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PERTURBING LIGNIFICATION

Abstract. Perturbing lignification is possible in multiple and diverse ways. Without obvious growth/development phenotypes, transgenic angiosperms can have lignin levels reduced to half the normal level, can have compositions ranging from very high-guaiacyl/low-syringyl to almost totally syringyl, and can even have their lignins incorporate significant levels of novel monomers. Less has been accomplished in gymnosperms, but lignin level reduction, and alterations in the *p*-hydroxyphenyl:guaiacyl ratio appear possible. Such alterations in lignin composition and structure have marked impacts on chemical processing, in processes such as alkaline pulping to produce fine paper or ethanolysis to provide cellulose for bioethanol production. However, the impacts on solid wood properties remain unknown. On the one hand, any structural changes in the polymer composite might be expected to influence wood properties. On the other hand, such properties are largely driven by non-lignin components and, importantly, by microfibril angle. The actual composition and structure of lignins may have little influence on certain solid wood properties. This chapter provides an overview into a diverse set of materials with extreme variations in lignin that should prove valuable to future research.

1. INTRODUCTION

Lignification appears to be remarkably flexible. The process that synthesizes plant lignin polymers from monomers, typically the monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols) is a purely chemical process, not controlled by proteins or enzymes (Boerjan *et al.*, 2003; Ralph, Lundquist *et al.*, 2004). As such, the lignin polymer is unlike the other plant polymers, the polysaccharides and proteins. It has no optical activity (even though optical centers are generated during each addition of a monomer to the polymer) and in fact no regular, repeating structure. Due to the combinatorial nature of the radical coupling reactions involved in lignification, there are a variety of interunit structures within the polymer and there is no defined primary structure, i.e. no defined sequence of units. In fact, due to the extreme structural and isomer variability, the likelihood of finding two identical lignin polymer molecules is extremely remote. The chemistry, reactivity, and properties of lignins are therefore determined by the ensemble's averaged composition/structure.

Often overlooked by non-chemists is how drastically the composition and structure of a polymer will affect its properties. It is increasingly clear that chemical processing (e.g. in chemical pulping or various biomass conversion schemes such as ethanolysis) may be substantially improved or compromised by the structural alterations that have been produced to date using up- or downregulation of various monolignol pathway genes. For example, kraft pulping is superior for CAD-deficient softwoods and hardwoods (Dimmel *et al.*, 2001; Lapierre *et al.*, 1999; O'Connell *et al.*, 2002; Pilate *et al.*, 2002) and F5H-upregulated hardwoods (Huntley *et al.*, 2003), but COMT-deficiency reduces kraft pulping efficiency (Jouanin *et al.*, 2000; Lapierre *et al.*, 1999). [Enzyme abbreviations are given in the caption to Figure 1]. The reasons are readily related to the altered chemistry of the lignins. However, the effects of lignin composition and structure on solid wood

properties have not been studied. Woody material from plants with sufficient maturity and sufficiently defined structural changes has simply not been available. With the accessibility now of transgenics in which relatively targeted perturbations in the lignin pathway have been achieved, materials will become available for such testing. That is not to say that the genetic methods are perfect. In science it is ideal if you can perturb one single parameter, independent of all others. As is becoming clear, however, perturbing a single gene can have consequences well beyond the lignin pathway. The physical structure of the wall is compromised by some of the perturbations. This chapter will therefore concentrate mainly on identifying the types of lignin changes that have been achieved without major growth/development phenotypic or pleiotropic consequences, but with little assessment on wood properties. Hopefully, materials will become available and will be more extensively tested in the future.

1.1. The Monolignol Pathway and the Genes/Enzymes Involved

The biosynthetic pathway for producing the monolignols, the primary precursor monomers for polymerization into lignins (via lignification), has been well studied. Indeed most of the enzymes and their genes have been long identified and reviewed (Boerjan *et al.*, 2003; Dixon *et al.*, 2001; Higuchi, 2003; Lewis & Yamamoto, 1990; Ralph, Lundquist *et al.*, 2004; Sederoff & Chang, 1991; Whetten & Sederoff, 1995), as shown in Figure 1. However, new studies on substrate specificities and pathway dynamics have resulted in significant revisions of the way the pathway is regarded. For example, although originally thought to be an extensive metabolic grid, the pathway from guaiacyl to syringyl precursors (and ultimately from coniferyl alcohol in gymnosperms to the sinapyl alcohol that is also a primary monomer in angiosperms) is now considered to occur predominantly at the aldehyde level (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999) (Figure 1).

There continue to be new discoveries. It is only recently, for example, that the actual substrates for the C3H enzyme have been clarified. Originally the hydroxylation was considered to occur on *p*-coumaroyl-SCoA. Now there is evidence that the hydroxylation is carried out via various esters, produced from the SCoA-precursor by an HCT (Schoch *et al.*, 2001); the 3-hydroxylated ester product is then returned back to the SCoA level by the same HCT enzymes. Consequently the HCT enzymes are new targets that can be used to perturb the lignification process. It has already been shown in several species that downregulating HCT has similar effects to downregulating C3H.

1.2. Lignification: the Polymerization Process

Lignification is the polymerization step, a process that is now well established to be under simple chemical control. Although enzymes, peroxidases and perhaps laccases, are implicated in generating the radicals required to achieve the radical coupling reactions that generate the polymer, the actual coupling steps are independent of enzymes or proteins (Ralph, Brunow *et al.*, 2007; Ralph, Lundquist

et al., 2004) Lignification should not be confused with the biochemical pathways that produce the building blocks, the lignin monomers or monolignols; all steps in the biosynthesis of the monolignols are catalyzed by enzymes for which genes have been identified, (Figure 1).

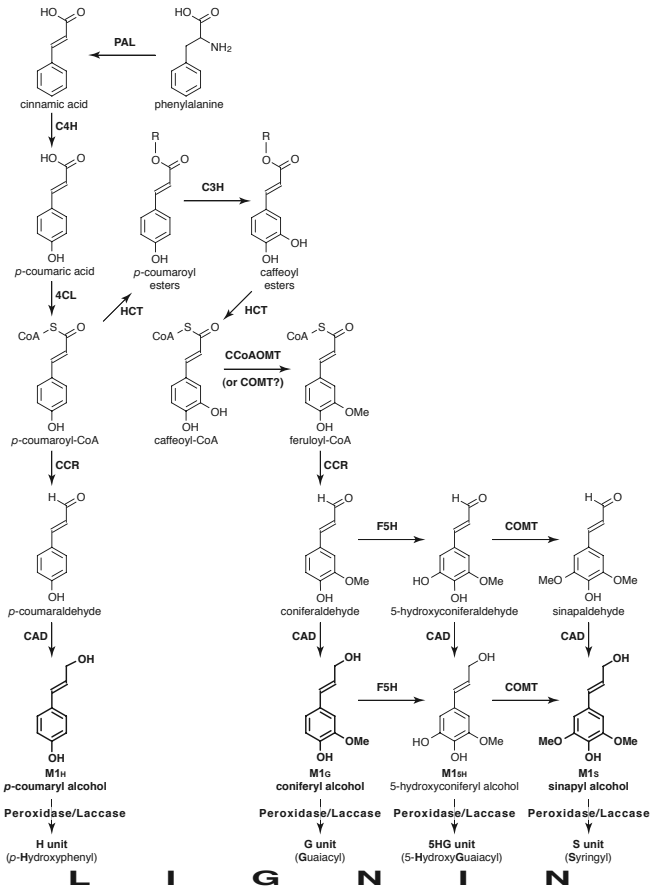


Figure 1. Monolignol biosynthetic pathway; the most favored routes toward the production of monolignols p-coumaryl **M1H**, coniferyl **M1G**, and sinapyl **M1S** alcohols from which p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in lignin derive. CAD, cinnamyl alcohol dehydrogenase; 4CL, 4-coumarate:CoA ligase; C3H, p-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid O-methyltransferase; HCT, p-hydroxy-cinnamoyl-CoA:d-quinate (R = quinate) or shikimate (R = shikimate) p-hydroxycinnamoyl-transferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase. Lignification is via peroxidase/H_2O_2 or laccase/O_2 to generate radicals (from the monolignols and, possibly indirectly, the growing polymer), but the enzymes do not direct subsequent coupling reactions which are under simple chemical control.

1.3. The "Theories" of Lignification

Casual readers of the lignin literature can be forgiven for concluding that there is now a new theory for lignification. Indeed publications claim that the half-century-old Freudenberg-based theory (Freudenberg & Neish, 1968) of combinatorial radical coupling under simple chemical control is nothing more than an "unsupported notion," and that the new, unilaterally designated "theory," involves absolute structural control of lignin biosynthesis on proteins bearing arrays of dirigent binding sites, and by template replication of lignin primary chains (Davin & Lewis, 2005). The actual situation is, however, strikingly different.

Misuse of the word "theory" abounds these days in the media and even in scientific literature. Researchers occasionally need to be reminded that a theory, unlike an hypothesis or an idea, must be consistent with all known facts and must be independently testable. The editor of *Science*, Dr. Alan Leshner recently did just that (Leshner, 2005). The existing lignification theory meets those conditions, being entirely consistent with everything that is known about lignification. Of course some dilemmas remain, but none that require the theory to be summarily dismissed. Proposing a new theory needs a *raison d'être*. It must be specified how the existing theory fails, and how the new theory meets those deficiencies. Any new challenger hypothesis must also support all the known facts.

As detailed in an in-press book chapter (Ralph, Brunow *et al.*, 2007), and partially addressed in a review on lignins (Ralph, Lundquist *et al.*, 2004), the challenger hypothesis fails on all counts. All of the issues raised against the current theory are invalid, and the dirigent hypothesis has extraordinary difficulties accommodating such aspects as lignins' lack of optical activity, its ability to incorporate a variety of monomers, how a growing polymer chain can cross-link with another such chain (forming the prevalent 5–5-linkages), etc.

The dirigent/replication hypothesis also suffers from a problem not well appreciated to date. A case has been made by the proponents for repeating macro-structures in the polymer, "such as an 18-mer" (Rouhi, 2001). Unfortunately, repeating sequences simply cannot be universally realized in a linear chain as the following example shows. Consider a high-syringyl lignin. There are essentially only two types of units possible, those formed by β–β- or by β–O–4-coupling reactions (Figure 2). (A small amount of β–1-coupling is also possible, but ignoring that here does not change the conclusions). The key point is that any polymer chain containing a syringaresinol (β–β-coupled) unit <u>must</u> start from that unit (Figure 2). This is a chemical constraint. It is simply not possible to obtain β–β-coupling from sinapyl alcohol and an already formed dimer, higher oligomer, or polymer; there is no way of getting single-electron density to the β-position of any dimer or oligomer to enable radical coupling at the β-position. Thus, any chain containing a syringaresinol (β–β-) unit (and most of them do since resinol units comprise some 5-10% of units in high-syringyl lignins) must <u>begin</u> with that β–β-coupling. The chain is then extended only by β–O–4-coupling of a new monolignol to the phenolic ends of this dimer or the polymer as it grows. This also means that any replication of that chain, which has even been denoted lately as "double-stranded replication" (Chen &

Sarkanen, 2006) simply cannot occur from either end of the chain as alluded to by the RNA analogy—it must start at the resinol unit.

