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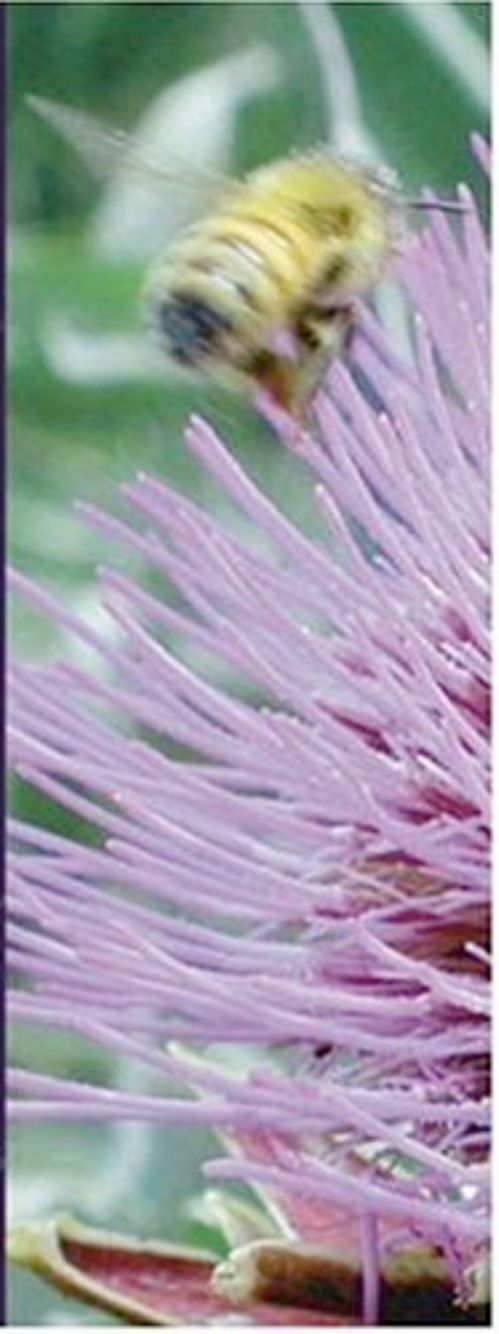

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Chapter 2

Lignification: are Lignins Biosynthesized via simple Combinatorial Chemistry or via Proteinaceous Control and Template Replication?

John Ralph, Gösta Brunow, Philip J. Harris, Richard A. Dixon, Paul F. Schatz and Wout Boerjan

2.1. Introduction

Lignification is the polymerization process generating lignins (complex hydroxyphenylpropanoid polymers) from phenolic monomers, notably the monolignols *p*-coumaryl, coniferyl and sinapyl alcohols. The current theory, based on original work by Freudenberg (Freudenberg & Neish, 1968), contends that the essential structural features of lignin could be mimicked by chemical processes involving oxidative coupling of phenols. All the structural features of lignin polymers are in accordance with the view that the actual polymerization step is solely under chemical control and that the radical coupling reactions are not, for example, directed by proteins or enzymes (Ralph *et al,*. 2004b). An alternative but ill-defined concept, attempting to displace the current theory, has been advanced; for a recent description see (Davin & Lewis, 2005). Without offering any experimental evidence the authors propose that lignin primary structure is dictated by 'proteins harboring arrays of dirigent (monolignol radical binding) sites,' and that lignin macromolecules may be replicated by template replication mechanisms using the lignin chain itself as the template. The authors contended that the current free-radical coupling theory is an 'unproven working hypothesis,' and unilaterally raised their own hypothesis to theory status. However, the supposed replacement paradigm lacks diagnostic supporting evidence and has difficulty in explaining many of the basic facts. Despite this, the new paradigm has come to some prominence, even appearing in a text book to the exclusion of the currently held theory (Croteau *et al*., 2000). This chapter is an attempt to bring balance to the current debate and to draw attention to the fact that the new contender has not earned the status of a theory.

The following are a small sample of the points that should be more openly discussed. Some have already been addressed in reviews and commentary articles on the subject (Sederoff *et al.*, 1999; Hatfield & Vermerris, 2001; Ralph *et al*., 2001b; Boerjan *et al*., 2003; Monties, 2003; Ralph *et al.,* 2004b).

2.2 The current theory

The current theory holds that the polymerization is a chemical process involving the radical coupling of phenols, incorporating available phenolic substrates subject to simple chemical and physical controls (Harkin, 1967; Freudenberg & Neish, 1968; Sarkanen & Ludwig, 1971; Harkin, 1973; Brunow *et al*., 1999; Ralph *et al.,* 2004b). As radicals from the monolignols and from the evolving polymers are resonance-delocalized and are capable of coupling with different regiochemistries (Plate 2.1), the process is 'combinatorial' (Ralph *et al.,* 2004b). The reaction of major importance in lignin polymerization is the so-called 'endwise' cross-coupling of a new monomer (radical) with the free-phenolic end of the growing polymer (Sarkanen & Ludwig, 1971). Coniferyl and sinapyl alcohols invariably couple at their sidechain β-positions, *in vivo* or *in vitro*. Such reactions for adding sinapyl and coniferyl alcohol monomers onto polymers bearing syringyl and guaiacyl phenolic end-units are shown in Plate 2.1d–e. In each coupling, as shown in more detail for β–O–4-coupling, for example (Plate 2.1g), the β-carbon becomes a new chiral carbon. Since no optical activity has been observed in lignins, this coupling must yield a completely racemic mixture (i.e. 50:50 *R:S*). Intermediate quinone methide structures generated by the radical coupling step are re-aromatized via post-coupling nucleophilic addition reactions, by water in the case of the β–O–4-coupled product (Plate 2.1g), and internally by the phenol on the 'B-unit' for β–5-coupling (not shown). A less important pathway, so far authenticated only for monolignol addition to β-ether units, is via β–1-coupling (Plate 2.1h) (Setälä *et al*., 1999; Zhang & Gellerstedt, 2001; Ralph *et al.,* 2004b; Zhang *et al*., 2006). Other important couplings, which create branch-points in the polymer, may occur via 5–5- or 5–O–4-coupling of oligomers (not monomers) to produce 5–5- and 4–O–5-units (**D** and **E**, Plate 2.1f); clearly at least one guaiacyl unit is needed in such pathways.

At the start of the polymerization some dimerization of monolignols occurs (Morreel *et al*., 2004a), the formation of β–β-coupled structures (resinol units **C**) or cinnamyl endgroups being a sign of this (Plate 2.1a,b). Formation of two or three coupling products obviously cannot proceed in a statistically random manner. The proportion of each product will be governed by the relative energies of the transition states (somewhat reflected in the product energies) which will not only be different but will be differentially altered by physical reaction conditions such as pH, solvent polarity, temperature, etc. The term 'combinatorial' is now therefore considered more suitable than the earlier term 'random coupling.' And nothing more than simple chemical coupling propensity (which is not always trivial to predict) is needed to explain why dimerization of sinapyl alcohol, with two combinatorial choices (β–β, β–O–4) strongly favors β–β-coupling. It is important to stress here that the coupling propensities between monolignols when forming dimers (Plate 2.1a,b), have little to do with the coupling propensity of a monolignol with the growing oligomer (Plate 2.1d,e).

2.3 Is there a need for a new theory?

Since there are no observations that are inconsistent with the existing theory, one may ask if there is a need for a new theory. The new hypothesis seems to arise from a belief that

lack of absolute structural control over lignification would constitute a unique biochemical anomaly (see section 2.4.1). As will be described, there are certain requirements to be met before a new hypothesis can come into contention as a replacement theory.

2.3.1 The challenge hypothesis (proteinaceous control and template replication)

Over several years, a challenge hypothesis has emerged (Lewis & Davin, 1998; Gang *et al*., 1999; Kwon *et al*., 1999; Davin & Lewis, 2000; Burlat *et al*., 2001; Davin & Lewis, 2005). It was diagrammatically illustrated in a recent paper (Davin & Lewis, 2005) and is shown in abbreviated from in Plate 2.2a. Basically, the idea is that primary lignin macromolecules are assembled via putative 'proteins harboring arrays of dirigent (monolignol radical binding) sites' (Davin & Lewis, 2005). It is proposed that this lignin macromolecule then acts as a replication template to create identical (or, by some unknown mechanism, mirror image) molecules. In a manner remarkably like DNA replication, the process has recently been hypothesized as being via 'an antiparallel double-stranded lignin template' (Chen & Sarkanen, 2006). Such a model is fraught with difficulties, as will be described below.

2.3.2 Has the challenge hypothesis become a theory?

The unwary may conclude that there is a new *theory* for lignification. Without debate and without evidence the challenge hypothesis has been unilaterally elevated to a competing theory and beyond.[1] The position seems to have arisen from seeking only supporting evidence,[2] an approach distinctly at odds with Popper's definition of a scientific method (see http://plato.stanford.edu/entries/popper/). The authors of the challenge hypothesis have chosen the risky undertaking of proclaiming it a competing theory even in the absence of supporting evidence.

2.4 Are criticisms of the current theory valid?

Challenges to the current combinatorial model have been put forward. Any facts that fail to be consistent with a theory puts that theory in jeopardy. But are the challenges valid? We will discuss the following challenges that purportedly falsify the current theory.

2.4.1 Lignification as a biochemical anomaly

The view that uncontrolled reactions are unnatural has been widely espoused by the proponents of the dirigent/replication hypothesis.[3] Firstly, it must be emphasized that the existing theory does not claim that lignification is uncontrolled. It is clear that the cell exerts exquisite control over the supply of monolignols (and other phenolics) to the lignifying zone, over the supply of H_2O_2 and, consequently, the radical-generation capacity, etc. Such control over monolignol supply is evident in the temporal progression from *p*-coumaryl through coniferyl through sinapyl alcohol incorporation (Terashima *et al*., 1993), in the enhanced incorporation of *p*-coumaryl alcohol into softwood compression wood lignins

(Timell, 1986), and in the differences in lignin composition in various cell types (Monties & Lapierre, 1981; Chesson *et al*., 1997; Hatfield *et al*., 1999b; Chen *et al*., 2000; Chabannes *et al*., 2001; Donaldson, 2001; Li *et al*., 2001; Patten *et al.,* 2003; Harris *et al*., 2005). 'Evidence' for control has often overlooked the fact that lignin composition could be controlled at the level of monolignol transport from the cytoplasm to the site of polymerization. The issue being debated, however, is in the control over the radical coupling steps involved in the polymerization (Ralph *et al.,* 2004b). We leave the discussion regarding the possible uniqueness of lignins' uncontrolled reactions to the end of this chapter, section 2.7.

2.4.2 The β-Ether frequency anomaly

A major criticism of the current theory espoused by the proponents of the dirigent/replication hypothesis has been that the high β-ether frequency observed in lignins is anomalous (Lewis & Davin, 1998; Lewis *et al.,* 1998a; Davin & Lewis, 2000).[4] Dehydrodimerization of sinapyl alcohol (Plate 2.1b), produces the β–β-coupling product syringaresinol typically in over 90% yield (Tanahashi *et al*., 1976). As noted above (Plate 2.1a), coniferyl alcohol produces the three combinatorial dimers from coupling of one monomer at its β-position with the other at the β-, 4–O-, or 5-positions in roughly equal amounts (Tanahashi *et al.,* 1976; Katayama & Fukuzumi, 1978). In neither case, therefore, does the β-ether frequency approach 50%. Yet lignins are reported to have 50-70% (or higher) β-ethers (Adler, 1977). How can such an apparent anomaly be explained?

This assumption of an anomaly is based on a misunderstanding of the lignification process. As explained above (section 2.2), the growth of the lignin molecule occurs by coupling of a monolignol radical to a radical formed on the phenolic end group of the polymer, a process termed 'end-wise coupling.' It has proved difficult to reproduce this type of polymerization *in vitro* because the end-wise coupling is repressed when higher concentrations of monolignols are present. Monolignol radicals preferentially couple with like monolignol radicals (when available) rather than cross-couple with dimers or higher oligomers (Syrjänen & Brunow, 1998; Syrjänen & Brunow, 2000; Syrjänen & Brunow, 2001). Dehydrodimerization reactions are therefore over-represented in synthetic lignins even when attempts are made to introduce the monolignol slowly (Freudenberg, 1956; Sarkanen, 1971; Terashima *et al*., 1995). Consequently, the β-ether frequency is low, considerably lower than in typical lignins. Limiting the diffusion rates (and therefore monolignol radical concentrations) to favor cross-coupling reactions reveals that β-ethers are strongly favored in cross-coupling reactions. In model reactions carried out via diffusion of coniferyl alcohol through dialysis tubing where coniferyl alcohol reacted with a lignin phenolic end-unit model, apocynol, the ratio of β–O–4- to β–5-product was 10:1 (Syrjänen & Brunow, 2000), and in experiments with coupling to a guaiacyl dimer, only the β–O–4-trimer was found (Syrjänen & Brunow, 1998).

