The Science and Lore of the Plant Cell Wall

Biosynthesis, Structure and Function

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Edited by

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What Makes a Good Monolignol Substitute?

John Ralph

Lignin and Lignification

Among the more intriguing components in the plant cell wall are the lignin polymers. Their formation, occurring after the polysaccharides are laid down, provides structural integrity to those lignified cell walls, facilitates water transport, and provides defensive functions. But what makes the polymers enigmatic is their mode of formation. Unlike the polysaccharides and proteins, no exact chemical "sequence" of units is dictated by the cell. Although there is considerable control by the cell over aspects of the structure by the supply of the various lignin monomers to the wall and the supply and control of oxidants (in the peroxidase-H_2O_2 system), the assembly of the polymer is a combinatorial process, under the auspices of simple chemical control, i.e. governed by normal chemical concerns such as the concentrations of reactants, their natural coupling and cross-coupling propensities, the matrix, and the physical conditions during the polymerization (Boerjan et al. 2003, Ralph et al. 2004). The process is not under the control of enzymes, for example, so results in racemic polymers with astronomical numbers of possible isomers; no two lignin molecules need have the same structure. This theory has been recently challenged (Gang et al. 1999), and even a text book has pronounced that lignification is a process under absolute structural control (Croteau et al. 2000), but this challenge is wholly without merit and should now be dismissed (Ralph et al. 2004).

In addition to providing a flexible system for the plant to respond to various challenges and stresses, such a polymerization mode provides unparalleled opportunities to re-engineer this component of the wall. Nature herself has explored many variations on the theme already. Certain plants use monomers that most texts would not consider to be lignin monomers; it is widely assumed that lignins derive from only three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols but, as reviewed recently (Boerjan et al. 2003, Ralph et al. 2004), other lignin monomers must be recognized. And nature of course experiments with genetic engineering. Mutations sporadically appear in which "crucial" lignin-biosynthetic-pathway

genes are knocked out. Some such plants have made substitutions for the monolignols whose biosynthesis has been negatively impacted.

The process of lignification has been well implemented. In what remains best described as a process of radical coupling of phenols (Harkin 1967), the monolignols are rather ideal monomers. Like most other chemical polymerizations, the primary polymerization reaction involves the coupling of a monomer with the growing polymer (Fig. 1a); monomer-monomer reactions certainly occur but are less prominent in the overall scheme. Polymer-polymer (or oligomer-oligomer) coupling reactions play a significant role in lignification, creating branching points in the polymer. The radical coupling process is what allows the polymerization to be somewhat combinatorial. The monolignols are potentially capable of coupling at various sites (β-, 4–O-, 1-carbons for coniferyl and sinapyl alcohols, and additionally the 5-carbon for coniferyl alcohol, but almost overwhelmingly react via their β-positions with phenolic ends on the growing polymer, Fig. 1a. This conveys a certain linearity to the polymer. The phenolic end can couple at its 4–O- and, less frequently, 1-positions, and/or (in the case of guaiacyl units only) the 5-position. In many ways it is the favored β–O–4-coupling of a monolignol with the growing polymer that defines lignins, but it is the other reactions including β–5- and β–1-coupling, as well as 5–5- and 4–O–5-coupling between oligomers, that characterizes them.

Non-monolignol Lignin Monomers

So what kinds of monomers can substitute for the primary monolignols? From studies to date, those that are capable of undergoing analogous β–O–4-coupling with the growing polymer seem to be the most successful. This may be the major requirement, although it still provides considerable variability, as will be seen below. Plants which have available, and apparently attempt to utilize, monomers that can not undergo this type of coupling reaction fare more poorly, presumably because their lignin substitutes do not adequately exhibit the properties required of them.

Acylated monolignols

A class of successful lignin precursors are the variously acylated monolignols implicated in an assortment of plants, Fig. 1b (Ralph et al. 2004). Sinapyl acetate has been demonstrated to be a monomer in kenaf bast fiber and palm lignification, and at low levels in other