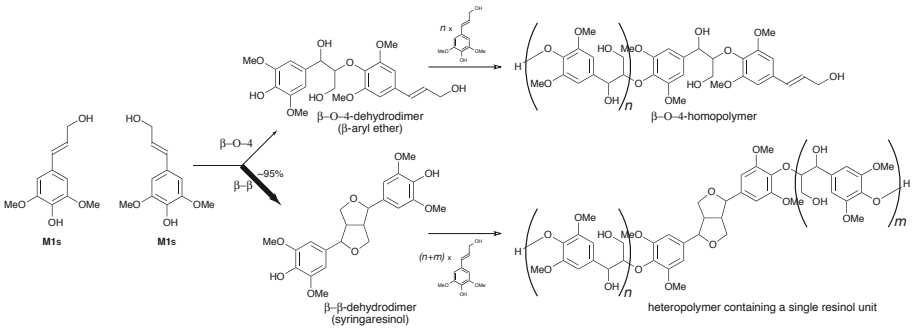


Figure 2. Lignification from sinapyl alcohol **M1s**. The first step, dehydrodimerization, can occur via one of two pathways, overwhelmingly via β–β-coupling to give syringaresinol. Further reactions, with either the β–O–4- or the β–β-dehydrodimer are almost exclusively via β–O–4-coupling of the sinapyl alcohol monomer to the phenolic end-unit; a small amount of β–1-coupling (not shown) is also possible, but further β–β-coupling is impossible. Thus the polymer chain can at most contain only a single resinol unit; two resinols in a chain is chemically impossible. Consequently, "sequences" containing a resinol can not be repeated in the same chain, dispelling any notion that lignins contain repeating macro-units.

An important corollary is that any linear chain can contain only one resinol unit (Ralph, Brunow *et al.*, 2007) (Figure 2). Simple chemical considerations make it absolutely impossible to ever have two resinol units in a chain. The idea, therefore, of have repeating macro-structures (containing resinols) in such lignins is chemistry-violating nonsense.

The future may change concepts and new hypotheses for lignification remain welcome. However, progression of the science in this field is not served by any further assertion of the dirigent-based notion. If its proponents want to keep it in contention, there is no choice but to deliver the protein harboring the array of dirigent sites (and its gene) (Davin & Lewis, 2000), to demonstrate its role in defining absolute lignin primary sequence, and to demonstrate actual replication. Now a decade since the notion was introduced, an inability to find a protein that bears multiple, putatively dozens of, radical binding sites demands explanation. After all, dirigent proteins that couple two monolignols to make optically active lignans have well-established structure, providing information on the necessary binding site motifs (Davin *et al.*, 1997).

Although enzymes and proteins are involved in the biosynthesis of the monomer, and the process of lignification is under exquisite control (monomer supply, radical concentration, etc.) by the cell, and although the "sequence" of any individual chain is influenced by physical and chemical factors, and the reaction matrix in the wall, there is no evidence for any proteinaceous control over the building of the lignin primary structure. Although it has been ridiculed by one group, the old tongue-in-cheek remark in a conference proceedings' that the exact chemical structure of

lignin is unimportant appears to be correct. The current theory of lignification, that primarily of combinatorial chemical coupling of monomer radicals with the growing polymer (radicals), continues to serve well for understanding aspects of lignin biosynthesis and lignin structure/chemistry.

2. ALTERING LIGNIN CONTENT

2.1. Typical Lignin Levels

Lignin levels range from about 26-33% in softwoods, 20-30% in temperate hardwoods, and possible up to 40% in tropical hardwoods (Fengel & Wegener, 1989), although the amount varies depending on species, maturity, environment, and other factors. There are also variations at the cellular level. Indeed, the lignins vary in composition and structure between cells and in various regions of the cell. Most measures therefore provide average composite levels for the entire sample.

2.2. Issues with Quantifying Lignin

It needs to be stressed from the outset that comparing lignin levels across sample types is fraught with difficulties. It is problematic enough to provide reliable data on "normal" plant materials; although a variety of lignin determination methods exist and are in common use, there are issues with all of them. Once the lignin structure is changed, perhaps dramatically and perhaps even by incorporating novel units into the polymer, it should be obvious that the measures become increasingly tenuous. Thus comparisons of lignin levels in a normal plant with its transgenic, or between plants (or transgenics), should not be over-interpreted. For example, the Klason method measures an acid-insoluble lignin gravimetrically and an acid-soluble component by UV. Modified lignins need not have the same reactivity toward acid, and the extinction coefficients for the UV determination will obviously be different for different structural types. Thus, noting that the lignin level of 32% in a natural CAD-deficient pine mutant, for example, is higher than the 31% level in the control may indeed be statistically significant by the measurements performed, but the drastic structural changes do not allow any significant inferences on the actual amounts of "lignins" to be made.

2.3. Transgenics with Lower Lignin Levels

Lowering the lignin level can be accomplished by downregulating some of the genes in the monolignol pathway (Figure 1), particularly those genes early in the committed pathway or, as is shown in at least one case, by co-suppression of two genes later in the pathway. The most successful has been with 4CL (Hu *et al.*, 1999; Li *et al.*, 2003). Downregulation of 4CL in aspen/poplar has resulted in lignin levels as low as 50% of the wild-type levels. Early investigations indicated that this reduction was without major alterations in lignin structure although this work needs

to be repeated with the more advanced methods available today. Whether cell wall polysaccharides are more elevated in a compensatory fashion is still being debated; in any compositional measurements, if lignin levels are down obviously the other components must be higher when summed to 100%. However, the approximate doubling in cellulose:lignin ratio is a potential boon to chemical pulping and ruminant digestibility. The plants initially appear to grow vigorously, but there are indications that prolonged growth and viability are compromised. As with other modifications, selecting levels that produce valuable processing gains while retaining plant vigor and growth rates remains under active research.

Studies into the ramifications on the many interacting pathways associated with cell wall biosynthesis and plant defense are only just beginning. Boerjan's group has been examining how perturbing a single lignin-pathway gene can induce alterations in a host of biochemical processes (Dauwe *et al.*, 2007; Leplé *et al.*, 2007). Such studies are extraordinarily complex and have only been carried out on a limited selection of genes at present; 4CL has not been examined in such a comprehensive way.

Nevertheless, such lignin reductions have obvious potential impacts on the efficiency of various processes ranging from natural ruminant digestibility and enzymatic saccharification for bioethanol production, to chemical pulping. However, to date the influence on solid wood properties has not been examined in any detail. Wood from plants with perturbed 4CL levels can provide valuable material for examining the influence of lignin quantity on wood properties, with minimal interference from competing influences. A preference to use mature woods for physical testing however requires that interesting lines be grown for extended periods.

3. ALTERING LIGNIN COMPOSITION (AND STRUCTURE)

Perturbing a single step in the monolignol biosynthetic pathway can produce dramatic compositional changes (and associated structural changes). Affecting the distribution of H:G:S units in lignin is accomplished by targeting the production of the precursor monolignols **M1**, *p*-coumaryl, coniferyl, and sinapyl alcohols. The key enzymes are the hydroxylases, notably C3H (the hydroxylase that effects the 3-hydroxylation that provides the entry point from H-based to G precursors) and F5H (the hydroxylase that effects the 5-hydroxylation that provides the entry point from G-based to S precursors) (Figure 1). To date, as far as the author is aware, C3H (or its associated HCT) has only been downregulated, but several studies describe both the down- and upregulation of F5H. The latter will be described first.

3.1. Altering S:G Ratios by Perturbing F5H

Angiosperms, unlike most gymnosperms, are characterized by having syringyl/guaiacyl lignins. That is, their lignins derive from the two monolignols, coniferyl and sinapyl alcohols. Altering S:G ratios can have a profound impact on the chemical reactivity of lignins. Syringyl moieties are heavily involved in β-aryl ether

linkages which are readily cleaved during alkaline cooking (e.g. soda and kraft pulping) or during acidolytic treatment (e.g. in ethanolysis to produce cellulose feedstocks for ethanol production). There is therefore interest in raising the S:G in hardwood feedstocks to allow less energy-intensive processing, and even toward introducing syringyl units into softwood lignins in the hope of retaining the superior fiber qualities while facilitating delignification.

Massive alterations in S:G can be realized by perturbing F5H. Indeed, in a single line, it is possible to produce plants having essentially only guaiacyl lignin (Marita *et al.*, 1999; Meyer *et al.*, 1998), which therefore resembles a softwood lignin, or essentially only syringyl lignin in plants far more extreme than those normally found in nature (Franke *et al.*, 1998; Huntley *et al.*, 2003; Marita *et al.*, 1999; Stewart *et al.*, 2007).

3.1.1. F5H-Downregulation (Reducing S:G)

Since lowering S:G has not been of much interest to the wood processing industries, downregulation of F5H on species of commercial interest has not been reported. However, the gene was originally discovered in *Arabidopsis* where the F5H knockout mutant produced its lignin devoid of syringyl components, i.e. essentially 100% G-lignin resembling the lignins in gymnosperms (Marita *et al.*, 1999; Meyer *et al.*, 1998).

3.1.2. F5H-Upregulation (Enhancing S:G)

Upregulating F5H can markedly increase the syringyl content of angiosperm lignins. For example, in poplar, up to ~97.5% S is achievable (Figure 3B) (Stewart *et al.*, 2007). Similar levels were initially noted in *Arabidopsis* (Marita *et al.*, 1999), but it was not recognized until later that the lignins in F5H-upregulated *Arabidopsis* incorporated a new monolignol, 5-hydroxyconiferyl alcohol, and therefore exhibited signs of COMT-deficiency (see below) (Ralph, Lapierre, Marita *et al.*, 2001). COMT is apparently not limiting in poplar/aspen (Li *et al.*, 2003). The 97.5% level noted in poplar transgenics puts this line among plants with the highest syringyl levels reported.

3.1.3. Combinatorial F5H-Upregulation and 4CL-downregulation

It is interesting to address whether perturbing two genes can be used to produce twin benefits in an additive fashion. Such an approach has been achieved in aspen. Downregulation of 4CL to lower the lignin level, and upregulation of F5H to enhance S:G successfully produced aspen transgenics with up to 52% less lignin and a 66% higher S:G (Li *et al.*, 2003).