Selective formation of β–O–4-units in lignins can therefore be achieved by limiting the supply of monolignol radicals. In the cell, simple diffusion control may suffice. No other agents are necessary to 'explain the mystery of specificity of radical precursor coupling in lignin biosynthesis' (Davin & Lewis, 2000). This feature was recognized early on as evidenced by eloquent passages in various treatises (Sarkanen & Ludwig, 1971; Adler, 1977), and has been emphasized repeatedly, by, for example, Lundquist (1973); Syrjänen & Brunow

(1998); Boerjan *et al.* (2003); Ralph *et al.* (2004b). The notion that the β-ether frequency is an anomaly that the current theory cannot accommodate must therefore be dismissed.

2.4.3 'Obligatory' linkages?

Softwood resinols (β–β-linked structural units) have been claimed to be 'obligatorily 5-linked' (Chen & Sarkanen, 2003; Sarkanen, 2005). The claim arises from extrapolating various published observations.[5] If pinoresinol units in softwood lignins were obligatorily 5-linked, an explanation under the combinatorial theory would be needed. But again, what are the facts?

First, the level of resinol units in softwood lignins is very low, typically about 2% (Adler, 1977; Zhang *et al.,* 2003). Second, such units obviously have two phenolic ends, each of which is capable of radical coupling at its 4–O- or 5-positions. Third, and rather importantly, pinoresinol is produced by dehydrodimerization of the monolignol coniferyl alcohol. At the time it is formed, early in the process of lignification, it is likely that there will be other dehydrodimers and small oligomers present. The 5-coupling of pinoresinol with another pinoresinol (Freudenberg & Sakakibara, 1959), or its cross-coupling with a β-ether dimer, have been shown (Kim, 2006, unpublished) to be efficient reactions. The idea therefore that at least one end of a pinoresinol unit becomes 5-coupled is hardly surprising.

But are such units *obligatorily* 5-linked? The answer appears to be no. Although early reports are that the β–β-dimer derived from pinoresinol is not released from acidolysis or thioacidolysis, it is released by another milder ether cleaving reaction, derivatization followed by reductive cleavage (the DFRC method). As illustrated in a recent review (Ralph *et al.,* 2004b, Fig. 8), the level of the DFRC dimer from pinoresinol in spruce or pine lignin is about a quarter the level of the secoisolariciresinol product. Secoisolariciresinol units are present in lignins at about half the level of pinoresinol units (Zhang *et al.,* 2003) and are also capable of coupling at both phenolic ends. Regrettably, neither we nor others have yet quantified the release of such units but they are not difficult to detect. Quantification is now required to determine just what fraction of the pinoresinol can be released. There may also be a need to determine why thioacidolysis and DFRC appear to differ here.

In summary, evidence is that pinoresinol units are *not* obligatorily 5-linked and it is not difficult to come up with reasonable explanations under the current theory (different coupling propensities, coupling with other oligomers present in the lignifying zone at the same time) to rationalize why only a fraction of the particularly minor (~2%) pinoresinol components in softwood lignins can be released by ether-cleaving reactions. The claim that resinols are obligatorily 5-linked, and that proteinaceous control over lignification is therefore demanded, is not supported.

2.5 Is there anything wrong with the dirigent hypothesis?

2.5.1 Monomer substitution

The remarkable statement that 'There is, however, no known precedent for the free interchange of monomeric units in any biopolymer assembly, then or now…' (Lewis, 1999), was in response to suggestions that plants finding themselves with reduced capacity to supply traditional monolignols appeared to respond by (partial) substitution with other available

phenolics, for example, monolignol pathway intermediates such as the hydroxycinnamaldehydes, or 5-hydroxyconiferyl alcohol (Ralph *et al*., 1997; Ralph *et al.,* 2001b). Although this evidence continues to be denied (see Section 2.5.1.1), monomer substitution has now been compellingly established, as reviewed by Ralph *et al.* (2001b*,* 2004b). Monomer substitution in more highly structurally controlled polysaccharide biosynthesis had already been unequivocally illustrated. Fucose-deficient Arabidopsis *mur1* mutants substitute L-galactose for L-fucose in their xyloglucans (Zablackis *et al*., 1996; Sederoff *et al.,* 1999). Since the dirigent/replication hypothesis had already been declared to be incompatible with the concept of monomer substitution (Lewis 1999),[6] establishing monomer substitution should be sufficient to dispel the challenge hypothesis.

2.5.1.1 Has monomer substitution been disproven?

It has been claimed that 'Furthermore, most – if not all – claims of non-lignin monomers acting as lignin monomer surrogates have now been unequivocally disproven (Anterola & Lewis, 2002)' (Patten et al., 2005). 5-Hydroxyconiferyl alcohol's unmistakable substitution for sinapyl alcohol in COMT-deficient angiosperms (see Section 2.5.1.2) was carefully avoided. In each case addressed, the proof offered was simply inaccurate. Three of the most criticized examples will suffice here to make the point that monomer substitution has not been disproven and is in fact continuing to gain validation.

Hydroxycinnamaldehyde incorporation into lignins

The hydroxycinnamaldehydes coniferaldehyde and sinapaldehyde incorporate integrally into angiosperm lignins, as reviewed by Boerjan *et al.* (2003) and Ralph *et al.* (2004b). The evidence is from several sources. First, NMR reveals additional aldehyde peaks at different chemical shifts from simple cinnamaldehyde endgroups (Ralph *et al*., 1999b; Kim *et al*., 2000; Ralph *et al.,* 2001b; Kim *et al*., 2003). Diagnostic 2D NMR experiments reveal the nature of such aldehydes and, in particular, demonstrate that coniferaldehyde monomers β–O–4-cross-couple with syringyl phenolic end-units on the growing polymer whereas sinapaldehyde β–O–4-cross-couples with both guaiacyl and syringyl units. Incidentally, such cross-coupling of coniferaldehyde with guaiacyl units cannot be attained *in vitro* either. Second, the occurrence of hydroxycinnamaldehyde β–O–4-coupled units in lignins is validated by diagnostic marker compounds released from such units by analytical thioacidolysis (Kim *et al*., 2002; Lapierre *et al*., 2004). The marker levels increase in conjunction with the degree of CAD-deficiency in transgenic poplar.

How can this data be reconciled with attempts to impugn the notion of hydroxycinnamaldehyde incorporation? For example, the ability to extract the red coloration from CAD-deficient fiber using methanolic-HCl was recently presented (Laskar *et al*., 2004). The implication was that such extraction removed aldehyde components ('polyaldehydes') which could, therefore, not have been integrated into lignin polymers. A simple test would have revealed that such color removal had nothing to do with the aldehyde components in the lignin.[7] As noted above, thioacidolysis produces diagnostic marker compounds from hydroxycinnamaldehydes that are incorporated end-wise into lignins by β–O–4-coupling. Methanolic-HCl extraction, even after multiple such extractions, 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 are: 7.0 μM/g of cell wall for unextracted material and 8.7 μM/g for MeOH/HCl-extracted cell walls *vs*. 213 and 247 μM/g of normal syringyl and guaiacyl monomers (Ralph, Kim *et al*., 2008).

It is therefore obvious that the hydroxycinnamaldehydes that are incorporated into cell wall lignins in CAD-deficient plants are integral components of the polymer. They have been established to be coupled via end-wise β–O–4-coupling with the phenolic end of the growing polymer, by pathways completely analogous with the coupling of the monolignols, qualifying them as authentic lignin monomers in these plants. Since they replace monolignols (to an extent depending on the degree of CAD-deficiency), it is hard to refute the notion of monomer substitution during lignification. Clearly, the monolignol substitution by hydroxycinnamaldehydes has not been unequivocally disproven.

DHCA incorporation into lignins

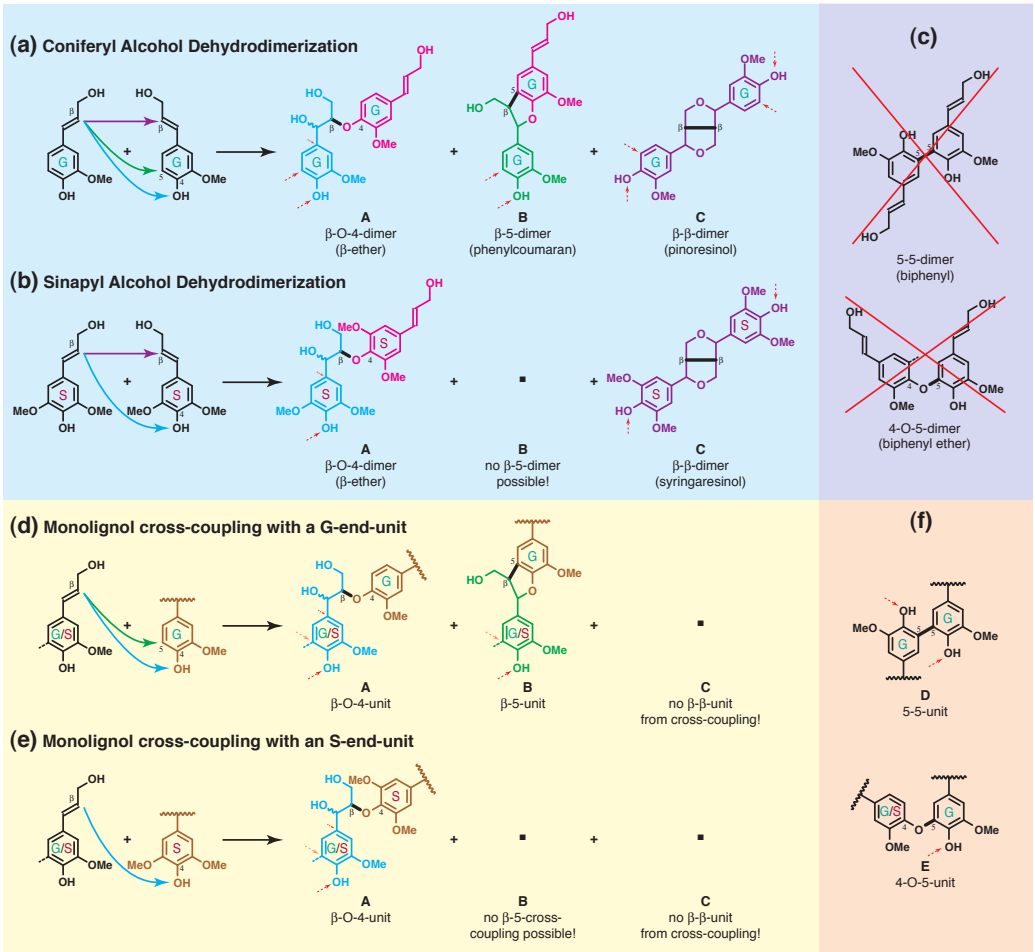
Dihydroconiferyl alcohol (DHCA) is a well-established minor component (of unknown origin) of gymnosperm lignins (Ralph *et al*., 1999a; Ralph *et al.,* 1999b; Sederoff *et al.,* 1999). It is far from an ideal lignin monomer — without the cinnamyl alcohol double bond, the only coupling possible is to 4–O- and 5-positions on the aromatic ring, so DHCA units are always end-units. A CAD-deficient pine lignin contained enormously enhanced levels of this component (Ralph *et al.,* 1997; Sederoff *et al.,* 1999; Lapierre *et al*., 2000a; Dimmel *et al*., 2001; MacKay *et al*., 2001). As was clearly indicated in the initial report (Ralph *et al.,* 1997), it was found cross-coupled with coniferyl alcohol, the normal monolignol whose availability for lignification was severely curtailed in the mutant, in products such as the β–5-coupled dihydro-dehydrodiconiferyl alcohol. But about half of the DHCA units were 5-coupled, including in dibenzodioxocin structures (Sederoff *et al.,* 1999). Coniferyl alcohol monomers have never been found to 5–5-couple, so the idea that 5–5-coupled DHCA units could arise from post coupling benzylic reduction reactions of coniferyl alcohol is illogical. Ignoring all such evidence, DHCA units continue to be ascribed to post-coupling transformations (Lewis *et al.,* 1998a;[8] Lewis, 1999).Nevertheless, additional evidence continued to accumulate to support the original premise that DHCA is an authentic monomer. This includes data from thioacidolysis and pyrolysis (Lapierre *et al.,* 2000a; Dimmel *et al.,* 2001; MacKay *et al.,* 2001), along with the significant observation that the derived guaiacylpropane-1,3-diol is also incorporated into gymnosperm lignins (Ralph *et al.,* 1999a; Ralph *et al.,* 2001b).[9] These diols are found incorporated into normal gymnosperm lignins and at significantly elevated levels in the CAD-deficient pine lignin. Logically guaiacylpropane-1,3-diol is a monomer derived from DHCA under the oxidative conditions. When formed and present during lignification, it is incorporated into the polymer like any other phenolic component present in the lignifying zone, subject to simple chemical principles. All of the above provide unequivocal proof that DHCA is an authentic monomer that is incorporated into the lignification process.