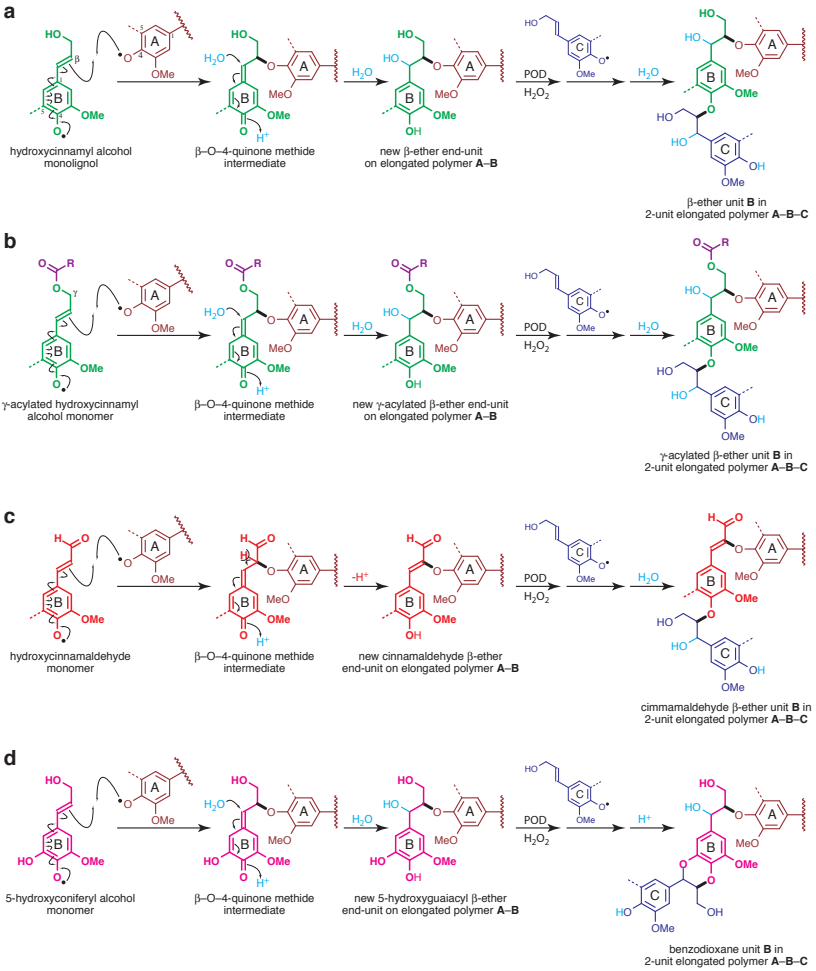

Fig. 1 Differences in cross-coupling and post-coupling reactions for various well-suited "monomers" incorporated into lignification. Illustration is for the major β–O–4-coupling only. a) Normal hydroxycinnamyl alcohol radicals **B** cross-couple with the phenolic end of the growing polymer **A**, mainly by β–O–4-coupling, to produce an intermediate quinone methide which rearomatizes by nucleophilic water addition to produce the elongated lignin chain **A**–**B**. The subsequent chain elongation via a further monolignol radical **C** etherifies the unit created by the prior monomer **B** addition, producing the 2-unit-elongated polymer unit **A**–**B**–**C**. b) Various γ-acylated monolignols (*p*-coumarate, *p*-hydroxybenzoate, and acetate) cross-couple equally well producing analogous products but with the β-ether unit **B** γ-acylated in the lignin polymer unit **A**–**B**–**C**. c) Hydroxycinnamaldehydes **B** may also cross-couple with the phenolic end of the growing polymer **A**, again mainly by β–O–4-coupling, to produce an intermediate quinone methide again, but

one which rearomatizes by loss of the acidic β-proton, producing an unsaturated cinnamaldehyde-β–O–4-linked **B** end-unit. Incorporation further into the polymer by etherification is analogous to a). The unsaturated aldehyde units **B** give rise to unique thioacidolysis markers. d) 5-Hydroxyconiferyl alcohol monomer **A** also cross-couples with the phenolic end of the growing polymer **A**, mainly by β–O–4-coupling, to produce an intermediate quinone methide as usual which rearomatizes normally by nucleophilic water addition to produce the elongated lignin chain **A**–**B** bearing a novel 5-hydroxyguaiacyl phenolic end-unit. The subsequent chain elongation via a further monolignol radical **C** coupling β–O–4 to the new phenolic end of **A**–**B**, but this time the rearomatization of the quinone methide (not shown) is via internal attack of the 5-OH producing novel benzodioxane units **B**–**C** in the 2-unit-elongated polymer unit **A**–**B**–**C**. 5-Hydroxyconiferyl alcohol incorporation produces a lignin with a structure that deviates significantly from the "normal" lignin. The bolded bonds are the ones formed in the radical coupling steps.

hardwoods. Sinapyl *p*-hydroxybenzoate is similarly a monomer in palm, aspen, poplar and willow lignification. And sinapyl *p*-coumarate (as well as lower levels of coniferyl *p*-coumarate) analogously contribute to lignification in grasses. What benefit the plant receives from lignin acylation is little understood. *p*-Hydroxybenzoate and *p*-coumarate are excellent substrates for many of the peroxidases that only slowly oxidize sinapyl alcohol. And, since they form less stable radicals, they readily undergo radical transfer with sinapyl alcohol, generating the sinapyl alcohol radical required for its incorporation into the polymer (Takahama et al. 1996). Their roles as radical transfer agents make them a sort of catalyst for lignification, especially with respect to sinapyl alcohol monomers. These phenolic esters then may have a role in facilitating the oxidation of sinapyl alcohol and oligomer substrates. Despite being phenolic, they themselves do not undergo coupling reactions and are found adorning lignins as pendant free-phenolic entities. In model reactions, it is not until other phenolics in the system are depleted that these components couple (Ralph et al. 2004). Their presence in free-phenolic form in lignins provides evidence that the cell limits radical concentrations. The role of the over 50% levels of acetylated sinapyl alcohol in kenaf is even less understood. Presumably the resultant polymer is more hydrophobic than normal lignins, so the acetylation may be associated with drought tolerance.