3.1.4. F5H for S:G Perturbation—Conclusions

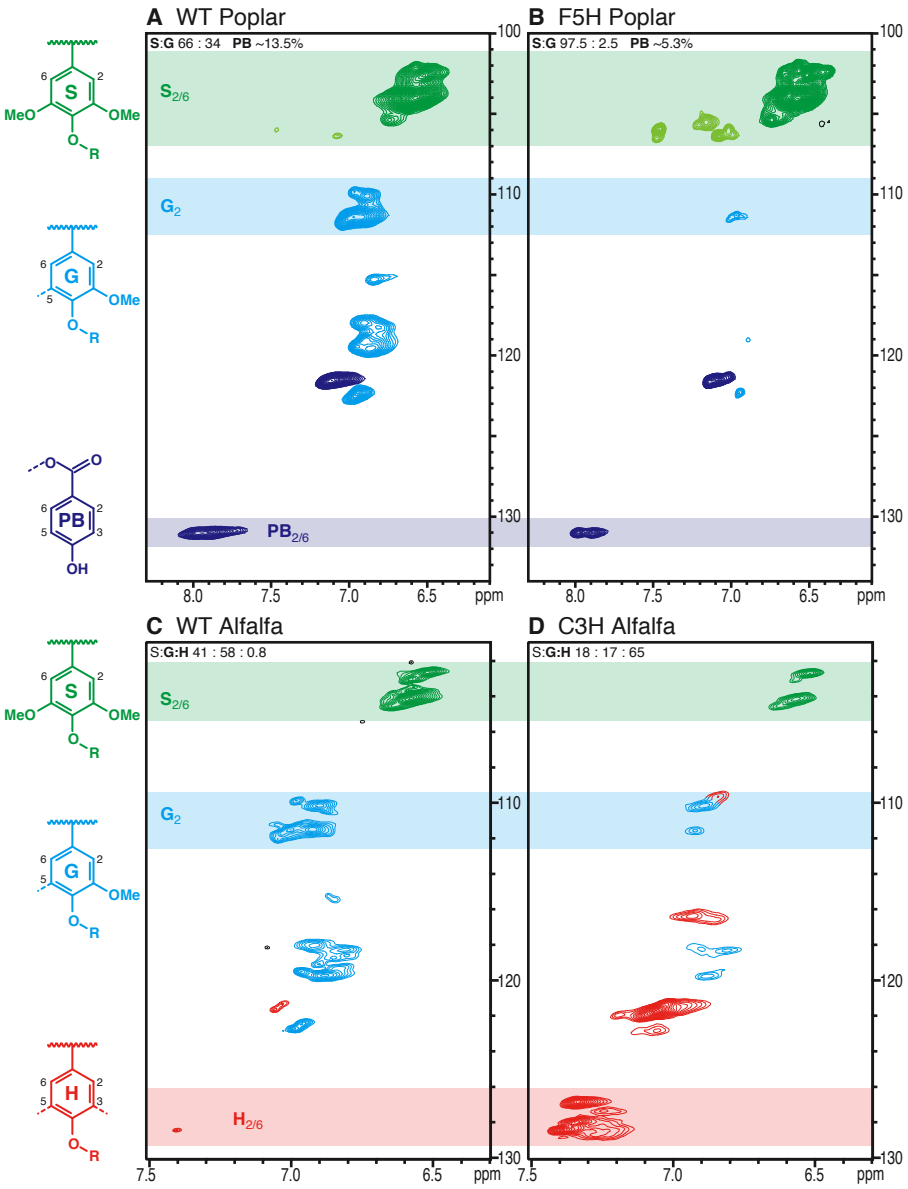
As is apparent, massive S:G compositional changes can be realized in angiosperm lignins by perturbations of the F5H-catalyzed hydroxylation step. Plants with little observable growth/development impacts can be delivered with essentially only guaiacyl lignin (100% G), or up to about 97.5% syringyl lignin (2.5% G). Such plants, mature poplars for example, should provide ideal materials for testing the influence of lignin composition on solid wood properties.

3.2. Altering H:G Ratios by Perturbing C3H or HCT

p-Hydroxyphenyl (H) units, derived from the monolignol *p*-coumaryl alcohol, typically account for less than a few percent of the units in lignins (Figure 3). An exception is in softwood compression woods where high-compression-wood lignin-enriched zones can reportedly be up to 30% H (Timmel, 1986). Compression wood is generally considered undesirable for wood properties and for pulping. Utilizing genetic methods to perturb H:G is beginning to allow one of the effects, the lignin structural changes, to be unraveled; compression woods are also characterized by enhanced galactan levels (Altaner *et al.*, 2006; Bouveng & Meier, 1959; Stockman & Hägglund, 1948). It is not yet clear whether the events leading to polysaccharide redistribution are influenced by the lignin pathway.

3.2.1. C3H-Downregulation (Enhancing H:G)

In Arabidopsis, where knockout mutants are available, the *ref8* C3H-deficient mutant had, as anticipated, no detectable S or G components, only H (Franke *et al.*, 2002). The plants were however particularly stunted and otherwise compromised. C3H-downregulation has been examined in alfalfa, where H-unit levels were elevated from ~1% in wild-type controls to up to 65% in a transgenic line (Ralph, Akiyama, Kim, Lu, Schatz *et al.*, 2006) (Figures 3C-D). Such plants had delayed flowering and reduced stature, but plants with a lower degree of downregulation (Reddy *et al.*, 2005), but still displaying over 20-fold (28% H-levels by thioacidolysis) had no obvious growth or development phenotype. Downregulation of C3H in poplar realized more modest elevations (from ~0.1% in the WT to 28% in the best transgenic, as determined by NMR) (Ralph, Akiyama, Kim, Lu, Ralph *et al.*, 2006) — manuscript in preparation.


A WT Poplar
S:G 66 : 34 PB ~13.5%
B F5H Poplar
S:G 97.5 : 2.5 PB ~5.3%
C WT Alfalfa
S:G:H 41 : 58 : 0.8
D C3H Alfalfa
S:G:H 18 : 17 : 65
S2/6
G2
PB2/6
H2/6
MeO
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OH
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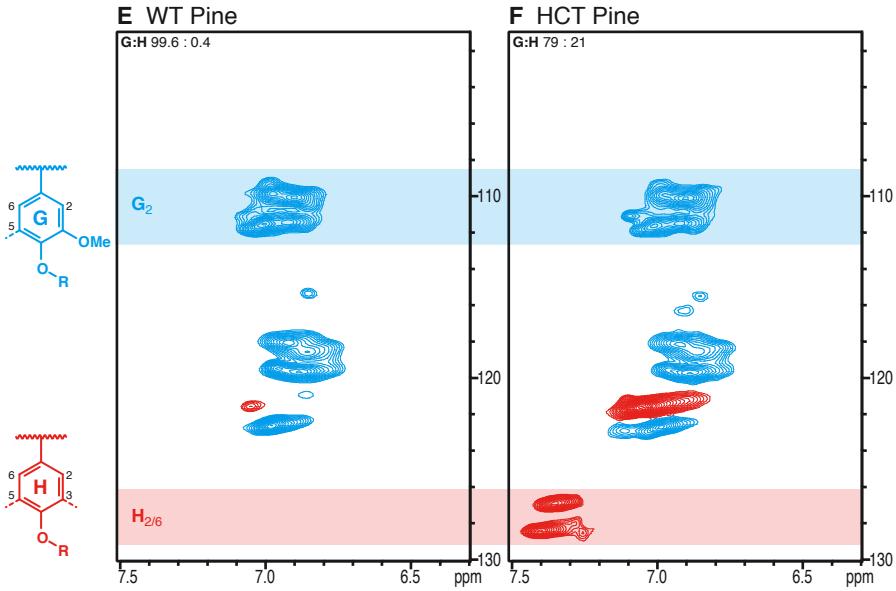

Figure 3. NMR spectra for determining S:G:H (and p-hydroxybenzoate) levels in lignins. A) Wild-type poplar, with a slightly S-rich S/G lignin. B) F5H-upregulated poplar in which the syringyl component reaches a record 97.5% (Stewart *et al.*, 2007). C) Wild-type alfalfa, a slightly G-rich lignin with only traces of H units. D) C3H-downregulated alfalfa in which H units become overwhelming, at the expense of S and G units (Ralph, Akiyama, Kim, Lu, Schatz *et al.*, 2006). E) Pine tracheary elements, like pine lignins themselves, are nearly 100% G, here showing some 0.4% of H units. F) HCT-downregulated pine elements with highly elevated H-levels (Wagner *et al.*, 2007). Ratios are determined by volume-integrating the S2/6, G2, and H2/6 contours in the aromatic regions (shown) of 2D HSQC (heteronuclear single-quantum coherence) spectra (Ralph, Akiyama, Kim, Lu, Schatz *et al.*, 2006)

3.2.2. HCT-Downregulation (Enhancing H:G)

The companion enzyme, HCT, producing the ester substrates upon which C3H acts, has also been downregulated in alfalfa and in a pine tracheary element system (Wagner *et al.*, 2007). Structural details from the alfalfa transgenics are not available, but the plants apparently respond similarly to HCT- and C3H-downregulation. A pine cultured tracheary element system is providing the first glimpses of the process in softwoods. HCT has been shown to also operate in such a system, where its downregulation enhanced H-levels by over 50-fold (from ~0.4% in the control to ~23% in the transgenic).

4. LIGNIFICATION WITH NOVEL MONOLIGNOLS

One of the most striking, and perhaps controversial, aspects of lignification is that the process of lignification involves more than just the two or three primary

monolignols, as reviewed (Boerjan *et al.*, 2003; Ralph, Lundquist *et al.*, 2004). Although long recognized, it has usually gone without comment that there are many other structures in lignins that implicate the incorporation of phenolics besides the monolignols. These historically included minor amounts of phenolic components such as dihydroconiferyl alcohol in gymnosperms and hydroxycinnamaldehydes — indeed, the traditional diagnostic stain (phloroglucinol-HCl) for lignin operates somewhat selectively on cinnamaldehyde endgroups (Adler *et al.*, 1948). More major contributions were from biosynthetic products derived from monolignols such as the acylated monolignols, monolignol acetates, *p*-hydroxybenzoates, and *p*-coumarates. The list of reasonably well-authenticated phenolics that appear to be incorporated into the lignification process is growing annually. Some of these monomers are shown in Figure 4.

Less appreciated is the fact that these phenolic monomers have to be exported to the wall in order to enter into the polymerization process. Even products of incomplete, or truncated, monolignol biosynthesis incorporate into lignins in a variety of mutants and transgenics. In most cases, with sufficiently sensitive methods, these same components can be found at very low levels in "normal" plants. The suggestion therefore is that there is some "slop" in the process and, importantly, that there are transport mechanisms that are able to accommodate structurally diverse phenolics, not just for the traditional monolignols.

This apparent ability of the lignification process to accommodate wide-ranging phenolic precursors portends enormous potential to engineer lignin toward simplified processing. Certainly, there are limitations on the kinds of phenolics that can be successfully introduced into lignins (Ralph, 2006), and on the phenolic precursors that the plant can provide for lignification. But the potential to engineer lignins beyond the relatively limited tinkerings on the monolignol pathway that have been examined to date could open up new avenues toward drastically improved processing of plant biomass for fuel production, for example.

How can the lignification "biopolymer assembly" accommodate a diverse array of phenolic precursors, presumably even some not previously encountered? The answer lies again in the nature of the lignification reaction. It is a polymerization process that is not controlled by proteins or enzymes. Every step in the production of the lignin monomers, the monolignols, is of course controlled by enzymes (for which the genes are known). But the actual polymerization is, from all evidence, merely a chemical reaction. Because of the combinatorial nature of the radical coupling reactions involved, there are a variety of structures that can be produced even when lignification is from a single monomer. For example, polymerization with just coniferyl alcohol will yield β–β-, β–5- and β–O–4-structural units in the polymer, and polymer-polymer coupling reactions can additionally produce 5–5-, and possibly 4–O–5-coupled units.