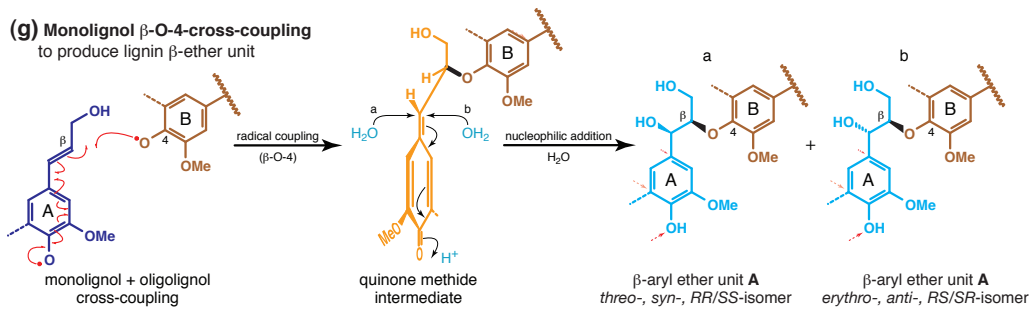
Tyramine ferulate incorporation into lignins

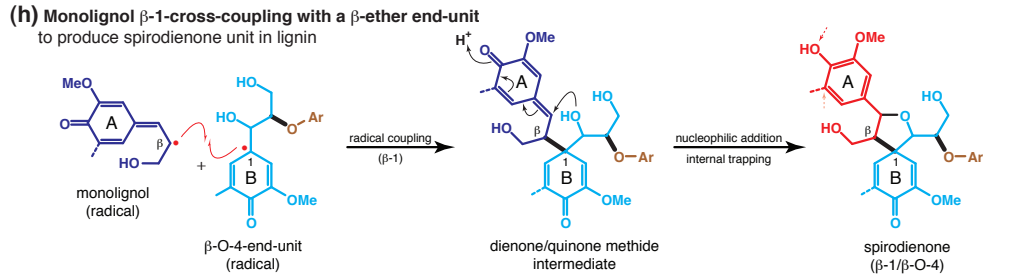
The levels of tyramine ferulate appeared to be enhanced in tobacco down-regulated in cinnamoyl-CoA reductase (CCR) (Piquemal *et al*., 1998; Ralph *et al*., 1998b). Substrates

Plate 2.1 The current theory of lignification is it takes place via combinatorial radical coupling. (**a**)/(**c**) Dehydrodimerization of monolignols, the basis of lignan formation but a minor reaction in lignification. (**a**) Dehydrodimerization of coniferyl alcohol produces three dehydrodimers **A**–**C**, all of which involve coupling of at least one monolignol at its β-position, in comparable amounts. (**b**) Dehydrodimerization of sinapyl alcohol produces only two products, **A** and **C**. (**c**) Dehydrodimerization does not produce the 5–5- and 4–O–5- structures often shown in texts. (**d**)–(**e**) Cross-coupling of a monolignol with the growing polymer, the major reaction in lignification. (**d**) Cross-coupling of a hydroxycinnamyl alcohol with a guaiacyl (**G**) unit gives only two main products **A** and **B**, the β-ether **A** being major. (**e**) Cross-coupling of a hydroxycinnamyl alcohol with a syringyl (S) unit has only one major pathway leading to β-ether units **A**. When the polymer phenolic end unit is a β-ether, β–1-coupling may also occur to a relatively minor extent. (**f**) Coupling of preformed oligomers is the source of the 5–5- and 4–O–5-units, **D** and **E**. (**g**)–(**h**) More detailed mechanisms for β–O–4- and β–1-cross-coupling reactions. (**g**) Cross-coupling of a monolignol (at its β-position) with a phenolic end-unit (G or S, at its 4–O-position) produces a new β–O–4-bond and introduces a new chiral center, the β-carbon. The intermediate quinone methide produced undergoes rearomatization via nucleophilic addition to the α-carbon, usually by water, producing another new chiral center at the α-carbon. The product is completely racemic, indicating an independence from proteinaceous control. The unit's two possible isomers (from the two racemic chiral centers), the *RR/SS* and the *RS/S*R isomers, also termed *syn* and *anti* or *threo* and *erythro*, are produced in ratios indicating kinetically control (see text). (**h**) Cross-coupling of a monolignol (at its β-position) with a phenolic β-aryl ether end-unit (G or S, at its 1-position) can produce a new β–1-bond. In this case, the intermediate quinone methide is trapped internally by the α-OH on the original β-ether unit, forming a novel spirodienone structure in the lignin (Setälä *et al.,* 1999). Such structures, now readily found by NMR at low levels in most lignins (Zhang & Gellerstedt, 2001; Marita *et al.,* 2003; Ralph *et al.,* 2004b; Zhang *et al.,* 2006), will cleave in mild acid rearomatizing the dienone to produce the 'traditional β–1-unit' with a phenolic B-ring and a glyceraldehyde-2-aryl ether. The existence of β-1-units in lignins as spirodienones explains why the B-rings in released β–1-structures are never phenol-etherified.

In all products, the new bonds formed by the radical coupling reactions are drawn bolder. Red arrows indicate sites at which further radical coupling can occur during lignification; the lighter arrows to the 5-positions in (**d**)–(**e**) and (**g**)–(**h**) structures show where coupling can occur in G-units but not in S-units (where the 5-position is occupied by a methoxyl group).


(a) Coniferyl Alcohol Dehydrodimerization
A
β-O-4-dimer
(β-ether)
B
β-5-dimer
(phenylcoumaran)
C
β-β-dimer
(pinoresinol)
(b) Sinapyl Alcohol Dehydrodimerization
A
β-O-4-dimer
(β-ether)
B
no β-5-dimer
possible!
C
β-β-dimer
(syringaresinol)
(c)
5-5-dimer
(biphenyl)
4-O-5-dimer
(biphenyl ether)
(d) Monolignol cross-coupling with a G-end-unit
A
β-O-4-unit
B
β-5-unit
C
no β-β-unit
from cross-coupling!
(e) Monolignol cross-coupling with an S-end-unit
A
β-O-4-unit
B
no β-5-cross-
coupling possible!
C
no β-β-unit
from cross-coupling!
(f)
D
5-5-unit
E
4-O-5-unit


(g) Monolignol β-O-4-cross-coupling
to produce lignin β-ether unit
radical coupling
(β-O-4)
nucleophilic addition
H2O
monolignol + oligolignol
cross-coupling
quinone methide
intermediate
β-aryl ether unit A
threo-, syn-, RR/SS-isomer
β-aryl ether unit A
erythro-, anti-, RS/SR-isomer


(h) Monolignol β-1-cross-coupling with a β-ether end-unit
to produce spirodienone unit in lignin
monolignol
(radical)
β-O-4-end-unit
(radical)
radical coupling
(β-1)
dienone/quinone methide
intermediate
nucleophilic addition
internal trapping
spirodienone
(β-1/β-O-4)

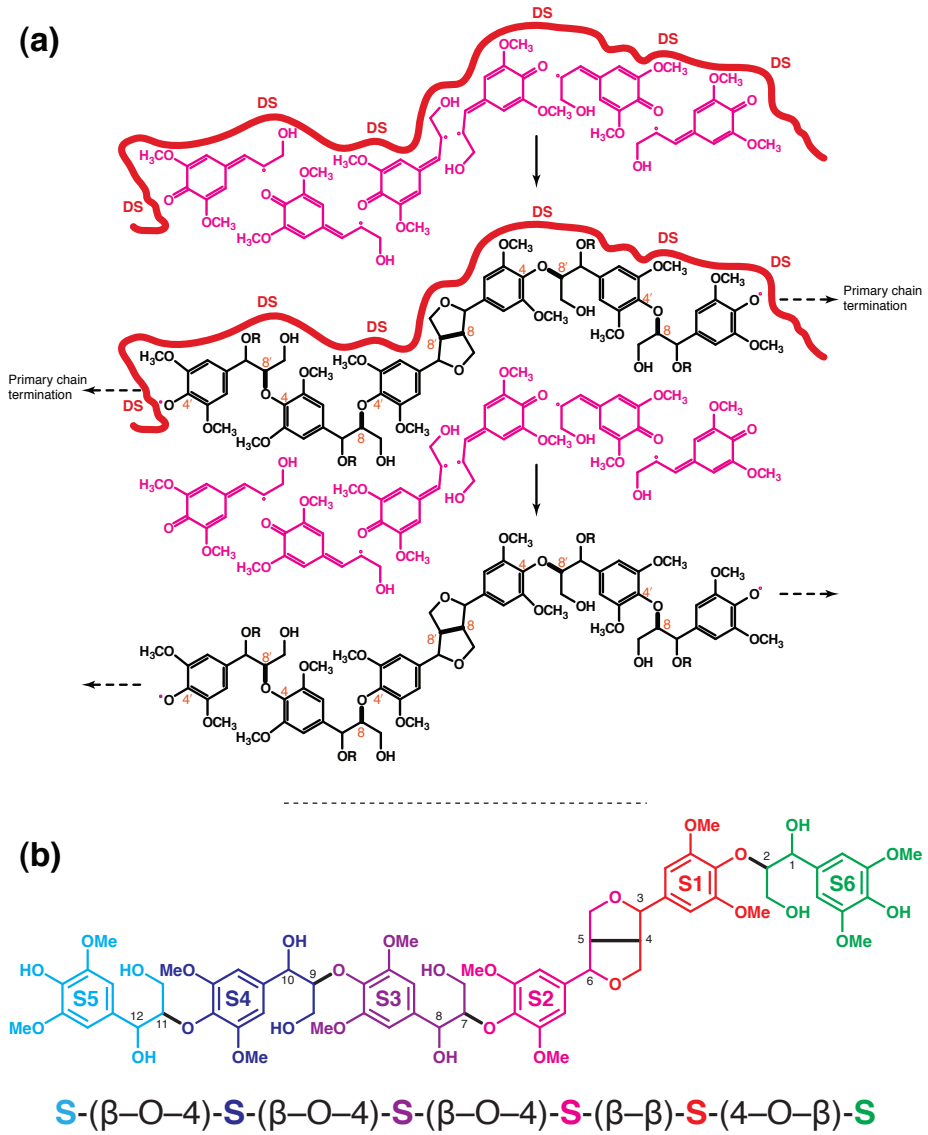
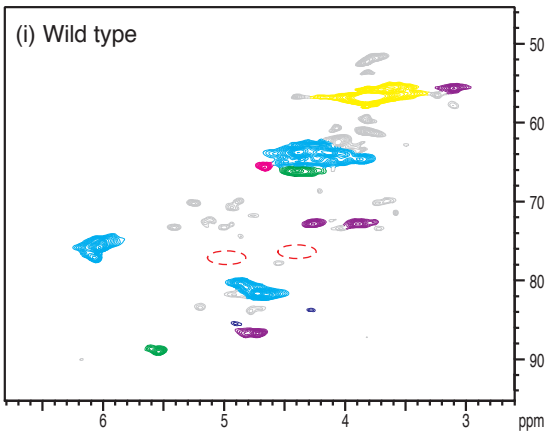
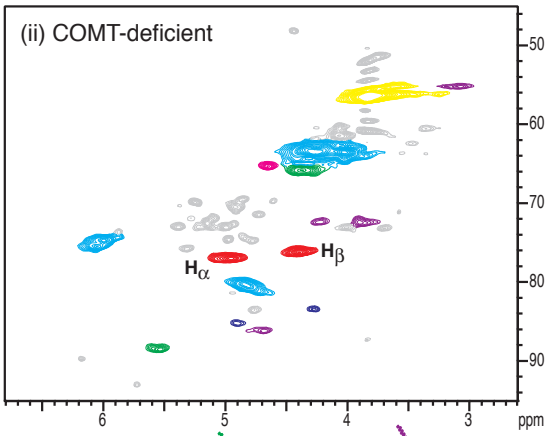
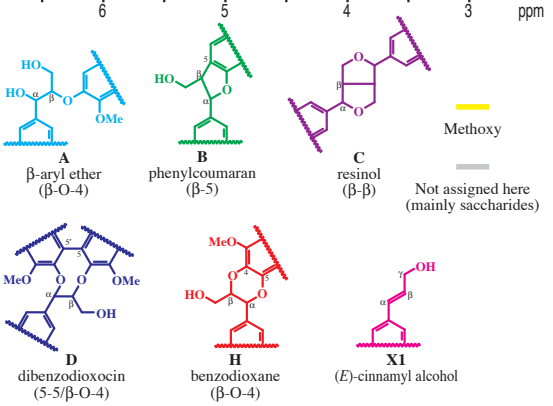


Plate 2.2 (**a**) The dirigent array/replication model, redrawn from (Davin & Lewis, 2005). It is suggested that a protein putatively harboring an array of dirigent (monolignol radical binding) sites assembles primary lignin chains. Such chains are then hypothesized to act as templates for replication of the chain. Note that, in the scheme shown, for either the primary lignin synthesis or for template replication, mechanistic chemical principles demand that the only starting point is with the β–β-coupling in the middle. (**b**) An example of a hexamer, S-(β–O–4)-S-(β–O–4)-S-(β–O–4)-S-(β–β)-S-(4–O–β)-S, cf. the all-syringyl analog of the hexamer in Fig. 4 in Davin & Lewis (2005) that has been putatively sequenced (see text, Section 2.5.2.1). The 12 chiral carbons are labeled.