Whatever the reasons for these acylated components, the three types of acylated monolignols (acetate, *p*-hydroxybenzoate, and *p*-coumarate) are now well implicated as authentic lignin precursors in their respective plants. It is just becoming apparent by *in vitro* studies that the acylation

does not significantly affect the course of the coupling reactions (Fig. 1b). That means that the acylated monolignols also behave nicely as lignin monomers, most importantly undergoing the β–O–4-coupling reactions that incorporate them into the chain of the polymer. Acylated monolignols alter the structure of the lignins by more than just adorning them with pendant groups, however. This is because the γ-OH group on a monolignol, Fig. 1a, functions in some post-coupling reactions, internally trapping the quinone methide following β–β-coupling, for example (not shown). With the γ-OH group acylated, Fig. 1b, such internal reactions are no longer possible and the quinone methide must be rearomatized by trapping an external nucleophile, usually water, and forming quite different products in the lignin as a result (Lu and Ralph 2002). Additionally, the stereochemistry of water attack on the quinone methide intermediate following β–O–4-coupling is altered. The lignins therefore differ from "normal" lignins in both substantial and subtle ways.

Hydroxycinnamaldehydes and dihydroconiferyl alcohol in CAD-deficient plants

A pertinent example of poor monomer substitution is in a viable but unhealthy CAD-deficient mutant pine (MacKay et al. 1997, Ralph et al. 1997). CAD is the last enzyme on the monolignol biosynthetic pathway, reducing coniferaldehyde to the monolignol coniferyl alcohol in softwoods. Coniferaldehyde might be anticipated to build up in this plant, and evidence is that it does. On paper, coniferaldehyde appears to be a good candidate for a lignin monomer, Fig. 1c. It doesn't have the alcohol group, but it has the requisite conjugated double bond of the cinnamyl unit, and it was early on shown that it undergoes the same range of monomer-monomer coupling reactions as coniferyl alcohol, forming its own versions of β–O–4-, β–5-, and β–β-dehydrodimers (Connors et al. 1970). It could even make a dehydrogenation polymer. The drawback, as was not revealed until later studies on angiosperms (Ralph et al. 2001, Kim et al. 2003), is that coniferaldehyde simply will not chemically β–O–4-cross-couple with phenolic guaiacyl units in the polymer. It is purely a chemistry problem; it will undergo such coupling with suitably unsaturated units, but with a normal guaiacyl unit, the reactants are not sufficiently chemically compatible to react either *in vitro* or, apparently, *in vivo*. Since the CAD-deficient pine still makes some coniferyl alcohol, the polymer needs to incorporate monomers compatible with

a normal guaiacyl lignin. Coniferaldehyde simply does not comply. It therefore becomes relegated to homo-coupling reactions and cross-coupling reactions solely with the monolignol coniferyl alcohol, and is therefore left adorning only the periphery of the lignin molecule (as end-groups) and not significantly incorporating into the polymer chain. Interestingly, this pine also produces, for unknown reasons, high levels of dihydroconiferyl alcohol (Ralph et al. 1997, Lapierre et al. 2000). It also finds itself in the lignin. Obviously dihydroconiferyl alcohol cannot couple at its β-position — there is no way to get the required single electron density to the β-position without the presence of the double bond. It therefore is also limited to a set of reactions that do not incorporate it into the lignin chain, again relegating it to the periphery of the structure. Nevertheless, it is found in the lignin at striking levels due to its ability to still 5- and 4–O-couple with guaiacyl units, as well as with the monolignol, coniferyl alcohol. This pine then appears to have attempted to augment its polymer by substituting available coniferaldehyde and dihydroconiferyl alcohol monomers for some of the deficient coniferyl alcohol. It remains viable, but not vigorous.