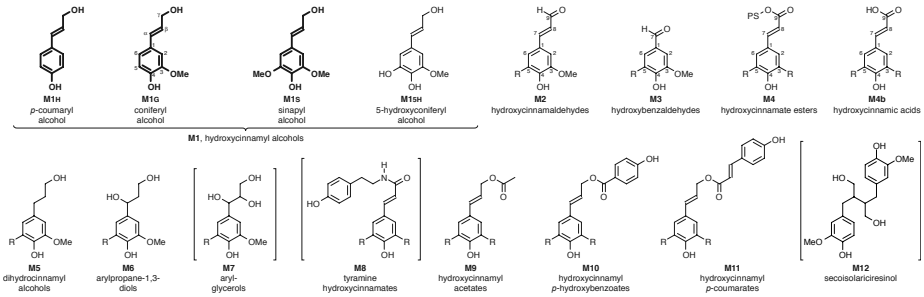

Figure 4. Lignin precursors/monomers (Boerjan *et al.*, 2003). Lignins derive primarily from the three monolignols **M1**, namely **M1H**, **M1G**, and **M1S**. (where the subscripts indicate the type of aromatic nucleus, p-Hydroxyphenyl, Guaiacyl, or Syringyl, in the polymer). **M15H** is a monomer in COMT-deficient plants resulting in 5-hydroxyguaiacyl units in the form of benzodioxanes in the polymer. Other precursors **M2**-**M12** incorporate into lignins to varying degrees. Bracketed compounds have not been firmly established as authentic monomers or, in the case of **M12**, are of unknown derivation. Aldehydes **M2** and **M3** are in normal lignins and more prevalent in CAD-deficient plants. Units **M4**, particularly ferulates **M4G** in grasses, incorporate into lignin. Ferulic acid **M4bG** itself has just been authenticated in lignins via NMR and thioacidolysis markers derived from products of its incorporation. Dihydroconiferyl alcohol **M5G** is found at low levels in all gymnosperms and at elevated levels in a CAD-deficient mutant pine (Ralph *et al.*, 1997), as are the 1,3-diols **M6G** that derive from it. Glycerols **M7** may be produced from monolignols under the oxidation conditions of lignification; they are commonly found in synthetic lignins. Tyramine ferulate **M8G** is found incorporated into the polymers in tobacco (Ralph, Hatfield, Piquemal *et al.*, 1998). Acylated monolignols **M9**–**M11** are prevalent in certainly classes of plants. Finally, secoisolariciresinol and derived structures can be found in angiosperm lignins (Zhang *et al.*, 2003); the unit is not optically active so it does not arise from incorporated lignans.

So what happens when a new phenolic finds itself in the lignifying zone? As long as it is capable of forming a phenolic radical via the peroxidase-H_2O_2 radical generating system, that radical can undergo coupling and, more importantly, cross-coupling reactions based simply on its chemical propensity to do so. This is not without limitation. Just because a coupling reaction can be envisaged "on paper" does not mean that the coupling will occur in practice. Until molecular modeling becomes a great deal more sophisticated than is readily available at present, the only way to determine compatible coupling reactions is to test them empirically, or to deduce them from detailed structural analysis of the polymers produced. An example here, to be illustrated in context below, explains why CAD-deficiency in gymnosperms fails to introduce much coniferaldehyde into the lignin, whereas CAD-deficiency in angiosperms results in the incorporation of both coniferaldehyde and sinapaldehyde into the lignin. The reason is simply because coniferaldehyde does not cross-couple well with guaiacyl units (essentially the only units present in gymnosperm lignins). It is however chemically matched for coupling with the syringyl units found in angiosperms. Sinapaldehyde, as it turns out, couples well with either guaiacyl or syringyl units.

Chemical coupling compatibility means that there are limitations on the types of phenolics that can readily incorporate, at relatively low levels, into lignins. A recent book chapter explored "What Makes a Good Monolignol Substitute" (Ralph, 2006). For now, the key aspects are logically those that make monolignols good lignin monomers. In particular, endwise β–O–4-coupling to the phenolic end of the growing polymer chain remains paramount. Monomers that do not possess the conjugation into the sidechain (i.e. without the α,β-double bond) can undergo coupling reactions with each other and with monolignols at their ring-positions, but because the sidechain cannot couple, remain relegated to terminal units on the polymer.

The following introduces how various novel monomers may be exported into the cell wall and may be incorporated during lignification.

4.1. Ferulate-polysaccharide Esters

Ferulate esters of polysaccharides are present in the cell wall, particularly in grasses (Hatfield *et al.*, 1999; Ralph, Bunzel *et al.*, 2004; Ralph, Hatfield, Grabber *et al.*, 1998), but now also implicated in gymnosperms (Carnachan & Harris, 2000). They can radically couple with each other ("dehydrodimerize") to result in polysaccharide-polysaccharide crosslinking. Indeed, the entire anticipated array of ferulate coupling products (Figure 5), is now well authenticated in a variety of plants (Ralph, Bunzel *et al.*, 2004), including in cereal grains (Bunzel *et al.*, 2007; Bunzel *et al.*, 2004; Bunzel *et al.*, 2001), and even fruits and vegetables (Bunzel & Ralph, 2006), where they may have nutritional and health consequences. As evidenced by the multitude of coupling products (and products resulting from post-coupling reactions) this is also a combinatorial coupling reaction independent of any enzymatic/proteinaceous control.

Ferulates are electronically compatible with lignification. They have been shown in biomimetic systems to couple integrally into the polymer (Ralph *et al.*, 1992), and there is ample evidence that they do so *in planta* (Grabber *et al.*, 1998; Grabber *et al.*, 2000; Micard *et al.*, 1997; Oosterveld *et al.*, 1997; Ralph, Bunzel *et al.*, 2004; Ralph *et al.*, 1995; Ralph, Quideau *et al.*, 1994). The result is a cross-linking of two classes of structural polymers, polysaccharides and lignins. Presumably such cross-linking strengthens the wall in much the same way as disulfide cross-linking of rubber enhances the polymer; natural latex swells in gasoline and abrades easily, but vulcanization allows the production of tires that we will complain about if they don't survive our driving habits after 50,000 miles of contact with the road.

Unfortunately little physical testing has been done on materials with reduced or elevated cross-linking due primarily to the difficulty in obtaining defined materials.

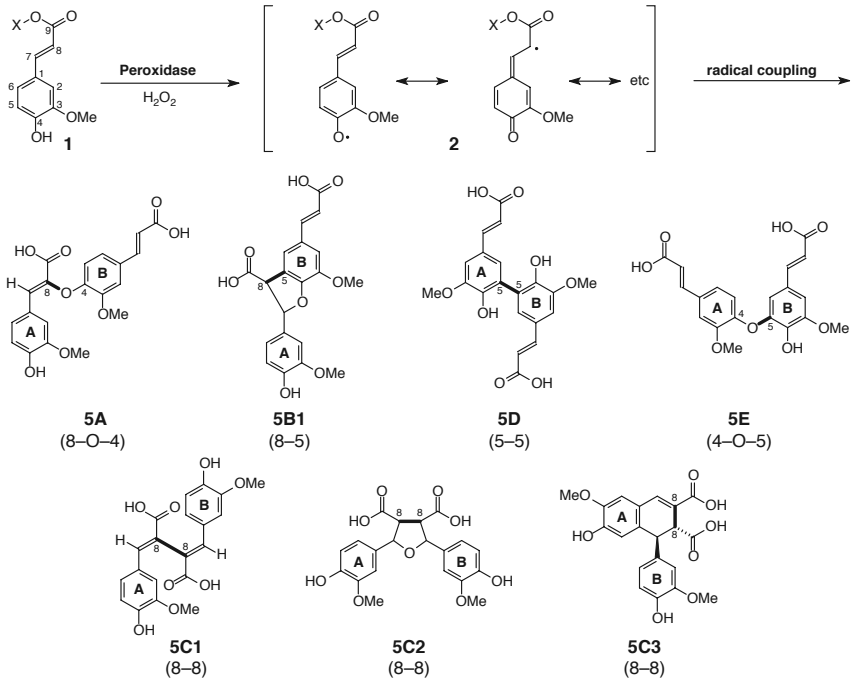

Figure 5. Ferulates **M4bG** (=**1** in this Figure) acylate wall polysaccharides (X) in grasses and gymnosperms. Like monolignols they dehydrodimerize combinatorially—all of the products **5** shown have been evidenced in the wall. Such dehydrodimerization provides a powerful mechanism for cross-linking cell wall polysaccharides. Ferulate and its dehydrodimers are also compatible with lignification. At least in grasses, there is compelling evidence that they enter into lignification, resulting in polysaccharide-lignin cross-linking mediated by ferulate and its dehydrodimers. Such ferulates then can also be classified as lignin "monomers".

4.2. Lignification with Products of Truncated Monolignol Biosynthesis

One aspect of perturbing the monolignol biosynthetic pathway that became clear early on was that products from incomplete monolignol biosynthesis could apparently be exported to the wall and could then become, subject to their chemical compatibility, incorporated into lignins. Examples include the hydroxy-cinnamaldehydes in CAD-deficient plants, and 5-hydroxyconiferyl alcohol in COMT-deficient plants.

4.2.1. Hydroxycinnamaldehydes (CAD-deficiency).

CAD catalyses the final step in the monolignol pathway, reducing coniferaldehyde to coniferyl alcohol and/or sinapaldehyde to sinapyl alcohol. Its downregulation might be expected to lead to an accumulation of the hydroxycinnamaldehydes.

Apparently, these can be exported to the wall as there is good evidence for their incorporation into lignins, particularly in angiosperms (Kim *et al.*, 2002; Kim *et al.*, 2003; Lapierre *et al.*, 2004).

The plant materials themselves often have striking coloration (Figure 6). The naturally CAD-deficient brown-midrib mutant (*bmr1*) of maize/corn has, logically enough, a brown midrib in its leaves. A naturally CAD-deficient loblolly pine mutant has dark reddish-brown xylem (MacKay *et al.*, 1997; Ralph *et al.*, 1997). And CAD-downregulated poplars can be quite red (Jouanin *et al.*, 2000). The coloration is certainly due to the presence of hydroxycinnamaldehydes — even synthetic lignins from coniferyl alcohol but incorporating coniferaldehyde are highly colored (Higuchi *et al.*, 1994). However, the red coloration itself is not diagnostic of its incorporation into the polymer. Indeed, all coupling and cross-coupling products isolated and characterized have only pale yellow coloration at most (Kim *et al.*, 2003). See the further discussion below.

It is difficult to measure the level of incorporation, although NMR methods are now available and this work is required. There is a useful marker compound in the thioacidolysis analytical method that allows the relative levels of hydroxy-cinnamaldehyde incorporation to be tracked with the degree of CAD-deficiency, for example. The marker arises from components in the lignin created specifically from the hydroxycinnamaldehydes' 8–O–4(cross)-coupling with the growing polymer. It is therefore a marker for the integral incorporation of hydroxycinnamaldehydes into the lignification process. The red coloration can be removed by treatment with MeOH/HCl, for example (Laskar *et al.*, 2004). The implication, however, that all hydroxycinnamaldehyde components can therefore also be extracted from the wall by such treatments is however in error. Removing the red coloration does not remove the components from the wall, as readily demonstrated by again measuring the thioacidolysis markers for such hydroxycinnamaldehyde incorporation into lignins (Ralph, Kim *et al.*, 2007)—the levels remain!

The way hydroxycinnamaldehydes incorporate into lignins can be deduced in some detail by NMR experiments *(Kim et al., 2003; Kim et al., 2000)*. Such experiments showed, for example, that coniferaldehyde would not 8–O–4-couple with guaiacyl units in the polymer, but could cross-couple with syringyl units. This observation explained why coniferaldehyde was only poorly incorporated into gymnosperm lignins, being relegated essentially just to end units (derived from coupling with a monolignol). Indeed coniferaldehyde would not cross-couple with polymer guaiacyl units in vitro either. [Coniferaldehyde will dehydrodimerize, and will cross-couple with coniferyl alcohol monomer]. Sinapaldehyde was shown by the same experiments to cross-couple with either guaiacyl or syringyl units, explaining its more ready incorporation into angiosperm lignins.