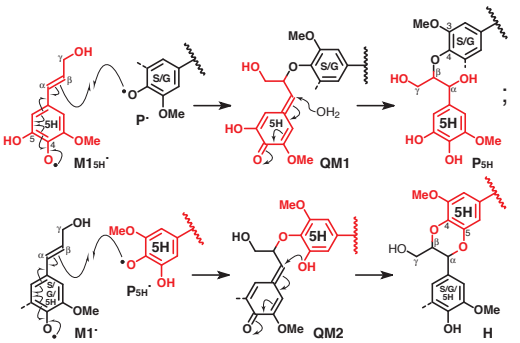
(a) HSQC (sidechain region)







(b) Mechanism: 5-hydroxyconiferyl alcohol to benzodioxane structures **H** in lignin



(c) HMBC (**A**α- and **H**α-correlations)

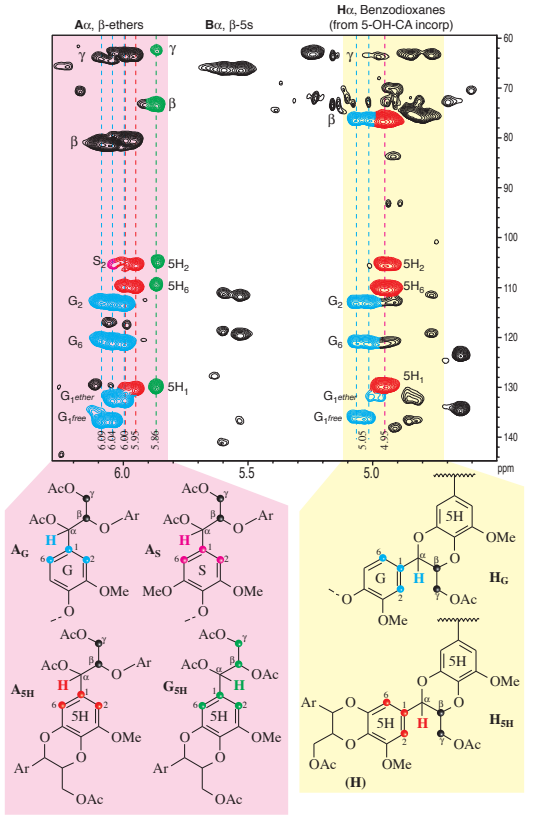


Plate 2.3 COMT-deficient plants, alfalfa in this case (Marita *et al.,* 2003), substitute 5-hydroxyconiferyl alcohol for sinapyl alcohol in the lignin polymerization. (**a**) Partial 2D ^{13}C-^{1}H correlative HMQC NMR spectra of lignins from (**i**) the wild-type and (**ii**) the COMT-deficient transgenic alfalfa. The major structural units (**A-D**, **X1**, and novel **H**) and color-coded chemical structures are shown. Dashed ovals in (**i**) delineate the areas in which benzodioxane units **H** would correlate (if they were present). (**b**) The mechanism by which 5-hydroxyconiferyl alcohol incorporates into the lignin to produce novel benzodioxane structures **H**. (**c**) Gradient-selected 2D HMBC sub-spectra showing α-proton correlations to carbons within 3 bonds in β-aryl ether units **A**, β–5-units **B** (not highlighted) and benzodioxane units **H**. These spectra demonstrate that all types of lignin monomers (S, G, and 5H) undergo β–O–4-coupling producing β-ethers **A** (pink shading: $\mathbf{A}_{G}$, $\mathbf{A}_{S}$ and $\mathbf{A}_{5H}$), and also glycerol units $\mathbf{G}_{5H}$ and, in the yellow shading, that coniferyl alcohol and 5-hydroxyconiferyl alcohol (and sinapyl alcohol at lower contour levels) all add to the new 5-hydroxyguaiacyl units formed after coupling of 5-hydroxyconiferyl alcohol, to form benzodioxanes $\mathbf{H}_{G}$, $\mathbf{H}_{5H}$ (and $\mathbf{H}_{S}$). 5-Hydroxyconiferyl alcohol is clearly acting as a surrogate lignin monomer in this polymerization.

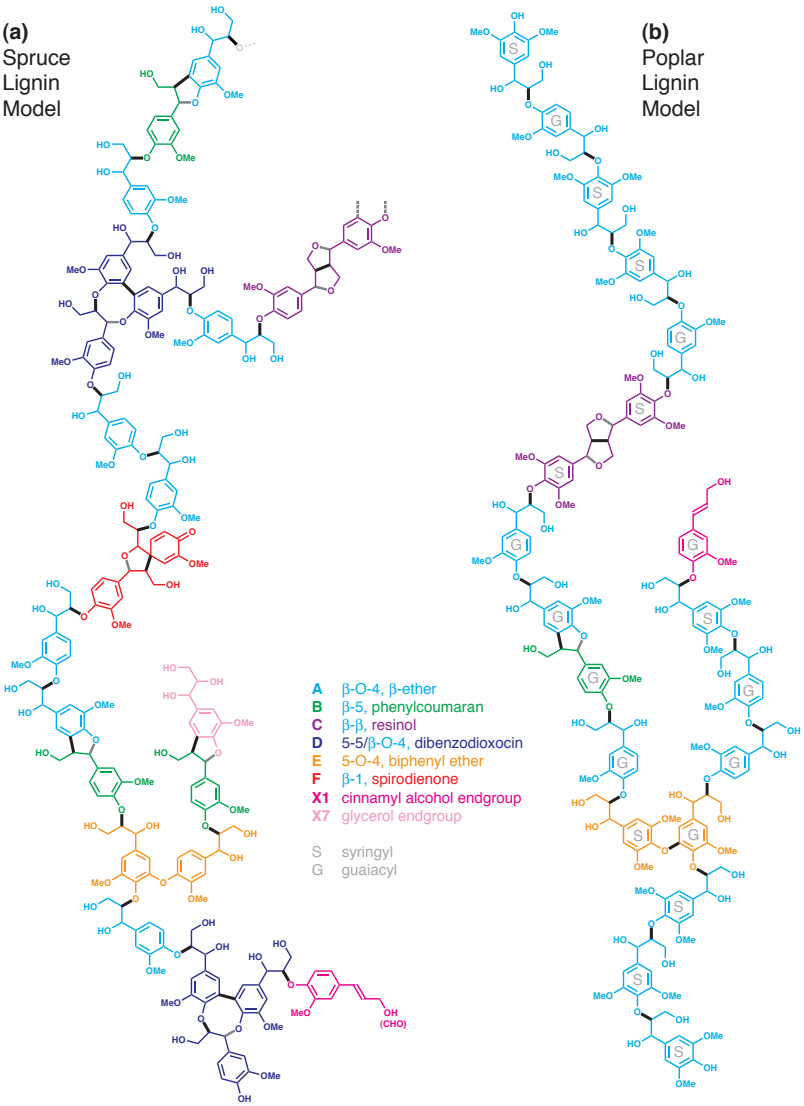


Plate 2.4 Lignin Polymer Models for (**a**) a softwood (spruce) isolated lignin with 25 units, redrawn from Brunow (2001), and (**b**) a hardwood (poplar) isolated lignin with 20 units, redrawn from Boerjan *et al.* (2003). Color coding is uniform across the two models. Bold black bonds indicate the bonds formed during lignification, from radical coupling; lighter gray bonds are from post-coupling internal rearomatization reactions; α-OH groups (and their bonds) from nucleophilically added water assume the colors of their parent structures. The softwood lignin is more branched and contains a lower proportion of β-ether units **A**. The branch points (4–O–5-units **E**, orange; dibenzodioxocin units **D**, dark blue) are differentiated by unique coloring, even though such units may also be β-ethers, for example. Note that each of these structures represents only one of billions of isomers. The softwood model (**a**) has 49 optical centers, as drawn. However, the relative stereochemistries of pairs of centers in ring structures (phenylcoumarans **B**, resinols **C**, and dibenzodioxocins **D**) are fixed; we frankly don't know about the relationships in spirodienones. There are therefore at least 40 'real' centers so we insinuate that there are at least 2^{39} (over 500 billion) physically distinct isomers for the structure shown. For the poplar model (**b**), we calculate that there are 2^{33} (over 8.5 billion) physically distinct isomers. Since lignins are not optically active, the optical centers must be generated randomly, i.e. under simple chemical control. Caution: these are ONLY MODELS! They do not imply any primary structure or sequencing in the lignins themselves but attempt to accommodate the main linkage types and their approximate relative frequencies.

at the oxidation level of an acid, ester, or amide might logically build up when reduction to the aldehyde was compromised. However, the knowledge that such amides were also well known wound-response products prompted caution about whether tyramine ferulate was really a component of the *lignin* in CCR-deficient or wild-type plants where the component is also readily observed (Ralph *et al.,* 1998b). It was however carefully established that tyramine ferulates were integral to the phenolic polymer component isolated in typical fashion (Ralph *et al.,* 1998b). More recently it has been claimed that such components are not part of the polymer fraction (Anterola & Lewis, 2002). The argument presented was that the tyramine peaks in 1D ^{13}C-NMR spectra were much sharper than those from the bulk lignin polymer and therefore were relegated to simple low-molecular-mass impurities.

The sharp peak argument is flawed, reflecting unfamiliarity with NMR relaxation phenomena. While it is true that solution-state NMR peaks broaden with increasing molecular mass owing to the slower molecular tumbling rate and consequent faster relaxation rates, other factors determine line-widths. Terminal units that are especially rotationally mobile also have lower relaxation rates and hence sharper peaks. This is exactly the situation with the terminal free-phenolic tyramine units attached to ferulates incorporated into the backbone of these isolated polymers (Ralph *et al.,* 1999b). Free-phenolic *p*-coumarate units are found acylating the γ-methylol of sidechain units in grass lignins (Ralph *et al*., 1994), and *p*-hydroxybenzoates are 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 & Lundquist, 2001; Lu & Ralph, 2003). All display analogously sharp peaks in their ^{13}C NMR spectra. A simple argument, beyond noting the fact that the lignins were molecular-mass-fractionated, as stated in the original CCR paper (Ralph *et al.,* 1998b), 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 had been well established in a number of studies (Ralph *et al*., 1995; Ralph *et al*., 1998a; Hatfield *et al*., 1999a; Grabber *et al*., 2002; Ralph *et al*., 2004a). 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 certainly be debated, but the contention that they are have been 'unequivocally disproven' as being incorporated into the polymer by combinatorial radical coupling reactions is not supported by the facts.

2.5.1.2 Malleability of lignification: what makes a good monolignol substitute?

Lignins are complex enough when considered to be derived from the three monolignols. However, it is abundantly clear that, in normal and transgenic plants, there may be many other lignin precursors (Boerjan *et al.,* 2003; Lu *et al*., 2004; Morreel *et al*., 2004b; Ralph *et al.,* 2004b). Combinatorial coupling reactions and the malleability of a process that is limited only by its chemistry (and the availability of phenolic monomers), under the current theory, allow researchers to contemplate substantial engineering of the polymer. Basi-

cally, any phenolic transported to the lignifying zone of the cell wall can, subject to simple chemical concerns, be incorporated into the polymer. There remain obvious limits, but we suggest that the perturbations that have been achieved to date by down- and up-regulating monolignol biosynthetic pathway genes merely portend what is possible.

