spectrum shows essentially only the normal thioacidolysis monomers, revealing that monomers released from syringyl units in the lignin are highly etherified (only ~1% free-phenolic) whereas about 12% of the monomers released from guaiacyl units were free-phenolic. In the CCR-deficient *Arabidopsis*, firstly the markers AG are again readily identified. The degree of methylation also shows that over 80% of the markers AG derived from etherified units, showing that they were integrated into the lignin polymer. The levels of free phenolic syringyl (1.4%) and guaiacyl (15.1%) units is similar to that in the WT control.

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**Figure S4.** Thioacidolysis of a dehydrotrimer A5 of ferulic acid (A5FFF, Figure 1 of the parent paper), containing the *bis*-ether moiety, efficiently releases the marker AG, as well as ferulic acid A1G and its EtSH addition product A1'G.

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