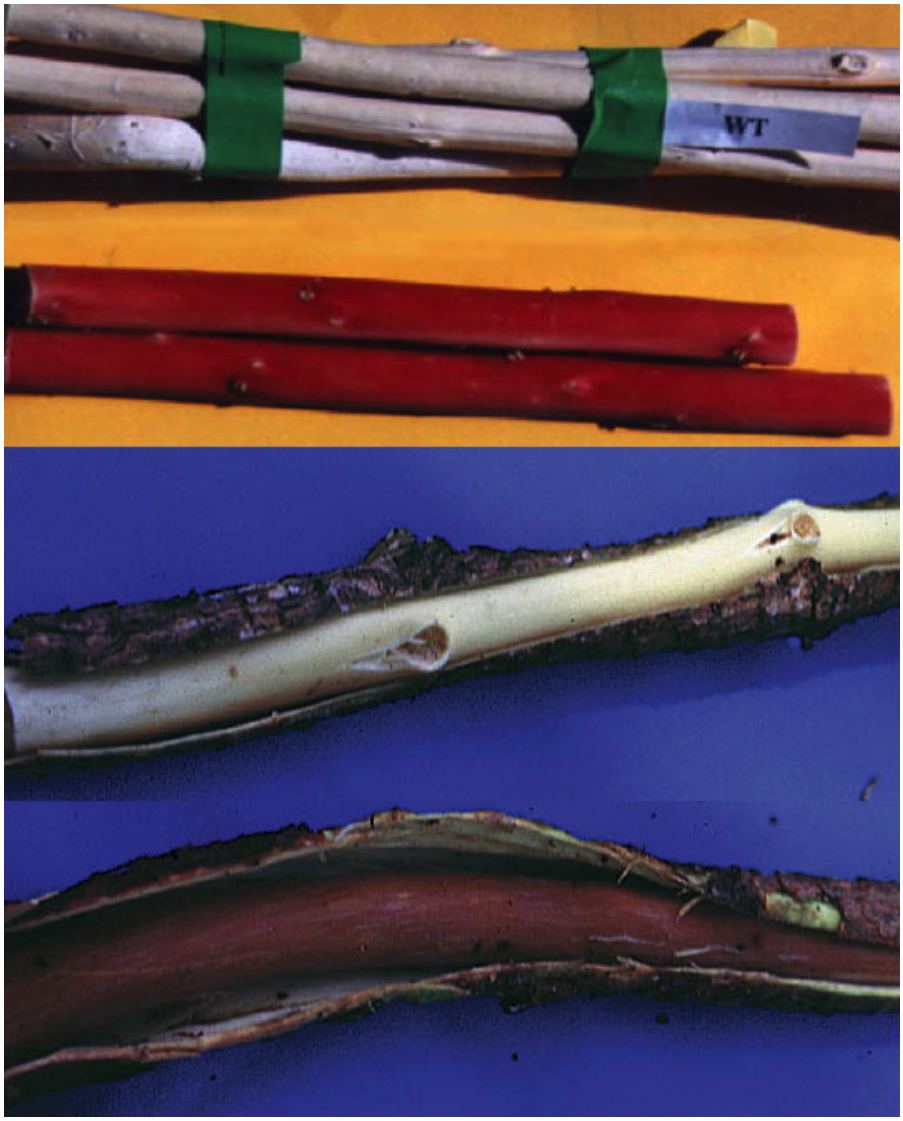

Figure 6. CAD-deficient plants often display red xylem coloration. The coloration is associated with the presence of elevated levels of hydroxycinnamaldehydes, but the hydroxycinnamaldehydes that become incorporated into the lignin polymer, at least the ones that have been identified, are NOT responsible for the coloration. Hydroxycinnamaldehydes that are incorporated into lignins by 8–O–4-coupling (c.f. β–O–4-coupling) are evidenced in 2D NMR spectra, and release diagnostic marker compounds by thioacidolysis; the markers are NOT lost when the red coloration is extracted out. Top: Wild-type control and CAD-deficient poplars from the European trials (Jouanin et al., 2000). Bottom: Normal and CAD-deficient mutant pine from Sederoff's group (MacKay et al., 1997).

4.2.2. 5-Hydroxyconiferyl Alcohol (COMT-deficiency).

A particularly striking example of a plant's response toward utilizing novel monomers comes from COMT deficiency. COMT is the enzyme that methylates the 5-OH on the pathway toward sinapyl alcohol (Figure 1). Its downregulation limits the rate at which the substrate, 5-hydroxyconiferaldehyde, can be methylated to sinapaldehyde. As the preceding hydroxylase reaction catalyzed by F5H is irreversible, and as apparently feedback does not substantially limit entry into the hydroxylation/methylation pathway, 5-hydroxyconiferaldehyde builds up. It is reduced, presumably by the same CAD that reduces coniferaldehyde and sinapaldehyde, to 5-hydroxyconiferyl alcohol. Again, it was not obvious *a priori* that 5-hydroxyconiferyl alcohol could/would be exported to the wall, but that is apparently the case. This novel monomer, 5-hydroxyconiferyl alcohol, functions quite ideally as a monolignol, at least for partial replacement of the normal monolignol sinapyl alcohol. COMT-downregulated poplar (Jouanin *et al.*, 2004; Marita *et al.*, 2001; Morreel, Ralph, Lu *et al.*, 2004; Ralph, Lapierre, Lu *et al.*, 2001; Ralph, Lapierre, Marita *et al.*, 2001) and Arabidopsis (Ralph, Lapierre, Marita *et al.*, 2001), and COMT-deficient maize mutants (Marita *et al.*, 2003) (Lapierre *et al.*, 1988), incorporate the 5-hydroxyconiferyl alcohol integrally into the lignin polymer (Figure 7). As revealed by detailed NMR experiments, the alcohol couples at its β-position to the phenolic end of the growing polymer (by 4–O- or 5-coupling), analogously with the normal monolignols. New hydroxycinnamyl alcohols (coniferyl, sinapyl, or another 5-hydroxyconiferyl alcohol) then couple at their β-positions with predominantly the 4–O-position of the new 5-hydroxyguaiacyl endgroup formed. The 5-OH however provides a new pathway for re-aromatizing the intermediate quinone methide; internal trapping by the 5-OH creates a new benzodioxane structure in the lignins.

The cyclic benzodioxane unit **J** (Figure 7), is presumably less rotationally mobile and flexible than the syringyl β-ether unit it displaces, so the polymer properties are anticipated to be altered. Certainly, the chemical reactivity is altered. COMT-deficient angiosperms pulp less readily (Jouanin *et al.*, 2000; Lapierre *et al.*, 2000). This is largely because, although the benzodioxane is an α,β-diether unit, the etherified ether is not cleaved under soda or kraft pulping conditions (Lu & Ralph, 2007). Therefore the ethers created by incorporation of 5-hydroxyconiferyl alcohol are stable to pulping, unlike the readily cleavable syringyl β-ethers produced from the sinapyl alcohol that it replaces.

Substitution of sinapyl alcohol with 5-hydroxyconiferyl alcohol, at least up to approximately 30% of the total monomer levels, does not appear to affect plant growth and development. Perturbation of COMT therefore provides material in which the lignin structural changes are dramatic. The impact on wood properties could be of considerable interest.

The following approach is a way to test how far normal monolignols can be replaced by novel monolignols. As can be anticipated from the biosynthetic pathway (Figure 1), downregulating COMT and concomitantly upregulating F5H might be a way to drive the levels of 5-hydroxyconiferyl alcohol towards dominance over the normal monolignols. Such materials have just become available in *Arabidopsis*;

preliminary indications are that 5-hydroxyconiferyl has successfully become the dominant monomer, but the plants are particularly stunted.

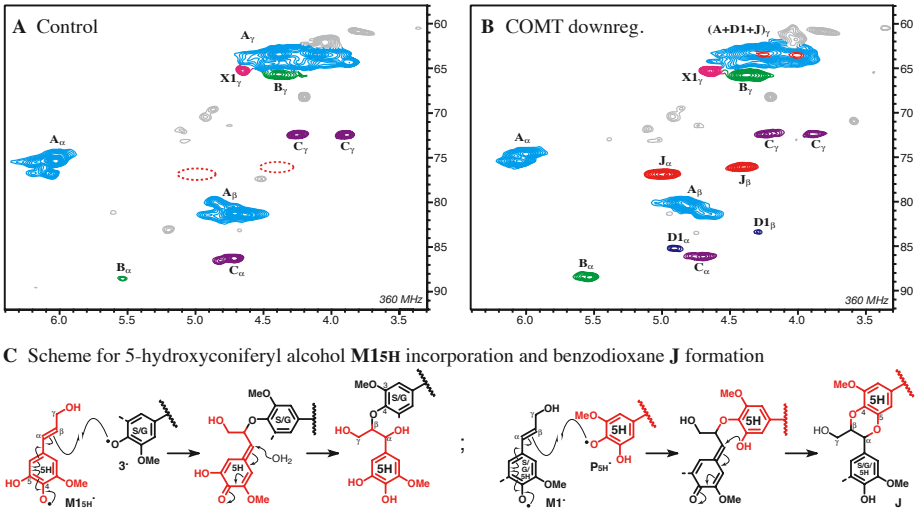

Figure 7. Benzodioxane units result from lignification with the novel monomer 5-hydroxyconiferyl alcohol **M15H**. Partial poplar lignin NMR HMQC spectra (at 360 MHz) showing major lignin peaks and highlighting new peaks for benzodioxane units **J** in COMT-downregulated plants. Acetylated lignins were from **A**) a control poplar, **B**) a COMT-downregulated poplar transgenic. **C**) Scheme for production of benzodioxanes **J** in lignins via incorporation of 5-hydroxyconiferyl alcohol **M15H** into a guaiacyl lignin. Lignin unit designations and coloration is now something of a standard (Ralph, Marita *et al.*, 1999): **A** = β-aryl ether (β–O–4), **B** = phenylcoumaran (β–5), **C** = resinol (β–β), **D** = dibenzodioxocin (5–5/β–O–4), **X1** = cinnamyl alcohol endgroup.

4.2.3. Ferulic Acid (CCR-deficiency)

We have recently found ways to authenticate the low levels of ferulic acid **M5G** (itself) that are incorporated into lignins in some normal plants, and at elevated levels into CCR-deficient plants (Ralph, Kim *et al.*, 2007). A recent report (Laskar *et al.*, 2006) declaring that no evidence could be found for ferulic acid incorporation into CCR-deficient Arabidopsis unfortunately overlooked the clear evidence that is now authenticated via thioacidolysis marker compounds and NMR data from poplar, tobacco, and Arabidopsis.

Ferulic acid, the activated SCoA form of which is the substrate for CCR, must now therefore also be regarded as a lignin monomer. The degree to which it can be incorporated into lignins remains unknown—currently estimated levels are certainly low. However, its incorporation mechanism has provided a novel new branching mechanism for lignins (Ralph, Kim *et al.*, 2007).

4.3. Lignification with Phenolics Beyond the Monolignols

Phenolics that are intermediates already synthesized on the pathway toward the monolignols (or logical products from them, such as 5-hydroxyconiferyl alcohol from 5-hydroxyconiferaldehyde) are not the only phenolics that appear to be incorporated into lignins. Phenolics beyond the pathway are also in evidence.