From what is now known about 'non-traditional monomers,' some insights into what makes a good monolignol substitute emerge. They are not particularly surprising. Those that function the best, allowing the plant to grow without obvious phenotypic alteration, are those that function like the monolignols themselves (Ralph, 2006). The most important reaction is the linear end-wise β–O–4-coupling of the monomer with the phenolic end of the growing polymer. Ideal candidates are the acylated monolignols, such as sinapyl acetate (in kenaf, palms), sinapyl and coniferyl *p*-coumarate (grasses) and sinapyl *p*-hydroxybenzoate (palms, poplars and willows). The acylation of the γ-hydroxyl does not significantly interfere with coupling reactions at the β-position. Post-coupling reactions may be significantly altered, however. For example, after β–β-coupling (or cross-coupling with a monolignol), the normal internal trapping of the quinone methide moieties by the γ-OH cannot occur when the γ-OH is acylated (Lu & Ralph, 2002). The coupling still occurs but the products are altered; re-aromatization of the quinone methide takes place via water addition and then may involve the newly formed α-OH in trapping of the other quinone methide moiety. More subtly, the presence of the acyl group alters the isomer-ratio of β-ethers arising from water addition to those quinone methides. Once again, the emerging observations of the same isomer distributions *in vitro* and *in vivo* in these diverse products strongly suggests that post-coupling reactions are not under proteinaceous control.

Partial substitution of sinapyl alcohol with 5-hydroxyconiferyl alcohol, in plants deficient in caffeate/5-hydroxyferulate *O*-methyl transferase (COMT), doesn't appear to adversely affect plant growth and development (Van Doorsselaere *et al*., 1995; Vignols *et al*., 1995; Morrow *et al*., 1997; Baucher *et al*., 1998; Lapierre *et al*., 1999; Osakabe *et al*., 1999; Boudet, 2000; Jouanin *et al*., 2000; Lapierre *et al*., 2000b; Li *et al*., 2000; Akim *et al*., 2001; Bajrovic *et al*., 2001; Guo *et al*., 2001a; Guo *et al*., 2001b; Marita *et al*., 2001; Parvathi *et al*., 2001; Pincon *et al*., 2001; Ralph *et al*., 2001a; Ralph *et al.,* 2001b; Guo *et al*., 2002; Pilate *et al*., 2002; Piquemal *et al*., 2002; Barrière *et al*., 2003; Boerjan *et al.,* 2003; Bout & Vermerris, 2003; Fontaine & Barrière, 2003; Higuchi, 2003; Jouanin *et al*., 2004; Morreel *et al.,* 2004b; Ralph *et al.,* 2004b). Again, 5-hydroxyconiferyl alcohol beautifully undergoes the end-wise β–O–4-coupling reaction (Plate 2.3b). β–O–4-Addition of another monomer to the new dihydroxylated end-unit **P5H** produces a quinone methide intermediate **QM2**, the re-aromatization of which now results in novel benzodioxane units **H** in the lignin, (Plate 2.3a,b. Such units presumably confer enhanced rigidity on the polymer. It has been clearly established that any of the monolignols (coniferyl alcohol, sinapyl alcohol, or further 5-hydroxyconiferyl alcohol) can and do add to the new *o*-diphenol end-unit produced after 5-hydroxyconiferyl alcohol addition to the polymer (Plate 2.3c) (Marita *et al*., 2003). 5-Hydroxyconiferyl alcohol is therefore readily incorporated into the lignification process, making it an excellent monolignol substitute.

Hydroxycinnamaldehydes present a more interesting case. As noted above (see section 2.5.1.1) both coniferaldehyde and sinapaldehyde will, like the monolignols, β–O–4-couple with the phenolic end of a growing lignin polymer (Ralph *et al.,* 2001b). Coniferaldehyde,

however, will only undergo such coupling with syringyl units. Thus both monomers can extend the chain in angiosperm lignification. It is not yet fully understood how the subsequent coupling reactions are affected by the more electron-deficient phenolic ends produced from the coupling of hydroxycinnamaldehydes, i.e. whether further extension of the polymer is attenuated, as may be suggested (Lapierre *et al.,* 2004). The inability of coniferaldehyde to β–O–4-cross-couple with guaiacyl end-units explains why the CAD-deficient pine did not have a lot of new aldehyde structures, despite the other evidence that the monomer was significantly elevated during lignification (MacKay *et al*., 1997; Ralph *et al.,* 1997; Ralph *et al.,* 1999b; Sederoff *et al.,* 1999; Lapierre *et al.,* 2000a; Kim *et al.,* 2003). Without being able to end-wise couple into the polymer, the coniferaldehyde in gymnosperms is relegated to end-units arising from coupling of coniferyl alcohol with the coniferaldehyde, and a few other reactions. One interesting but still minor reaction is coniferaldehyde's coupling with 'syringyl-like' units in softwoods, namely the 5-substituted guaiacyl units in 5–5- and/or 5–O–4-units. β–O–4-Cross-coupling of coniferaldehyde with such structures appear to be evidenced in NMR spectra (Kim *et al.,* 2003).

2.5.2 'Well-defined primary structure'

Notions of well-defined and repeating structures for lignin polymers have emerged from time to time. The most infamous is perhaps the Forss model in which repeating 18-mer units were postulated (Forss, 1966; Forss & Fremer, 2003). Structurally, the cyclic lignin structure proposed does not fit the known structural features in any way. For example, a common rendition (see http://www.lignin.fi/lignin.html) had some 12 of its 18 units as non-cyclic α-aryl ethers — there are few if any such structures in lignins (Ede & Kilpeläinen, 1995; Ralph *et al.,* 2004b). With the emergence of the dirigent hypothesis, and putative proteinaceous control of lignification, repeating units are again being entertained.[10] The 'evidence' comes from alleged sequencing of small oligomers. As will be shown (see section 2.5.2.1), such observations not only fall short of diagnostic evidence for well-defined primary structures in lignin, they are fully consistent with the current theory. More recent claims that the apparent linearity of monomer yield (on a cell wall basis) vs lignin content demand proteinaceous control are also preliminarily addressed in section 2.5.2.2.

2.5.2.1 Sequencing of lignin oligomers

A recent contention was that putatively 'sequenced' fragments, such as the hexamer S-(β–O–4)-S-(β–O–4)-S-(β–O–4)-S-(β–β)-S-(4–O–β)-G, the all-syringyl analog of which is shown in Plate 2.2b, (cf. Fig. 4 in Davin & Lewis, 2005), requires programmed assembly by protein templates (Davin & Lewis, 2005). It should, however, be pointed out that the structure of the hexamer is based on mass spectral evidence and no sequencing has been carried out; the position of the β–β-unit is not known. The reader is encouraged to read the paper by Evtuguin & Amado describing some beautiful mass spectrometric methods (Evtuguin & Amado, 2003), bearing in mind the following observations that relate to the programmed assembly notion. First, it is evident from Fig. 1 in this paper that the putative hexamer masses were chosen from a virtual continuum of low-abundance peaks. Second, note that the eucalyptus lignin sample chosen has a particularly high syringyl content.

The constitution of such a syringyl-rich hexamer is entirely in agreement with the combinatorial coupling theory. For example, since the only coupling mode of a monomer to a syringyl unit is β–O–4 (Plate 2.1e), the syringyl oligomers must be linear and can contain at most a single β–β-unit (resinol).[11] Thus, small oligomers containing all β-ethers or one resinol and a remainder of β-ethers are patently unremarkable — they are exactly what would be expected in combinatorial coupling.[12] There are, quite simply, few other choices in a high-syringyl polymer. The structures 'sequenced' therefore in no way repudiate the theory of combinatorial coupling and cannot be used as evidence for other levels of control during lignification. Concluding that the putative sequencing of such a hexamer rules out combinatorial coupling[13] overlooks the constraints placed on polymer formation by simple mechanistic chemical principles.

Moreover, the mass spectral evidence does not reveal anything about the possible occurrence of stereoisomers. When postulating that lignification is mediated by proteins the authors have evaded addressing the problem of explaining how a racemic polymer like lignin can be formed with protein templates when no racemic proteins are known. Instead of admitting this serious gap in the challenge hypothesis the authors question simple calculations of the possible numbers of isomers of lignin molecules. In polymeric lignin most linkages contain two chiral carbon atoms. And since a structure with *n* asymmetric carbons has 2^n possible stereoisomers (March, 1985), the hexamer in the above example (Plate 2.2b) has $2^{12} = 4096$ total stereoisomers but, taking into account that the β–β-unit has fixed relative stereochemistry, there are $2^9 = 512$ actual possible stereoisomers, $2^8 = 256$ of which are not enantiomers and are therefore physically distinct, and all of which have the same m/z and are indistinguishable by mass spectrometry. Ignoring the simple fact that there are many possible isomers to be considered for any of these oligomers or the polymer (see section 2.5.3.1) does not solve the problem. The fact is that there is no scientific evidence that the isolated oligomer is a single isomer.

Finally, the notion of repeating units should be re-addressed. If the putative protein encodes a chain, say an 18-mer, containing a syringaresinol (β–β-coupled) unit, then this can never repeat![11] The reason again is that the radical coupling mechanism simply does not allow for more that one syringaresinol unit in any linear chain, regardless of its length. The whole idea of repeating macro-units therefore also needs to be dismissed.

2.5.2.2 Monomer-independent sequences

Among the more intriguing recent claims[14] (based largely on unpublished data) is the following: evidence for control over lignin assembly is provided by the ~~fact~~ claim that putatively invariant and predetermined distributions of identical linkages with the primary lignin chains are independent of the degree of monomer methoxyl group substitution patterns and/or lignin contents (Davin & Lewis, 2005; Lewis & Davin, 2005). Evidence for ~~evidence for control over lignin assembly is provided by the claim that putatively invariant and predetermined distributions of identical linkages with the primary lignin chains are independent~~ the designation of well-defined primary structures was claimed from monitoring lignin deposition by thioacidolysis and nitrobenzene oxidation in *Arabidopsis* stems (Davin & Lewis, 2005). It is suggested that the linearity of plots of monomer yield (μM/g of cell wall) *vs*. lignin level (acetyl bromide lignin as a percent of the cell wall) supports the

notion of monomer-independent sequence (Davin & Lewis, 2005). This conclusion does not have any logical connection with the experimental results, which are, in themselves, open to different interpretations: for instance, the degree of syringyl or guaiacyl enrichment in the lignins is not stated.[15] The linearity of the monomer yield versus lignin content (the reality of which is open to doubt — the curve does not go through the origin![16]) is perfectly explainable with a constant ratio of end-wise to bulk polymerization in the time frame studied. The only physically relevant measure is the monomer yield to lignin level on a strictly molar basis; the moles of lignin units have not been calculated from the *p*-hydroxyphenyl:guaiacyl:syringyl (H:G:S) ratio, also confounding the analysis.

The structure of lignins is complex and there are many competing factors that contribute to thioacidolysis and nitrobenzene oxidation monomer yields, but the data refute any notion that there is mysterious monomer-independent control over the lignification. High-syringyl lignins are obviously depleted in β–5-units, but are elevated in β–β-units, even in the same transgenic lines. For example, down-regulation of F5H produces guaiacyl-rich lignins, and an F5H-deficient *Arabidopsis* mutant is, like softwoods, essentially guaiacyl-only (Meyer *et al*., 1998), whereas F5H-up-regulation produces lignins with high-syringyl and only very low guaiacyl contents (Marita *et al*., 1999; Huntley *et al*., 2003; Li *et al*., 2003). Furthermore, *p*-hydroxyphenyl-rich alfalfa lignins (deriving largely from the typically minor monomer, *p*-coumaryl alcohol) appear to be totally depleted in β–1-coupled products that are present in the control (Ralph *et al*., 2006a). In all cases, the distribution of lignin linkages is sufficiently disparate that the notion of sequence invariance is irreconcilable.

In summary, the purported linearity probably lacks a physical basis. But even if it is real, more diagnostic experiments and data are required if they are to provide evidence for the existence of well-defined primary (macro-)structure.

2.5.2.3 The 'urgent need to sequence lignin primary structure'

The espoused need to sequence lignin primary structure (Davin & Lewis, 2005) would only be valid if the dirigent/replication model were correct. Facts regarding lignin structure suggest, however, that lignins have no regularly repeating macro-structures. Attempts at sequencing extended structures are likely therefore Quixotian endeavors, for reasons that were covered in Section 2.5.2.1.