The above example would be unfulfilling if it were not for the fact that coniferaldehyde is a well-behaved monomer in angiosperms. How can this be? Again, it is simple chemistry. Angiosperms have guaiacyl/syringyl lignins (deriving from both coniferyl and sinapyl alcohols). Coniferaldehyde, which does not β–O–4-cross-couple with guaiacyl units, readily undergoes β–O–4-cross-coupling reactions with syringyl end-groups. As it turns out, sinapaldehyde readily undergoes cross-coupling with either guaiacyl or syringyl end-groups. As a result, both coniferaldehyde and sinapaldehyde couple in a similar way as the monolignols do, and can therefore incorporate integrally into the body of the polymer in CAD-deficient angiosperms. These details are revealed by NMR studies on the lignins (Kim et al. 2003), and by the release of thioacidolysis marker compounds specifically from these hydroxycinnamaldehyde-β–O–4-linked units (see Fig. 1c) (Kim et al. 2002). The plants appear to grow essentially normally, but the hydroxycinnamaldehydes may not be quite perfect monomers. It appears that coupling to the new type of conjugated β–O–4-phenolic end-units that these create, the next step in the polymerization, becomes difficult. The result is that many of the incorporated coniferaldehyde/sinapaldehyde

units remain as free-phenolic end-groups (Lapierre, unpublished), limiting the degree of polymerization of the lignin. In addition to the property changes caused by the structural changes, these lower molecular weight lignins are presumably less ideal for the plant. An interesting side-benefit however is that the lignins are much more easily broken down and removed in chemical pulping (Pilate et al. 2002), so plants with limited CAD-deficiency are being pursued for their enhanced pulping potential.

5-Hydroxyconiferyl alcohol in COMT-deficient plants

Beyond the (partial) substitution of the hydroxycinnamaldehydes for their hydroxycinnamyl alcohol monolignol analogs in CAD-deficient angiosperms is a particularly successful substitution in the case of COMT-deficiency (Ralph et al. 2001). COMT is a methyl transferase enzyme necessary for the biosynthesis of sinapyl alcohol and ultimately syringyl groups in lignins. Knock-out mutants are essentially or totally devoid of syringyl components, and COMT down-regulation will reduce the syringyl content. As CAD-deficient angiosperms incorporated the immediate CAD precursor (the hydroxycinnamaldehydes), COMT-deficient plants must deal with the un-methylated 5-hydroxy-coniferaldehyde precursor. Apparently CAD is able to reduce this aldehyde as it is 5-hydroxyconiferyl alcohol, not the aldehyde, that is exported to the wall and incorporated into lignins. 5-Hydroxyconiferyl alcohol has all the makings of an ideal monolignol substitute (Fig. 1d). It beautifully β–O–4-couples with guaiacyl, syringyl, or new 5-hydroxyguaiacyl phenolic endgroups, integrating into the polymer as would a primary monolignol. The lignin structure becomes strikingly different however. The presence of the extra phenolic-OH, the 5-OH, drastically affects the post-coupling reactions. Novel benzodioxane units are formed in the polymer as a result of incorporating 5-hydroxy-coniferyl alcohol, at striking levels (essentially replacing the syringyl units in the control plants). The plant does not seem to mind; COMT-deficient plants appear to grow normally. In this case, the severe structural changes are a serious detriment to chemical pulping. Despite still being β-ethers, these units will not efficiently cleave under pulping conditions, as do the syringyl units which they displace. However, COMT-deficient plants appear to be more digestible (Guo et al. 2001). A possible reason is that, by providing a rapid alternative internal pathway for rearomatizing the quinone methide intermediate,

these units cannot cross-link with polysaccharides in the wall (via addition to quinone methides). Lignin-polysaccharide cross-linking has been shown to have a detrimental effect on cell wall digestibility.

Conclusions

A small range of monomers are now known to substitute for the conventional monolignols in various natural and transgenic plants. Monolignol substitution appears to be most successful when the novel monomer behaves, in its chemical radical coupling and cross-coupling reactions, like a normal monolignol. Most important is the β–O–4-coupling reaction with the phenolic end of the growing polymer to extend the polymer chain, as shown in Fig. 1. The post-coupling reactions that may be altered by the different functionality on the monomer seem to have less effect. Thus massive changes in the lignin structure occur when 5-hydroxyconiferyl alcohol substitutes for sinapyl alcohol, for example — the coupling reactions are analogous, but post-coupling steps produce novel benzodioxane structures that drastically change the lignin. Observations that plants with monolignol substitution and profoundly altered lignin structure can fare well supports the heretical tongue-in-cheek idea expressed at a conference some time back that the exact structure of lignins is not that important to the functioning of the plant (Ralph 1997). The plant requires certain properties and functionality of its lignins, but does not expend resources dictating those properties by exactly stipulating lignin primary structure. Such biosynthetic malleability functions well for the plant, and also provides significant opportunities for engineering the polymer. Already it has been demonstrated that natural and industrial processes ranging from ruminant digestibility to chemical pulping can be both positively and negatively impacted by alterations to lignin composition and structure. It is also apparent that phenolic components from beyond the monolignol pathway itself (such as the acylated monolignols) may be incorporated into lignins if they have compatible reaction chemistry and are transportable to the wall. Future work should reveal opportunities beyond the interesting deviations achieved by up- and down-regulating genes on the monolignol pathway to date.

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