4.3.1. Reduced phenolics

Dihydroconiferyl alcohol **M5G** is found ubiquitously in gymnosperm lignins. Its mode of biosynthesis is not unclear; i.e. whether it arises from effective hydrogenation of coniferyl alcohol, or from a 1,4-reduction of coniferaldehyde followed by 1,2-reduction of the resultant dihydroconiferaldehyde to dihydro-coniferyl alcohol. It is found, for currently inexplicable reasons, at high levels in lignins from a CAD-deficient pine mutant.

Dihydroconiferyl alcohol is not an ideal monomer as it can not undergo coupling at its sidechain. It is therefore relegated to endgroups on the polymer. Evidence for its incorporation into the polymer come from observing products of its 5- and 4–O-coupling with coniferyl alcohol (at its β-position) and from its 5–5-coupling with like units or guaiacyl units in the polymer (Ralph, Lapierre, Marita *et al.*, 2001; Ralph *et al.*, 1997). It is also demonstrated to be involved in radical reactions by its conversion, *in vitro* and *in planta*, to guaiacylpropane-1,3-diol units **M6G**—two dihydroconiferyl alcohol radicals can disproportionate to one dihydroconiferyl alcohol monomer and one quinone methide; addition of water to the quinone methide produces the 1,3-diol **M6G** (Ralph, Kim *et al.*, 1999). The diol and its derived ketone can be readily found in gymnosperm lignins by NMR methods.

Another reduced compound, secoisolariciresinol **M12**, is also found in softwood lignins (Zhang *et al.*, 2003). Its source is unknown. Clearly it must have originally derived from a β–β-coupling of monolignols, but how it was reduced and how it got to the cell wall remains mysterious. Secoisolariciresinol is a lignan, but this is formed independently in lignan pathways that do not interact with lignin pathways. The lignan is always optically active; the product in the lignin is clearly not. A logical explanation is that the intermediate β–β-quinone methide is non-enzymatically reduced, e.g. by NADPH. Attempts to find biomimetic conditions that can trap and reduce this quinone methide have so far failed, although the β-ether quinone methide can be trapped and reduced this way (Holmgren *et al.*, 2006). We have recently found evidence for such β-ether quinone methide reduction products in lignin (Kim and Ralph, 2007, manuscript in preparation), so the pathway still remains in contention. Where and how such reactions occur, and how the racemic secoisolariciresinol produced is exported to the wall, remain to be determined.

4.3.2. Acylated Monolignols ("Adorning Lignins with substituents")

A particularly successful class of lignin monomers that is often overlooked is the variously acylated monolignols **M9-11** (Figure 4). They can account for a huge fraction of the monomers *in planta*. For example, fully 60% of the sinapyl alcohol

units incorporated into kenaf bast fiber lignins are acetylated (Ralph, 1996), deriving from sinapyl acetate **M9S** (Lu & Ralph, 2002). Recently, many other plants have been shown to also have high levels of acetylation (Del Rio *et al.*, 2007). Grasses are also *p*-coumaroylated, again mainly on syringyl units (Grabber *et al.*, 1996; Lu & Ralph, 1999; Ralph, Hatfield *et al.*, 1994), and various species (including palms, willows, and poplars) are *p*-hydroxybenzoylated (Landucci *et al.*, 1992; Li & Lundquist, 2001; Lu & Ralph, 2003; Meyermans *et al.*, 2000; Nakano *et al.*, 1961; Smith, 1955; Sun *et al.*, 1999). Cross-coupling products of sinapyl alcohol **M1S** and sinapyl *p*-hydroxybenzoate **M10** have recently been found in lignifying poplar xylem (Lu *et al.*, 2004; Morreel, Ralph, Kim *et al.*, 2004).

The acylation has now been quite compellingly demonstrated to occur at the monomer level (Lu & Ralph, 2002; Lu *et al.*, 2004); i.e. the monolignols are acylated to produce new monomers for lignification. Limited *in vitro* studies, and investigations into lignin structure, show that the presence of the acylating group does not substantially interfere with radical coupling reactions, but can influence the products via post-coupling reactions that occur in lignification. Thus the structures of β–β-coupled units are quite different, and predictably so, and β-ethers have different *erythro/threo* isomer ratios. The observations have lead this author to speculate the it is the coupling reactions that a monomer undergoes that are most important in the ability of the phenolic to function as a good monomer in lignification, and that post-coupling reactions (that generate the final structures found in the lignins, are less important (Ralph, 2006)). This is consistent with the notion that the *exact* structure of the lignin polymer is not that important!

With the exception of *p*-hydroxybenzoates and rather minor levels of acetates in some hardwoods, heavily acylated wood lignins are not known and have not been generated transgenically. In large part this is because the implicated putative transferase enzymes (that acylate monolignols using a presumably activated acid) have not been characterized, although our lab is making progress on the enzyme that is responsible for monolignol *p*-coumaroylation in grasses (Marita *et al.*, 2007). There is therefore an opportunity in the future to imbue both softwoods and hardwoods with the ability to create lignins from various acylated monolignols. The impact on wood properties is again unknown, but the following are noted. First, adding *p*-coumarates to lignins significantly increases the fuel value (to the extent that burning lignins is valuable); for example, using the calculator at http://home.fuse.net/clymer/rq/, the *p*-coumarate ester of a sinapate β-ether unit is has some 76% higher heat of combustion per mole than the parent β-ether, and 56% higher for the *p*-hydroxybenzoate analog. Also, acetates presumably make the lignins more hydrophobic and may therefore help with drought-tolerance. The reasons why some natural plants make the investment into producing acylated lignins will hopefully become clear once it is possible to downregulate such production in plants that produce them and/or introduce such functionality into plants that currently don't.

5. IMPACT OF LIGNIN COMPOSITION/STRUCTURE ON WOOD PROPERTIES?

What do all of these advances in understanding lignin structure and biosynthesis spell for understanding, and perhaps influencing, solid wood properties? As a chemist, this author confesses to expecting that all properties are going to be related to structure in some way. Certainly, chemical processing of wood (by alkaline pulping, or by acidolytic breakdown of the lignin component) is heavily influenced by the nature of the polymer. Indeed, it is important to recognize that structure, not just crude determinations of the amount of lignin or even some measures of composition, is crucial.

However, what determines the physical properties of solid wood? It was eye-opening, at the stimulating symposium that lead to this book, to learn that wood properties might in fact be relatively independent of the nature of the lignin. They appear to be driven by the cellulose fibers and, importantly, by microfibril angle (as addressed by other authors in this book). It is conceivable that the changes made to the lignin, dramatic though they are, may not have much of an impact. One can't help but feel that the reason exact lignin structure is of little concern to the plant is that the plant really only needs this polymer to have certain properties, properties within a range that can be met by lignins with considerably varying compositions and structure. Such notions are not inconsistent with the likely reason for the evolution of the polymer as plant forms emerged from their aquatic environment onto the land where they required UV protection and to deal with gravitational forces.

After reading this far in the Chapter, the reader may feel let down at having no insight into how lignin composition and structure address the topic of this book. These aspects are however documented here in the hope that the labs undertaking the genetic engineering of woody plants and those looking at wood and material properties will get together to address these aspects in the future. The idea has been to delineate the types of changes in the lignin that may be important for consideration, for processes including pulping, biomass conversion (to ethanol, for example) and for their solid wood properties.

In summary, Table 1 highlights materials should be recognized for their potential to address the aspects noted.

Table 1. Summary of important transgenics with diverse lignin composition and structure for conversion and solid wood properties testing.

Factor	Target	Result	Norm	SW	HW
4CL↓	Lignin↓	50% lignin	✓	?	✓
F5H↑	S/G↑	Up to 97% S	✓	–	✓
F5H↓	S/G↓	Low S, but how low?	?	–	✓
C3H↓ or HCT↓	H/G↑	Up to 30% H?	✓ ?	✓	✓
COMT↓	New monomer	Up to 30% 5HG	✓	–	✓
F5H↑ & COMT↓	New monomer↑	Higher levels of 5HG	☒	–	✓
F5H↑ & 4CL↓	Lignin↓ & S/G↑	50% lignin, higher S/G	✓	–	✓
CAD↓	Ald. monomers	Low level SAld, CAld	✓	☒	✓

Norm = normal growth and development (no obvious growth phenotype)
SW = softwoods (gymnosperms, e.g. pine)
HW = hardwoods (angiosperms, e.g. poplar)
S = syringyl (from sinapyl alcohol), G = guaiacyl (from coniferyl alcohol), 5HG = 5-hydroxyguaiacyl (from 5-hydroxyconiferyl alcohol)
✓= yes, ? = don't really know yet, ☒ = no, – = not relevant (in softwoods—no gene/enzyme to perturb)

6. REFERENCES

Adler, E., Björkquist, K. J., & Häggroth, S. (1948). Über die Ursache der Farbreaktionen des Holzes. *Acta Chemica Scandinavica, 2*, 93-94.

Altaner, C., Apperley, D. C., & Jarvis, M. C. (2006). Spatial relationships between polymers in Sitka spruce: Proton spin-diffusion studies. *Holzforschung, 60*(6), 665-673.

Boerjan, W., Ralph, J., & Baucher, M. (2003). Lignin Biosynthesis. *Annual Reviews in Plant Biology, 54*, 519-549.

Bouveng, H. O., & Meier, H. (1959). Studies on a galactan from Norwegian spruce compression wood (Picea abies Karst).terification on wood pulp fibers during bleaching. *Acta Chemica Scandinavica, 13*, 1884-1889.

Bunzel, M., Funk, C., Dobberstein, D., Steinhart, H., & Ralph, J. (2007, 29-31 August 2007). *Ferulate Trimers and Tetramers in Cereal Grains.* Paper presented at the Euro Food Chem XIV, Paris, France.

Bunzel, M., & Ralph, J. (2006). NMR Characterization of Lignins Isolated from Fruit and Vegetable Insoluble Dietary Fiber. *Journal of Agricultural and Food Chemistry, 54*(21), 8352-8361.

Bunzel, M., Ralph, J., Lu, F., Hatfield, R. D., & Steinhart, H. (2004). Lignins and ferulate-coniferyl alcohol cross-coupling products in cereal grains. *Journal of Agricultural and Food Chemistry, 52*(21), 6496-6502.

Bunzel, M., Ralph, J., Marita, J. M., Hatfield, R. D., & Steinhart, H. (2001). Diferulates as structural components in soluble and insoluble dietary fibre. *Journal of the Science of Food and Agriculture, 81*(7), 653-660.

Carnachan, S. M., & Harris, P. J. (2000). Ferulic acid is bound to the primary cell walls of all gymnosperm families. *Biochemical Systematics and Ecology, 28*, 865-879.

Chen, Y.-r., & Sarkanen, S. (2006, 3/26/2006). *On the configuration of a double-stranded template implicated in lignin biosynthesis.* Abstracts of Papers of the American Chemical Society, Atlanta, GA.

Dauwe, R., Morreel, K., Goeminne, G., Gielen, B., Ralph, J., Boudet, A.-M., Rochange, S., Kipka, J., Halpin, C., Messens, E., & Boerjan, W. (2007). Molecular phenotyping of lignin-modified tobacco

reveals associated changes in cell wall metabolism, primary metabolism, stress metabolism and photorespiration. *The Plant Journal*, in press.

Davin, L. B., & Lewis, N. G. (2000). Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. *Plant Physiology, 123*(2), 453-461.

Davin, L. B., & Lewis, N. G. (2005). Lignin primary structures and dirigent sites. *Current Opinion in Biotechnology, 16*(4), 407-415.

Davin, L. B., Wang, H.-B., Crowell, A. L., Bedgar, D. L., Martin, D. M., Sarkanen, S., & Lewis, N. G. (1997). Stereoselective biomolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science, 275*, 362-366.