2.5.3 Lignins' racemic nature

In a variety of studies over decades, using a variety of methods, fragments released from the lignin structure (under conditions which have been proven to retain optical activity) have never been found to be optically active (Ralph *et al*., 1999c; Akiyama *et al*., 2000). And entire isolated polymer fractions have no detectable optical activity. Until these papers were published, reservations continued to be expressed about the lack of optical activity in lignins.[17]

The lack of optical activity is entirely expected from the current combinatorial chemical coupling theory, but is a major problem for the dirigent/replication hypothesis. To address the lack of optical activity, two arguments have been advanced. First, it was suggested that there could be two sets of dirigent protein arrays, equally represented, encoding opposite

antipodes (Lewis & Davin, 1998; Gang *et al.,* 1999). It should not be necessary to point out that encoding the opposite enantiomer of a product requires a *different protein*. For example, a dirigent protein that produces (+)-pinoresinol has a primary structure that must obviously be different from a dirigent protein involved in producing (–)-pinoresinol. A different protein will have different kinetics. But even if the kinetics were the same, the two proteins would have to be available at the same time and space to produce racemic products. This is why, even when both enantiomers of a lignan are produced *in planta*, the lignans are not racemic — they have a net optical activity. The putative protein harboring arrays of dirigent sites would have to have an antipode in which *all* of those dirigent sites were re-engineered to produce the opposite enantiomer. Applying Occam's razor that it is the aim of science to present the facts of nature in the simplest and most economical conceptual formulations (Thorburn 1918), this proposal must be dismissed. The second proposed solution was to have template replication produce the opposite isomers, the implications of which will be addressed in section 2.5.4.

2.5.3.1 The 'number of isomers' problem

A consequence of racemic polymerization is extraordinary isomeric complexity that results in lignins and the implication that a lignin molecule has a low probability of being identical to other lignin molecules. Lignin monomers do not have any optical activity — unlike the protein and polysaccharide monomers, they possess no chiral centers. Chiral centers are however created in each coupling reaction involving the monolignol side-chain β-position, and another is created when the intermediate quinone methide is re-aromatized (Plate 2.1). The result is that the number of isomers of any 'randomly' formed lignin structure increases with its degree of polymerization, quickly becoming astronomical. This topic was fully addressed in a recent review (Ralph *et al.,* 2004b) and only the following will be noted here. The models illustrated in Plate 2.4 are not lignins, nor are they assumed 'sequences' of any kind, but are useful models conforming as closely as possible to available unit frequency data. The pine model, as drawn, with 25 units has over 500 billion possible isomers, all of which are physically distinct. The poplar model, with 20 linkages, has over 8.5 billion possible isomers.

The implications of the fact that lignins are racemic polymers needs to be more widely appreciated. This challenge has not been met by proponents of the dirigent/replication hypothesis, who continue to question this unequivocal chemical fact[18] (Davin & Lewis, 2005; Lewis & Davin, 2005) instead of providing experimental evidence. A priority before 'sequencing lignin primary structure' should be to determine whether released lignin oligomers comprise only a single enantiomeric pair, as predicted by the dirigent/replication hypothesis, or many isomers, as predicted by the combinatorial theory. If the number of isomers is found to be consistent with combinatorial coupling, the dirigent/replication hypothesis can be unequivocally dismissed.

2.5.4 Comments on template replication

The hypothesis that lignin primary chains might replicate by template polymerization (Guan *et al*., 1997; Sarkanen, 1998), where one chain dictates the exact mirror opposite

chain in the next assembled molecule (Lewis & Davin, 1998), is problematic. The coupling mechanism proposed is not compatible with the known mechanism of oxidative phenolic coupling. Identification of the forces that govern the replication has been attempted. But there is no discernible hierarchy among the dimer-dimer attractions that are proposed in the article (Chen & Sarkanen, 2003). To have real replication, attractions that are clearly stronger than the alternatives need to be identified, and what is being replicated must be clearly defined. Replication means replication, and that means that identical structures are being formed. This leads to a regular structure with identical units, and must be chiral (see Section 2.5.3).

The template hypothesis of lignin biosynthesis has no precedent. Of the 'several thousand papers' proclaimed as describing template effects (Davin & Lewis, 2005), not one was cited that pertains to lignin biosynthesis, nor was a documented example from nature of a protein template producing racemic polymers provided. Additionally, the problems facing the concept of template replication as a mechanism of lignin formation were evaded.

One concept that has not been appreciated again has to do with the presence of resinol units in a lignin chain. The polymerization of the chain shown in the dirigent/replication model (Plate 2.2a), must begin with β–β-coupling to first form the resinol unit. After that, coupling can occur sequentially from either end of this dimer. There is no alternative method that can produce the same polymer. In the same way, template replication of the chain shown in Plate 2.2a cannot begin at either of the ends of a lignin molecule — the only place it can begin is again with the resinol somewhere within the chain. It is therefore difficult to understand the concept of 'double-stranded template replication' – certainly it cannot operate in anything like the manner of DNA replication, which proceeds from one end to the other. To be taken seriously, the template replication hypothesis has to be brought into accordance with the mechanism of oxidative phenolic coupling, and it must be demonstrated that lignin indeed consists of two enantiomers in equal amounts.

2.5.5 Polymer branching

Only two structures, containing 5–5- and 4–O–5-linkages, are known to result in lignin branching. Neither are formed during end-wise monolignol coupling reactions; they require the coupling of the phenolic ends of two pre-formed oligomers or polymers. And neither can be formed without the participation of at least one guaiacyl unit. For that reason, high-syringyl lignins are logically more linear. The oldest estimates are that 4–O–5- and 5–5-structures in softwood guaiacyl lignins comprise a total of about 13–15% of the linkages (Adler, 1977). Some doubt is cast on the levels of 4–O–5-linkages as they cannot be authenticated in NMR spectra of softwood lignins, either isolated lignins or from dissolution of the entire cell wall fraction (Ralph, 2005). And there are suggestions that dibenzodioxocin structures, formed following addition of a monolignol to a 5–5-linked unit, may terminate the chain; for reasons that need to be examined, the chemical propensity for monolignols to couple to the phenolic unit in a dibenzodioxocin may be low. As a result of these factors, the structural picture is evolving to recognize lignins as more linear polymers, as reflected by recent models that have been proposed, e.g. the two shown in Plate 2.4. The criticism recently leveled (Davin & Lewis, 2005), that 'none of these data are in agreement with the unproven random coupling assembly hypothesis depicted as leading to highly cross-linked

three-dimensional polymers' is therefore outdated. Nevertheless, branching is a characteristic feature of lignin polymers, particularly in guaiacyl lignins, and must be accommodated by any lignification model.

Branching of the lignin chains is not envisaged in the replication theory. In the discussion of this topic, two explanations appear to be offered (Chen & Sarkanen, 2003). First, that there is in fact no branching in the native lignin macromolecule, although in this work the authors have used kraft lignin, derived from cooking wood at 170 °C in caustic soda and sodium sulfide, instead of native lignin. The second is that, if there is branching, it is 'incidental.' Neglecting for now the problems in validating 4–O–5-structures in softwood lignins, there is no debate over the existence of 5–5-coupling between guaiacyl units nor the dibenzodioxocin structures that result in the polymer. Biphenyls (5–5-linkages) are generally accepted as accounting for ~9.5-11% of the C9-units in softwood lignins (Adler, 1977). Dibenzodioxocins, readily detected in all but the most syringyl-rich lignins by NMR, have been estimated to involve ~11% of the C9-units in softwood lignins (Argyropoulos *et al*., 2002). They can hardly therefore be dismissed as being incidental. There is also mention of 'errant' monolignol radicals in (Chen & Sarkanen, 2003). These explanations of uncontrolled oxidative coupling in lignin biosynthesis sound dangerously close to the Freudenberg concept!

2.6 Why the new hypothesis is not in contention

Promoting ambiguous observations and ideas without direct experimental support is not what is required to bring a hypothesis toward theory status. If lignification is in fact under proteinaceous control, as suggested many years ago now, by putative proteins harboring arrays of dirigent sites, the evidence required is clear. Where is this protein or its gene? It should not be hard to find the gene for a protein harboring perhaps 18 or more dirigent binding sites, assuming that dirigent sites that bind monolignols would share some sequence identity with dirigent proteins, and also have features of externally exported proteins. Do such proteins appear in analysis of the cell wall proteome? An in-depth informatic analysis of the *Arabidopsis* genome sequence, coupled with analysis of the large amount of publicly available microarray data for co-expression of candidates with other lignin biosynthetic genes, might also reveal candidates. Has such an analysis been carried out? Furthermore, forward genetic analysis of Arabidopsis T-DNA insertion and/or activation tagged lines might be expected to reveal mutants in the presumed protein template with severe lignin phenotypes. Have such screens been performed? The standard tests then are the traditional loss-of-function/gain-of-function for the gene of interest. Such tests may be lacking even for the dirigent protein/genes themselves.[19]

Prolonging the case for the competing hypothesis by continuing to publish non-diagnostic data that may or may not support the hypothesis does not convince the scientific community. It is time to call for delivery of the protein and its gene. And, if replication on a lignin template is to be advanced, real replication of lignin structure needs to be demonstrated. We suggest that replicating a β-ether homopolymer, generated by recently published methods (Kishimoto *et al*., 2006), would be a good start.

2.7 Is lignification a biochemical anomaly?

Is lignification, the polymerization step, under absolute biochemical control? Or is it, as the current theory maintains, simply a chemical process? And is it true that 'Such claims (that the monolignols are combinatorially coupled according to chemical principles), if ever shown to be correct, would place lignin formation—Nature's second most abundant polymer, no less—with no biochemical equivalent of any kind' (Lewis *et al.,* 1998b)? We suggest that there is useful elegance in chaotic processes, and in combinatorial chemistry. Among the many natural products that could be considered here, we choose just two to illustrate the concepts. The first, cell wall cross-linking by ferulate dehydrodimerization, is a process related to lignification in that it involves combinatorial radical coupling processes. The second, polysaccharide biosynthesis, is used to illustrate that even highly enzymatically controlled processes can have random elements that result in enormously variable primary structure.

2.7.1 Ferulate dehydrodimerization is combinatorial

Ferulates acylating polysaccharides, particularly arabinoxylans in grasses, are now well implicated in vital cell wall cross-linking mechanisms (Ralph *et al.,* 1998a; Hatfield *et al.,* 1999a; Ralph *et al.,* 2004a). Polysaccharide-polysaccharide cross-linking is accomplished by ferulate dehydrodimerization as well as dehydrotrimerization (Bunzel *et al*., 2003; Rouau *et al*., 2003; Bunzel *et al*., 2004; Bunzel *et al*., 2005; Funk *et al*., 2005; Bunzel *et al*., 2006) and perhaps even higher oligomerization (via radical coupling mechanisms). And polysaccharide-lignin cross-linking occurs when ferulates and their dehydrodimers incorporate into lignins via radical reactions with lignin monomers and oligomers.

The simple point to be made here is that the range of combinatorial dehydrodimers (**1A**–**E**, Fig. 2.1) found in plant materials is the same range as produced by chemical radical coupling *in vitro*. Notably, all three 8–8-coupling products **1C** are found *in vivo*, implicating several post-coupling rearomatization pathways. Bearing Occam's razor ('Pluralitas non est ponenda sine neccesitate') in mind again, is it more logical to conclude that ferulate dehydrodimerization takes place via a simple combinatorial chemical process or via a carefully controlled biochemical process with enzymes or proteins specific for all of the coupling modes, to give the same array of products as an uncontrolled reaction?