Del Rio, J. C., Marques, G., Rencoret, J., Martinez, A. T., & Gutierrez, A. (2007). Occurrence of naturally acetylated lignin units. *Journal of Agricultural and Food Chemistry, 55*(14), 5461-5468.

Dimmel, D. R., MacKay, J. J., Althen, E., Parks, C., & Boon, J. J. (2001). Pulping and bleaching of CAD-deficient wood. *Journal of Wood Chemistry and Technology, 21*, 1-18.

Dixon, R. A., Chen, F., Guo, D. J., & Parvathi, K. (2001). The biosynthesis of monolignols: a "metabolic grid", or independent pathways to guaiacyl and syringyl units? *Phytochemistry, 57*(7), 1069-1084.

Fengel, D., & Wegener, G. (1989). Chemical composition and analysis of wood. In *Wood. Chemistry, Ultrastructure, Reactions* (pp. 26-65). Berlin-New York: Walter De Gruyter.

Franke, R., Hemm, M. R., Denault, J. W., Ruegger, M. O., Humphreys, J. M., & Chapple, C. (2002). Changes in secondary metabolism and deposition of an unusual lignin in the *ref8* mutant of Arabidopsis. *The Plant Journal, 30*(1), 47-59.

Franke, R., McMichael, C. M., Meyer, K., Shirley, A. M., Cusumano, J. C., & Chapple, C. (1998). Modified lignin in tobacco and poplar plants over-expressing the Arabidopsis gene encoding ferulate 5-hydroxylase. *The Plant Journal, 22*(3), 233-234.

Freudenberg, K., & Neish, A. C. (1968). *Constitution and Biosynthesis of Lignin*. Berlin-Heidelberg-New York: Springer-Verlag.

Grabber, J. H., Quideau, S., & Ralph, J. (1996). *p*-Coumaroylated syringyl units in maize lignin; implications for β-ether cleavage by thioacidolysis. *Phytochemistry, 43*(6), 1189-1194.

Grabber, J. H., Ralph, J., & Hatfield, R. D. (1998). Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *Journal of Agricultural and Food Chemistry, 46*(7), 2609-2614.

Grabber, J. H., Ralph, J., & Hatfield, R. D. (2000). Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. *Journal of Agricultural and Food Chemistry, 48*(12), 6106-6113.

Hatfield, R. D., Ralph, J., & Grabber, J. H. (1999). Cell wall cross-linking by ferulates and diferulates in grasses. *Journal of the Science of Food and Agriculture, 79*(3), 403-407.

Higuchi, T. (2003). Pathways for monolignol biosynthesis via metabolic grids: coniferyl aldehyde 5-hydroxylase, a possible key enzyme in angiosperm syringyl lignin biosynthesis. *Proceedings of the Japan Academy. Series B-Physical and Biological Sciences, 79*(8), 227-236.

Higuchi, T., Ito, T., Umezawa, T., Hibino, T., & Shibata, D. (1994). Red-brown color of lignified tissues of transgenic plants with antisense CAD gene: Wine-red lignin from coniferyl aldehyde. *Journal of Biotechnology, 37*(2), 151-158.

Holmgren, A., Brunow, G., Henriksson, G., Zhang, L., & Ralph, J. (2006). Non-Enzymatic Reduction of Quinone Methides during Oxidative Coupling of Monolignols: Implications for the Origin of Benzyl Structures in Lignins. *Organic and Biomolecular Chemistry, 4*(18), 3456-3461.

Hu, W.-J., Lung, J., Harding, S. A., Popko, J. L., Ralph, J., Stokke, D. D., Tsai, C.-J., & Chiang, V. L. (1999). Repression of lignin biosynthesis in transgenic trees promotes cellulose accumulation and growth. *Nature Biotechnology, 17*(8), 808-812.

Humphreys, J. M., Hemm, M. R., & Chapple, C. (1999). Ferulate 5-hydroxylase from Arabidopsis is a multifunctional cytochrome P450-dependent monooxygenase catalyzing parallel hydroxylations in phenylpropanoid metabolism. *Proceedings of the National Academy of Sciences, USA, 96*(18), 10045-10050.

Huntley, S. K., Ellis, D., Gilbert, M., Chapple, C., & Mansfield, S. D. (2003). Significant increases in pulping efficiency in C4H-F5H-transformed poplars: Improved chemical savings and reduced environmental toxins. *Journal of Agricultural and Food Chemistry, 51*(21), 6178-6183.

Jouanin, L., Goujon, T., de Nadaï, V., Martin, M.-T., Mila, I., Vallet, C., Pollet, B., Yoshinaga, A., Chabbert, B., Petit-Conil, M., & Lapierre, C. (2000). Lignification in transgenic poplars with extremely reduced caffeic acid *O*-methyltransferase activity. *Plant Physiology, 123*(4), 1363-1373.

Jouanin, L., Gujon, T., Sibout, R., Pollet, B., Mila, I., Leplé, J. C., Pilate, G., Petit-Conil, M., Ralph, J., & Lapierre, C. (2004). Comparison of the consequences on lignin content and structure of COMT and CAD downregulation in poplar and *Arabidopsis thaliana*. In C. Walter & M. Carson (Eds.), *Plantation Forest Biotechnology in the 21st Century* (pp. 219-229). Kerala, India: Research Signpost.

Kim, H., Ralph, J., Lu, F., Pilate, G., Leplé, J. C., Pollet, B., & Lapierre, C. (2002). Identification of the structure and origin of thioacidolysis marker compounds for cinnamyl alcohol dehydrogenase deficiency in angiosperms. *Journal of Biological Chemistry, 277*(49), 47412-47419.

Kim, H., Ralph, J., Lu, F., Ralph, S. A., Boudet, A.-M., MacKay, J. J., Sederoff, R. R., Ito, T., Kawai, S., Ohashi, H., & Higuchi, T. (2003). NMR Analysis of Lignins in CAD-deficient Plants. Part 1. Incorporation of hydroxycinnamaldehydes and hydroxybenzaldehydes into lignins. *Organic and Biomolecular Chemistry, 1*, 268-281.

Kim, H., Ralph, J., Yahiaoui, N., Pean, M., & Boudet, A.-M. (2000). Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Organic Letters, 2*(15), 2197-2200.

Landucci, L. L., Deka, G. C., & Roy, D. N. (1992). A ^{13}C NMR study of milled wood lignins from hybrid *Salix* Clones. *Holzforschung, 46*(6), 505-511.

Lapierre, C., Pilate, G., Pollet, B., Mila, I., Leplé, J. C., Jouanin, L., Kim, H., & Ralph, J. (2004). Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins. *Phytochemistry, 65*(3), 313-321.

Lapierre, C., Pollet, B., Petit-Conil, M., Pilate, G., Leple, C., Boerjan, W., & Jouanin, L. (2000). Genetic engineering of poplar lignins: Impact of lignin alteration on kraft pulping performances. *ACS Symposium Series, 742* (Lignin : Historical, Biological, and Materials Perspectives), 145-160.

Lapierre, C., Pollet, B., Petit-Conil, M., Toval, G., Romero, J., Pilate, G., Leple, J. C., Boerjan, W., Ferret, V., De Nadai, V., & Jouanin, L. (1999). Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid *O*-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiology, 119*(1), 153-163.

Lapierre, C., Tollier, M. T., & Monties, B. (1988). A new type of constitutive unit in lignins from the corn *bm3* mutant. *C. R. Acad. Sci., Ser. 3, 307*(13), 723-728.

Laskar, D., Choi, J. W., Kim, M. R., Davin, L. B., & Lewis, N. G. (2004). Cinnamyl alcohol dehydrogenase downregulation in tobacco: Seeing red. *Abstracts of Papers of the American Chemical Society, 228*, U238-U238.

Laskar, D. D., Jourdes, M., Patten, A. M., Helms, G. L., Davin, L. B., & Lewis, N. G. (2006). The Arabidopsis cinnamoyl CoA reductase *irx4* mutant has a delayed but coherent (normal) program of lignification. *Plant Journal, 48*(5), 674-686.

Leplé, J.-C., Pilate, G., Morreel, K., Dauwe, R., Christensen, J. H., Storme, V., DeRycke, R., Messens, E., Boerjan, W., Andersson, S., Sundberg, B., Lapierre, C., Pollet, B., Ruel, K., Lefèbvre, A., Josseleau, J.-P., Petit-Conil, M., Ralph, J., Kim, H., Grima-Pettenati, J., Erban, A., Fehrle, I., Kopka, J., Mansfield, S. D., Kang, K.-Y., Polle, A., & Neumann, A. (2007). Down regulation of cinnamoyl coenzyme A reductase in poplar (Populus tremula x P. alba); multiple level phenotyping reveals effects on cell wall polymer biosynthesis, degradation and structure. *Plant Cell*, submitted.

Leshner, A. I. (2005). Redefining science. *Science, 309*, 221.

Lewis, N. G., & Yamamoto, E. (1990). Lignin: occurrence, biogenesis and biodegradation. *Annu. Rev. Plant Physiology Plant Mol. Biol., 41*, 455-496.

Li, L., Zhou, Y., Cheng, X., Sun, J., Marita, J. M., Ralph, J., & Chiang, V. L. (2003). Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proceedings of the National Academy of Sciences, 100*(8), 4939-4944.

Li, S., & Lundquist, K. (2001). Analysis of hydroxyl groups in lignins by ^{1}H NMR spectrometry. *Nordic Pulp and Paper Research Journal, 16*(1), 63-67.

Lu, F., & Ralph, J. (1999). Detection and determination of *p*-coumaroylated units in lignins. *Journal of Agricultural and Food Chemistry, 47*(5), 1988-1992.

Lu, F., & Ralph, J. (2002). Preliminary evidence for sinapyl acetate as a lignin monomer in kenaf. *Journal of the Chemical Society, Chemical Communications*(1), 90-91.

Lu, F., & Ralph, J. (2003). Non-degradative dissolution and acetylation of ball-milled plant cell walls; high-resolution solution-state NMR. *The Plant Journal, 35*(4), 535-544.

Lu, F., & Ralph, J. (2007, June 10-14, 2007). *Why COMT-deficient plants have poor pulping performance.* Paper presented at the 10th International Congress on Biotechnology in the Pulp and Paper Industry, Madison, WI, USA.

Lu, F., Ralph, J., Morreel, K., Messens, E., & Boerjan, W. (2004). Preparation and relevance of a cross-coupling product between sinapyl alcohol and sinapyl *p*-hydroxybenzoate. *Organic and Biomolecular Chemistry, 2*, 2888-2890.

MacKay, J. J., O'Malley, D. M., Presnell, T., Booker, F. L., Campbell, M. M., Whetten, R. W., & Sederoff, R. R. (1997). Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Proceedings of the National Academy of Sciences, USA, 94*(15), 8255-8260.

Marita, J., Ralph, J., Hatfield, R. D., & Chapple, C. (1999). NMR characterization of lignins in *Arabidopsis* altered in the activity of ferulate-5-hydroxylase. *Proceedings of the National Academy of Sciences, USA, 96*(22), 12328-12332.

Marita, J. M., Grabber, J., Lu, F., Kim, H., Krantz, F., Ralph, J., & Hatfield, R. D. (2007). Development of a specific assay for *p*-coumaroylation of lignin monomers. In preparation.