2.7.2 Polysaccharide primary structure is NOT absolutely dictated

The (1→3),(1→4)-β-glucans from the cell walls of barley grains are a good example of polysaccharides in which there is a tight control over subunit structure, but a random distribution of the subunits within the molecule. These linear polysaccharides contain approximately 30% (1→3)- and 70% (1→4)-linkages. Approximately 90% of the polysaccharides consists of cellotriosyl and cellotetraosyl subunits composed of three and four glucosyl residues, respectively, joined by (1→4)-linkages; these subunits are joined by single (1→3)-linkages. Blocks of 5-11 (1→4)-linked glucosyl residues are also present in significant proportions (Woodward *et al*., 1983). However, the distribution of the cellotriosyl and cellotetraosyl

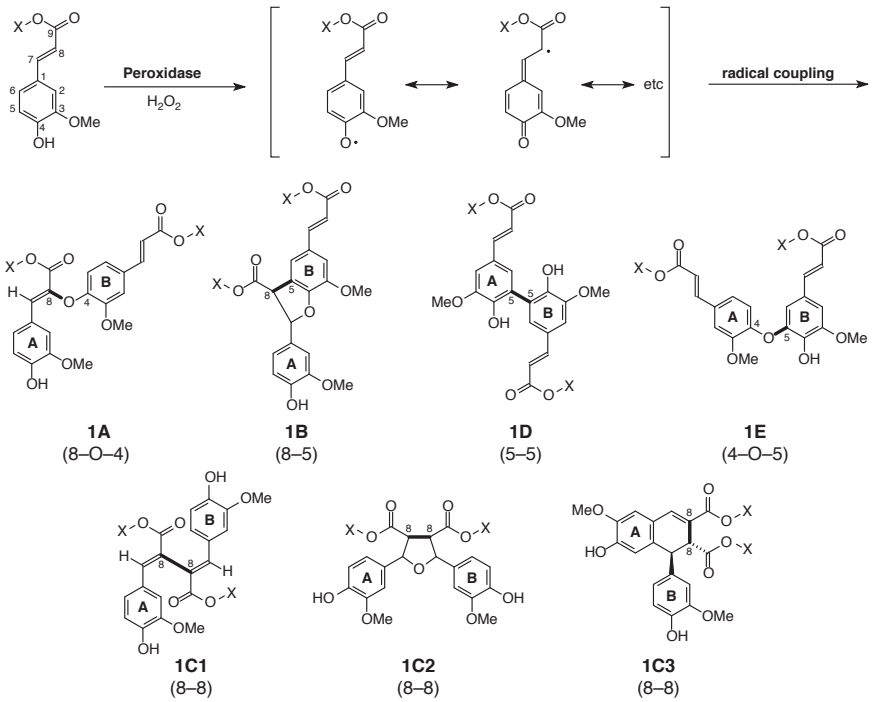


Fig. 2.1 Ferulate dehydrodimerization is an important mechanism for polysaccharide-polysaccharide cross-linking in grasses. All of the dimers **1A-E** have been authenticated, indicating that combinatorial coupling (8–O–4, 8–5, 8–8, 5–5, and 4–O–5) all occur *in planta*, and that post-coupling rearomatization reactions follow at least three courses, *in vitro* and *in planta*, giving distinct 8–8-coupling products **1C1**, **1C2** and **1C3**.

subunits within the molecule is random (Staudte *et al*., 1983). Mechanisms for the biosynthesis of (1→3),(1→4)-β-glucans must account for these features in the molecule. Interestingly, *in vitro* studies showed that substrate concentration influenced the structure of the polysaccharides (Henry & Stone, 1982). The concentration of UDP-glucose significantly affected the relative proportions of cellotriosyl and cellotetraosyl subunits. With the recent demonstration that proteins encoded by *CSLF* genes are involved in the biosynthesis of (1→3),(1→4)-β-glucans, further understanding of the biosynthesis of these molecules may soon be at hand (Burton *et al*., 2006).

2.8 Summary comments on the dirigent/replication hypothesis

The dirigent/replication hypothesis as it has been put forward (Chen & Sarkanen, 2003; Davin & Lewis, 2005) falls short of being a 'constructive challenge' for several reasons. A challenge-hypothesis must offer a solution to a problem, to something not explained by the current theory, and offer a stimulus for further research. What is the problem for which the dirigent/replication hypothesis is a solution? There is no clear statement of such a valid

problem in these articles. The hypothesis is instead offered as a 'conceivable mechanism whereby particular sequences of interunit linkages in lignin macromolecules can be replicated' (Chen & Sarkanen, 2003).

A constructive challenge should suggest new experiments that allow the hypothesized mechanism to be tested, and falsified if necessary. This is where the dirigent/replication hypothesis fails completely. The Freudenberg concept of oxidative coupling in lignin biosynthesis has enabled researchers to explain many features of the structure of lignin. Any alternative hypothesis has to be better than the accepted one to be taken seriously.

Synthesis of lignin on a protein array of dirigent sites that direct the coupling was initially an intriguing idea to consider. But there is a problem: all proteins are chiral and exist as single enantiomers, i.e. as only one of two mirror images. This fact constitutes a serious impediment to the acceptability of the dirigent hypothesis. If lignin is racemic, as researchers all agree, surely the simplest explanation is that the coupling occurs without the intervention of chiral dirigents.

Regarding the replication aspect itself, the idea of molecular attraction between the growing lignin polymer and the monolignol radical is quite plausible and undoubtedly of some importance. But the replication hypothesis as it is stated poses problems of its own. Replication means that identical structures will be formed. This leads to lignin macromolecules with a regular structure and identical units in identical sequences. In order to explain the lack of optical activity, the authors claim that replication inexplicably produces enantiomeric structures. What regularities of the replicating process have the authors observed that necessitate this complicated hypothesis?

In addition to the structural aspects of the polymers that have been revealed over many decades of research, more recent studies with transgenic plants indicate that lignification is a particularly malleable process. The dirigent/replication hypothesis does not tolerate monomer substitution, a process that is now well authenticated with several compelling examples in plants with reduced capacities to biosynthesize the traditional lignin monomers. By contrast, the existing theory readily accommodates monomer substitution along with the plethora of non-monolignol monomers that are becoming increasingly well authenticated in various lignins. Bonding patterns in lignins have been successfully replicated in laboratory cross-coupling experiments, demonstrating that cross-coupling propensities of a number of monomers observed *in vitro* can explain the couplings observed *in vivo*. Overall the oxidative coupling concept as originally formulated by Freudenberg still offers the best basis for understanding the lignification process.

2.9 Conclusions

The examples documented here, and countless more, illustrate how ostensible support for the dirigent array/template replication hypothesis hinges on carefully selected data. For a new hypothesis to be considered viable, it has to stand the scrutiny of experiment. An idea or a hypothesis is not a theory, as Science Editor Alan L. Leshner eloquently reminded researchers recently (Leshner, 2005).[20]

To date, presentations such as that of Davin & Lewis (2005) have failed to specify (in terms of experimental data) the shortcomings of the Freudenberg mechanism of lignifica-

tion, and failed to show how the dirigent/replication hypothesis resolves these shortcomings. Presenting ambiguous or irrelevant observations as evidence cannot supplant diagnostic experiments that validate or invalidate hypotheses. And declaring that 'recent progress has provided crucial evidence to support the *theory* that lignin primary structure is controlled at the proteinaceous level' and that 'evidence for control over lignin assembly has been demonstrated…' (Davin & Lewis, 2005) without producing the evidence, simply does not make it so.

Regarding the controversy, it has been said so well previously that another quote is warranted. Gary Taubes wrote the following in an article on 'The Soft Science of Dietary Fat' (Taubes, 2001). 'To the outside observer, the challenge in making sense of any such long-running scientific controversy is to establish whether the skeptics are simply on the wrong side of the new paradigm, or whether their skepticism is well founded. In other words, is the science at issue based on sound scientific thinking and unambiguous data, or is it what Sir Francis Bacon, for instance, would have called 'wishful science,' based on fancies, opinions, and the exclusion of contrary evidence? Bacon offered one viable suggestion for differentiating the two: the test of time. Good science is rooted in reality, so it grows and develops and the evidence gets increasingly more compelling, whereas wishful science flourishes most under its first authors before going downhill.'

Defending a 50 year old theory is hardly a highlight in a scientist's career. Our aim in this Chapter has been to avoid the potential tragedy whereby a perfectly good theory that remains consistent with all the current facts is overthrown, without challenge, by an unsupported runaway hypothesis. The danger to unwary researchers is that adherence to a principle that may not be correct alters experimental design approach and conclusions.[21] The logical conclusion from examining the facts is that the dirigent/replication hypothesis fails to challenge the current theory and should cease to be entertained. Further research into determining the number of lignin oligomer isomers, for example, may be worthwhile to confirm that lignins are truly combinatorial polymers. Heeding recommendations that lignin primary structure needs urgent sequencing would then be futile.

Finally, lignin should not be labeled an enigma because its exact primary structure is not absolutely stipulated. Lignins are certainly *not* unique in this regard. It is logical that their irregular structure has biological significance (Sederoff *et al.,* 1999). An ordered structure would be more sensitive to destructive influences. As succinctly stated by Denton: 'Biological defense is well served by loosely ordered chemistry. Where many randomly linked products are needed, sloppy pathways are economical' (Denton, 1998). A role of lignin is to fill available space in the cell wall, making the wall strong and hydrophobic. If the cell wall is already largely established by cellulose microfibrils and various complex hemicelluloses, it is difficult to imagine filling up the available space with rigid and ordered molecules in which the primary structure is defined. Indeed, the process is most facile either with small molecules, or with large molecules if the structure of the latter is flexible and able to respond to the local needs. The combinatorial coupling of monolignols is a superb solution of Nature for accommodating this task. Perhaps it is opportune to reevaluate the elegance in chaotic systems, particularly for plant defense. Plants have evolved a route to the crucial cell-wall component lignin that allows them tremendous flexibility to respond to environmental factors, and even to the tinkerings of genetic engineers bent on impeding monolignol production.

2.10 Notes

1 'Thus more and more evidence is gathering that reveals that lignin formation is under full biochemical control, in harmony with formation of all other known biopolymers' (Kwon *et al.,* 1999). 'In summary, there is now little doubt that the biochemical control of lignification is implemented at the levels of both monomer deposition and linkage specification' (Gang *et al.,* 1999). 'Taken together, this evidence suggests that lignin assembly *in vivo* is subject to biochemical regulation, whereby the appropriate monomers are linked in a specific manner to yield a limited number of coupling modes in characteristic proportions' (Croteau *et al.,* 2000). 'This is further evidence for full biochemical control of lignin assembly, i.e. via monomer transport, oxidation, and directed polymerization' (Patten *et al.,* 2003). '…which serve to underscore the fact that complexity of assembly need not be confused any further with random formation' (Anterola & Lewis, 2002). 'Recent progress has provided crucial evidence to support the *theory* that lignin primary structure is controlled at the proteinaceous level' (Davin & Lewis, 2005).

2 Lewis is quoted as stating: [In hypothesis-driven research, the way to move forward is] 'not to look for evidence that doesn't support it. You look for evidence that will support your hypothesis, and you do the controls' (Rouhi, 2001).

3 'It is inconceivable that lignin formation would be left to the vagaries of such a wide range of enzymes or be realized in a haphazard manner' (Davin *et al.,* 1997). 'This supposition represented a departure from *all* other known biochemical processes, since no explicit control of the final configuration of the product was envisaged' and 'which has *no* counterpart elsewhere in biochemistry' and 'the prevailing dogma has insisted that lignin biosynthesis occurs in a manner whereby… no direct control of macromolecular assembly is exercised at the enzymatic level' (Lewis *et al.,* 1998a). 'Such claims, if ever shown to be correct, would place lignin formation—Nature's second most abundant polymer, no less—with no biochemical equivalent of any kind' (Lewis *et al.,* 1998b). 'The one favored by the author is that of full biochemical control over the outcome of phenoxy radical coupling in vivo, in harmony with that of all other biological systems' (Lewis, 1999). 'For decades, the perceived formation of lignins *in vivo* has been biochemically incongruous' (Croteau *et al.,* 2000). 'If this (random coupling) were correct, then the formation of approximately 20-30% of all plant organic matter would have been left essentially to chance' (Davin & Lewis, 2000).

4 'This early working hypothesis did not, however, explain the preponderance of 8–O–4′ linkages in lignin biopolymers' (Lewis *et al.,* 1998a). 'The perspective could not, however, explain many biological aspects of lignification, including … the observed regiospecificity in coupling resulting in approximately 50% to 70% of all interunit linkages being 8–O–4′ bonded' (Davin & Lewis, 2000). *[Note: 8–O–4′ is equivalent to the* β*–O–4-terminology used throughout this chapter].*

5 Pinoresinol is not significantly released from β-ether cleaving reactions. Thus acidolysis of spruce did not release detectable pinoresinol (Lundquist, 1970); thioacidolysis released only traces of the same dimeric products as were produced from pinoresinol (Lapierre *et al.,* 1991; Jacquet *et al.,* 1997; Önnerud & Gellerstedt, 2003). When thioacidolysis trimers (following Raney Ni desulfurization) were examined, most of the β–β-products appeared to be 5–O–4-linked (Önnerud & Gellerstedt, 2003), explaining why β–β-dimers were released in such low quantities. This group suggested, as Lundquist had earlier (Lundquist, 1992), that pinoresinol units might derive not from monolignol-monolignol coupling reactions but from the 5-coupling of a monolignol to the growing oligomer, leading to an in-chain resinol formation (Zhang *et al.,* 2003). At this point, evidence for 5-coupling of monolignols is not compelling and it is not currently possible to distinguish this route from one in which preformed pinoresinol dimers couple at their 5-positions with other lignin oligomers (Ralph *et al.,* 2004b).

6 '…this concept of the free-interchange of lignin monomeric units is diametrically opposite to the working hypotheses that guide our own research undertakings' (Lewis, 1999).

7 Higuchi was the first to note that the red coloration of CAD-deficient plants could be attributed to products derived from the hydroxycinnamaldehydes (Higuchi *et al.,* 1994). Indeed, synthetic lignins incorporating hydroxycinnamaldehydes are distinctly reddish in color. The basis for the

color was not however conjectured. Dehydrodimers of hydroxycinnamaldehydes are at most only pale yellow colored (Kim *et al.,* 2003). The products from β–O–4-cross-coupling with guaiacyl or syringyl units are also not highly colored (Kim *et al.,* 2003). Interestingly, the latter structures do not stain with phloroglucinol-HCl either – this stain has long been used as a test for lignins due to the diagnostic staining coloration with hydroxycinnamaldehyde end-groups. Since hydroxycinnamaldehydes incorporated into lignins by β–O–4-coupling do not stain, however, this stain can not reliably be used to infer anything about the incorporation of hydroxycinnamaldehydes into angiosperm lignins (Kim *et al.,* 2003).

8 This reference also utilized cyclic arguments to ascribe DHCA components to minor impurities. 'Careful evaluation of the reported data revealed that only about 17% of this 'lignin' had been extracted from the tissues for analysis, and that the total contribution to the plant lignin from the dihydroconiferyl alcohol unit was < 5-6%' (Lewis *et al.,* 1998a). The DHCA component was erroneously assumed to be a fully-extractable impurity (and this assumption was then used in the proof that it was an impurity); subsequent analyses showed that significant levels of DHCA remained in the lignin that was not extracted and in the total lignin fraction (Ralph *et al.,* 1999b; Lapierre *et al.,* 2000a; Dimmel *et al.,* 2001; MacKay *et al.,* 2001). The accusation 'The researchers had failed to recognize that formation of dihydrodehydrodiconiferyl alcohol results from dehydrogenative dimerization of at least one, and more probably two, coniferyl alcohol moieties' (Lewis, 1999) is verifiably false. It was clearly stated in the original reference (Ralph *et al.,* 1997) that coniferyl alcohol was involved in the formation of this product. The claim that it arose from the reduction of *preformed* coniferyl alcohol dimers apparently ignored evidence that DHCA was undeniably a monomer.

9 If DHCA monomer is present under peroxidase-H_2O_2 conditions, not only will it undergo radical coupling reactions, but it can be converted to guaiacylpropane-1,3-diol (Ralph *et al.,* 1999a, 2001b). As has been seen previously with benzyl monomers (Zanarotti 1982, 1983), single-electron oxidations produce quinone methides by apparent double H•-abstraction; the mechanism is more likely to be disproportionation of two phenolic radicals to yield the one molecule of the original DHCA and one quinone methide. Under the aqueous conditions, the quinone methide derived from DHCA adds water, producing guaicylpropane-1,3-diol which will then incorporate into lignin.

10 'Both [Sarkanen and Lewis] say they wouldn't be surprised to find some repeating unit, such as an 18-mer' (Rouhi, 2001). 'Current evidence would indicate that there are only a few native lignin primary structures' (Davin & Lewis, 2005).

11 The restriction to only a single syringaresinol unit in a chain derives from the chemistry of lignification. Although β–β-coupling can and does occur between sinapyl alcohol lignin monomers (Plate 2.1b), the resulting syringaresinol dimer, like any other syringyl phenolic end-unit on the polymer, can only couple 4–O- (Plate 2.1e), (and, to a minor extent, 1-). And, since there is never single electron density that can be delocalized from a phenolic radical to the β-carbon of any lignin oligomer, it is simply not possible to produce another syringaresinol unit in the growing chain.

12 Contemplation of simple distributions is even possible here for the all-syringyl analog. For example, if it is assumed that a chain initiates with β–β-coupling to form a resinol R, a hexamer will have 5 interunit linkages, 4 of which will be β-ethers B, i.e. structures B4R. If adding sinapyl alcohol to a phenolic end was random then, in free solution, the distribution of the three possible hexamers B4R:B3RB:B2RB2 will be 1:4:3. Of course, the addition propensities are not equal; adding to a resinol phenol is electronically different than adding to the phenol of a β-ether unit. In the wall, one end of the resinol may also be less accessible. Finally, the hexamers available for MS study were not from interrupted biosynthesis but follow ball-milling which breaks β-ether linkages, so rationalizing the distribution of hexamers, were that data available, would remain complex.

13 'Thus recent claims … that lignins have no primary sequences have no sound scientific basis. Even the most cursory consideration that a lignin-derived fragment has already been sequenced might temper such claims' (Davin & Lewis, 2005).

14 'Evidence for control over lignin assembly has been demonstrated with the discovery of monomer-invariant aryl-O-ether linkages in lignins that upon alkaline cleavage release the corresponding monomers in equimolar amounts, regardless of monolignol composition' (Davin & Lewis, 2005); '…the existence of invariant and predetermined distributions of identical linkages with the primary lignin chains that are independent of the degree of monomer methoxyl group substitution patterns and/or lignin contents. Accordingly, the main primary chains of the biopolymers are considered to be of a predetermined regularity' (Lewis & Davin, 2005). 'We have identified severe restrictions imposed in vivo on lignin macromolecular configuration indicative on non-random assembly. Specifically, there is a predesignated template periodicity in placement of dominant (alkali-cleavable) inter-unit linkages in lignins invariant of methoxyl group substitution pattern (Jourdes *et al.,* 2006).

15 Existing data clearly and predictably show that (syringyl-rich) hardwood lignins give significantly higher yields of thioacidolysis monomers than (guaiacyl-rich) softwood lignins (Lapierre, 1993). Guaiacyl lignins with non-cleavable β–5- and 5–5-linkages, comprising some 15–20% of the linkages and being irregularly distributed, typically yield monomers at ~1200 μM per g of lignin (e.g. 1230 μM/g-lignin in spruce). High-syringyl lignins, which are essentially linear β-ether polymers with typically 10% non-cleavable β–β-units (resinols), and only one resinol per chain (see above, section 2.5.2.1), of course release significantly higher levels of monomers, typically twice those of softwoods (e.g. 2460 μM/g-lignin for a Birch lignin that is ~76%-syringyl). These data, predictable from what has been long known about the structure of guaiacyl vs syringyl lignins, simply cannot be reconciled with a model in which the sequence of units is independent of lignin composition, i.e. independent of the extent to which the polymers were derived from monomers *p*-coumaryl, coniferyl, or sinapyl alcohols.

16 If such plots are to have any basis in reality, obviously they must be forced through the origin — one can hardly debate that a plant with no lignin will not release any thioacidolysis monomers, or the corollary that zero release of monomers implies that there is no (true polymeric) lignin. Yet the projected line in the published plot (Davin & Lewis, 2005) implies that no monomers at all are released if the lignin content is below 6.5%. Constraining such fitting-curves to logically go through the origin suggests that they are not linear.

17 Davin and Lewis observed: 'Yet, although the questions of lack of optical activity in lignin formation had already been carefully addressed* by ourselves and others, several proponents of random coupling re-analyzed the enantiomeric composition of various lignin-derived dimeric products; as expected they were racemic' (Davin & Lewis, 2005). Response: to that point in time, these authors expressed doubt that lignins were racemic, as noted by Rouhi (2001) and as these examples show: 'This contrasts with the racemic linkages which are *believed* to be incorporated into lignin biopolymers' (Lewis *et al.,* 1998a); 'even the *apparent* lack of optical activity in lignins…' (Lewis, 1999); 'perhaps two of the most intriguing aspects about lignification is the *apparent* lack of any optical activity' and 'such a process could also explain the *perceived* lack of optical activity of lignins…' (Lewis & Davin, 1998); 'Indeed, such a process could also explain the *reputed* lack of optical activity in native lignins…' (Gang *et al.,* 1999); '…account for the *reported* lack of optical activity of polymeric lignins' (Davin & Lewis, 2000); 'In contrast to the lignans, lignins are *thought* to be racemic (optically inactive)' and '…the *presumed* lack of optical activity in lignins might result…'(Croteau *et al.,* 2000). *'Carefully addressed' apparently refers to the following distinctly anti-Occam suggestions: 'it could be that the primary chains, for example, form complementary 'mirror images' via template replication, or that there are two distinct types of proteins each encoding formation of complementary chains that effectively cancel out any measurable optical activity' (Lewis & Davin, 1998).

18 The following quotes illustrate how this basic chemical principle is not understood: 'For example, unproven assertions were made that a lignin molecule of relative molecular weight 21,500 could have as many as 10^{66} possible isomers, this being noted to approximate the number of atoms in the galaxy, whereas a lignin chain composed of 20 monomers has over 17 billion possible isomers. Such unproven assertions beg the question as to how much longer such extravagant claims will continue to be made' (Davin & Lewis, 2005) *[Response: as long as they*

continue to be true!]. 'Reinterpretation of previous (often error prone) claims of putative lignin random assembly/combinatorial biochemistry, including that of 20 mer lignin structures allegedly harboring 17 billion plus isomers' and 'these researchers take the unusual approach of developing a model with the scientifically incomprehensible suggestion of 17 billion plus isomers being possible in a short chain lignin' and 'it is timely to unequivocally refute the unparalleled notion that a 20-mer unit poplar lignin contains 17 billion plus isomeric forms. This scientifically untenable notion, being espoused in the 21st century, envisages nearly 1,000,000 times more isomers in a short chain lignin (i.e. 17,000,000,000 isomers!) than that, for example, of the total number of genes (28,000) present in the *Arabidopsis* genome. Such notions cannot be seriously entertained, not only because they fully lack scientific credibility, but because such pronouncements starkly run counter to the detailed and painstaking enzyme/protein work of ourselves and others as described above' (Lewis & Davin, 2005). *[Note: It was never stated that this was lignin, just a model, and not that it contains this many isomers, just that there are 17 billion possible isomers. Such calculations are factual and based on the principle, found in any elementary chemistry text, that a non-symmetrical molecule with n optical centers will have* 2^n *optical isomers,* 2^{n-1} *of which will be physically distinct compounds (March, 1985). A random 20-mer* β*-ether linear polymer has 38 optical centers leading to* 2^{38} *optical isomers and* 2^{37} *(over 137 billion) physically distinct isomers: The lesser number, 17 billion, arises because the model being considered was not a homo-*β*-ether polymer but incorporated structures in which certain of the centers were constrained relative to others (see Plate 2.4). The idea that there are not enough genes to account for such an astronomical number of isomers is absolutely correct, reinforcing the idea that biosynthesis of this racemic polymer cannot be under proteinaceous control!]*

19 What evidence has been obtained to rule out a role for dirigent proteins in lignin, as opposed to lignan, biosynthesis? Gene-array experiments by an independent group (Ralph *et al.,* 2006b) *[no relation to the author in this Chapter!]* suggest that dirigent proteins themselves are not involved in cell wall biosynthesis. What phenotypes are observed in *Arabidopsis* knock-outs of the various dirigent protein genes? Is there a lignin phenotype? Have any experiments been done to test dirigent function by loss or gain of function experiments in transgenics. This would not be difficult in *Arabidopsis*. The one issue that might arise would be genetic redundancy, such that there is no phenotype. Have the experiments been done, or do the comparisons of sequences among the dirigent protein family suggest that this will be a major problem? It should be possible to see if some do not have overlapping expression and should therefore give a phenotype if down-regulated.

20 'There is an important distinction between a belief and a theory. … In our business, a theory is not an educated guess nor, emphatically, is it a belief. Scientific theories attempt to explain what can be observed, and it is essential that they be testable by repeatable observations and experimentation. In fact, 'belief' is a word you almost never hear in science. We do not believe theories. We accept or reject them based on their ability to explain natural phenomena, and they must be testable with scientific methodologies' (Leshner, 2005).

21 For example, a study into wheat straw lignins recently concluded that the lignin was linear and regular, despite the data presented demonstrating just the opposite (Benjelloun-Mlayah *et al.,* 2005). The carbon NMR spectra are far more complex than would result from a lignin with a well-defined primary structure. Indeed, the extraordinary broadness and complexity of both proton and carbon spectra, compared to those from other natural polymers with well-defined primary structures, is attributable to the combinatorial nature by which the polymer was generated and the incredible stereochemical complexity resulting from racemic coupling reactions.

2.11 References *(Note: unlike in chapter, all authors here, and a few minor corrections!)*

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