Marita, J. M., Ralph, J., Lapierre, C., Jouanin, L., & Boerjan, W. (2001). NMR characterization of lignins from transgenic poplars with suppressed caffeic acid *O*-methyltransferase activity. *Journal of the Chemical Society, Perkin Transactions 1*(22), 2939-2945.

Marita, J. M., Vermerris, W., Ralph, J., & Hatfield, R. D. (2003). Variations in the cell wall composition of maize *brown midrib* mutants. *Journal of Agricultural and Food Chemistry, 51*(5), 1313-1321.

Meyer, K., Shirley, A. M., Cusumano, J. C., Bell-Lelong, D. A., & Chapple, C. (1998). Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA, 95*(12), 6619-6623.

Meyermans, H., Morreel, K., Lapierre, C., Pollet, B., De Bruyn, A., Busson, R., Herdewijn, P., Devreese, B., Van Beeumen, J., Marita, J. M., Ralph, J., Chen, C., Burggraeve, B., Van Montagu, M., Messens, E., & Boerjan, W. (2000). Modifications in lignin and accumulation of phenolic glucosides in poplar xylem upon down-regulation of caffeoyl-coenzyme A *O*-methyltransferase, an enzyme involved in lignin biosynthesis. *Journal of Biological Chemistry, 275*(47), 36899-36909.

Micard, V., Grabber, J. H., Ralph, J., Renard, C. M. G. C., & Thibault, J.-F. (1997). Dehydrodiferulic acids from sugar-beet pulp. *Phytochemistry, 44*(7), 1365-1368.

Morreel, K., Ralph, J., Kim, H., Lu, F., Goeminne, G., Ralph, S. A., Messens, E., & Boerjan, W. (2004). Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem. *Plant Physiology, 136*(3), 3537-3549.

Morreel, K., Ralph, J., Lu, F., Goeminne, G., Busson, R., Herdewijn, P., Goeman, J. L., Van der Eycken, J., Boerjan, W., & Messens, E. (2004). Phenolic profiling of caffeic acid *O*-methyltransferase-deficient poplar reveals novel benzodioxane oligolignols. *Plant Physiology, 136*(4), 4023-4036.

Nakano, J., Ishizu, A., & Migata, N. (1961). Studies on lignin. XXXII. Ester groups of lignin. *Tappi, 44*(1), 30-32.

O'Connell, A., Holt, K., Piquemal, J., Grima-Pettenati, J., Boudet, A., Pollet, B., Lapierre, C., Petit-Conil, M., Schuch, W., & Halpin, C. (2002). Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. *Transgenic Research, 11*(5), 495-503.

Oosterveld, A., Grabber, J. H., Beldman, G., Ralph, J., & Voragen, A. G. J. (1997). Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydrate Research, 300*(2), 179-181.

Osakabe, K., Tsao, C. C., Li, L., Popko, J. L., Umezawa, T., Carraway, D. T., Smeltzer, R. H., Joshi, C. P., & Chiang, V. L. (1999). Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. *Proceedings of the National Academy of Sciences, USA, 96*(16), 8955-8960.

Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leple, J. C., Pollet, B., Mila, I., Webster, E. A., Marstorp, H. G., Hopkins, D. W., Jouanin, L., Boerjan, W., Schuch, W., Cornu, D., & Halpin, C. (2002). Field and pulping performances of transgenic trees with altered lignification. *Nature Biotechnology, 20*(6), 607-612.

Ralph, J. (1996). An unusual lignin from Kenaf. *Journal of Natural Products, 59*(4), 341-342.

Ralph, J. (2006). What makes a good monolignol substitute? In T. Hayashi (Ed.), *The Science and Lore of the Plant Cell Wall Biosynthesis, Structure and Function* (pp. 285-293). Boca Raton, FL: Universal Publishers (BrownWalker Press).

Ralph, J., Akiyama, T., Kim, H., Lu, F., Ralph, S. A., Chapple, C., Nair, R. B., Wagner, A., Chen, F., Reddy, M. S. S., Dixon, R. A., Coleman, H. D., & Mansfield, S. D. (2006, August 22-25, 2006). *Lignification in transgenics deficient in 4-coumarate 3-hydroxylase (C3H) or the associated*

hydroxycinnamoyl transferase (HCT). Paper presented at the Polyphenols Communications 2006, XXIII International Conference on Polyphenols, Winnipeg, Manitoba, Canada.

Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P. F., Marita, J. M., Ralph, S. A., Reddy, M. S. S., Chen, F., & Dixon, R. A. (2006). Effects of coumarate-3-hydroxylase downregulation on lignin structure. *Journal of Biological Chemistry, 281*(13), 8843-8853.

Ralph, J., Brunow, G., Harris, P. J., Dixon, R. A., & Boerjan, W. (2007). Lignification: Are lignins biosynthesized via simple combinatorial chemistry or via proteinaceous control and template replication? In F. Daayf, A. El Hadrami, L. Adam & G. M. Ballance (Eds.), *Advances in Polyphenols Research* (in press). Oxford, UK: Blackwell Publishing.

Ralph, J., Bunzel, M., Marita, J. M., Hatfield, R. D., Lu, F., Kim, H., Schatz, P. F., Grabber, J. H., & Steinhart, H. (2004). Peroxidase-dependent cross-linking reactions of *p*-hydroxycinnamates in plant cell walls. *Phytochemistry Reviews, 3*(1), 79-96.

Ralph, J., Grabber, J. H., & Hatfield, R. D. (1995). Lignin-ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydrate Research, 275*(1), 167-178.

Ralph, J., Hatfield, R. D., Grabber, J. H., Jung, H. G., Quideau, S., & Helm, R. F. (1998). Cell wall cross-linking in grasses by ferulates and diferulates. In N. G. Lewis & S. Sarkanen (Eds.), *Lignin and Lignan Biosynthesis* (Vol. 697, Amer. Chem. Soc. Symp. Ser., pp. 209-236). Washington, DC: American Chemical Society.

Ralph, J., Hatfield, R. D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C., & Boudet, A.-M. (1998). NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. *Proceedings of the National Academy of Sciences, 95*(22), 12803-12808.

Ralph, J., Hatfield, R. D., Quideau, S., Helm, R. F., Grabber, J. H., & Jung, H.-J. G. (1994). Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *Journal of the American Chemical Society, 116*(21), 9448-9456.

Ralph, J., Helm, R. F., Quideau, S., & Hatfield, R. D. (1992). Lignin-feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *Journal of the Chemical Society, Perkin Transactions 1*(21), 2961-2969.

Ralph, J., Kim, H., Lu, F., Grabber, J. H., Boerjan, W., Leplé, J.-C., Berrio Sierra, J., Mir Derikvand, M., Jouanin, L., & Lapierre, C. (2007). Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and a *pseudo*-marker compound for cinnamoyl-CoA reductase deficiency). *The Plant Journal*, submitted.

Ralph, J., Kim, H., Peng, J., & Lu, F. (1999). Arylpropane-1,3-diols in lignins from normal and CAD-deficient pines. *Organic Letters, 1*(2), 323-326.

Ralph, J., Lapierre, C., Lu, F., Marita, J. M., Pilate, G., Van Doorsselaere, J., Boerjan, W., & Jouanin, L. (2001). NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of *O*-methyl-transferase-deficient poplars. *Journal of Agricultural and Food Chemistry, 49*(1), 86-91.

Ralph, J., Lapierre, C., Marita, J., Kim, H., Lu, F., Hatfield, R. D., Ralph, S. A., Chapple, C., Franke, R., Hemm, M. R., Van Doorsselaere, J., Sederoff, R. R., O'Malley, D. M., Scott, J. T., MacKay, J. J., Yahiaoui, N., Boudet, A.-M., Pean, M., Pilate, G., Jouanin, L., & Boerjan, W. (2001). Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochemistry, 57*(6), 993-1003.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P. F., Marita, J. M., Hatfield, R. D., Ralph, S. A., Christensen, J. H., & Boerjan, W. (2004). Lignins: natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochemistry Reviews, 3*(1), 29-60.

Ralph, J., MacKay, J. J., Hatfield, R. D., O'Malley, D. M., Whetten, R. W., & Sederoff, R. R. (1997). Abnormal lignin in a loblolly pine mutant. *Science, 277*, 235-239.

Ralph, J., Marita, J. M., Ralph, S. A., Hatfield, R. D., Lu, F., Ede, R. M., Peng, J., Quideau, S., Helm, R. F., Grabber, J. H., Kim, H., Jimenez-Monteon, G., Zhang, Y., Jung, H.-J. G., Landucci, L. L., MacKay, J. J., Sederoff, R. R., Chapple, C., & Boudet, A. M. (1999). Solution-state NMR of lignins. In D. S. Argyropoulos & T. Rials (Eds.), *Advances in Lignocellulosics Characterization* (pp. 55-108). Atlanta, GA: TAPPI Press.

Ralph, J., Quideau, S., Grabber, J. H., & Hatfield, R. D. (1994). Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *Journal of the Chemical Society, Perkin Transactions 1*(23), 3485-3498.

Reddy, M. S. S., Chen, F., Shadle, G. L., Jackson, L., Aljoe, H., & Dixon, R. A. (2005). Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.). *Proceedings of the National Academy of Sciences, 102*(46), 16573-16578.

Rouhi, A. M. (2001). Only facts will end the lignin war. *Chemical and Engineering News*(April 2), 52-56.

Schoch, G., Goepfert, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., & Werck-Reichhart, D. (2001). CYP98A3 from Arabidopsis thaliana is a 3'-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. *Journal of Biological Chemistry, 276*(39), 36566-36574.

Sederoff, R., & Chang, H. M. (1991). Lignin biosynthesis. In M. Lewin & I. S. Goldstein (Eds.), *Wood structure and composition* (pp. 263-285). New York: Marcel Dekker.

Smith, D. C. C. (1955). *p*-Hydroxybenzoates groups in the lignin of Aspen (*Populus tremula*). *Journal of the Chemical Society*, 2347.

Stewart, J. J., Akiyama, T., Chapple, C. C. S., Ralph, J., & Mansfield, S. D. (2007). Lignins with Extreme Syringyl Levels: The Effects of Over-Expression of Ferulate 5-Hydroxylase on Lignin Structure in Hybrid Poplar. *Journal of Biological Chemistry*, submitted.

Stockman, L., & Hägglund, E. (1948). Om granvedens träpolyoser och deras förhållande vid hydrolys (On the wood polyols in spruce wood and their behaviour on hydrolysis). *Svensk Papperstidnung, 51*, 269-274.

Sun, R. C., Fang, J. M., & Tomkinson, J. (1999). Fractional isolation and structural characterization of lignins from oil palm trunk and empty fruit bunch fibres. *Journal of Wood Chemistry and Technology, 19*(4), 335-356.

Timmel, T. E. (1986). *Compression wood in gymnosperms*. Heidelberg: Springer.

Wagner, A., Ralph, J., Akiyama, T., Flint, H., Phillips, L., Torr, K. M., Nanayakkara, B., & Te Kiri, L. (2007). Modifying lignin in conifers: The role of HCT during tracheary element formation in Pinus radiata *Proceedings of the National Academy of Sciences, USA, 104*(28), 11856-11861.

Whetten, R., & Sederoff, R. (1995). Lignin Biosynthesis. *Plant Cell, 7*, 1001-1013.

Zhang, L., Henriksson, G., & Gellerstedt, G. (2003). The formation of β-β structures in lignin biosynthesis - are there two different pathways? *Organic and Biomolecular Chemistry, 1*(20), 3621-3624.

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