# Lignin Formation in Plants. The Dilemma of Linkage Specificity

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Lignification is the process of forming the collective of phenylpropanoid macromolecules termed lignin. There are two ways to define lignin: 1) from a chemical point of view (i.e. its chemical composition and structure), and 2) from a functional view that stresses what lignin does within the plant. It has been recognized for 50 years now that lignin is a polymeric material composed of phenylpropanoid units derived from three cinnamyl alcohols (monolignols): *p*-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1). It was suspected that this view might be too simplistic (Sarkanen and Ludwig, 1971), and there are now many examples showing that other phenolics can be incorporated into lignins (for review, see Sederoff et al., 1999). From a functional point of view, lignins impart strength to cell walls, facilitate water transport, and impede the degradation of wall polysaccharides, thus acting as a major line of defense against pathogens, insects, and other herbivores.

The lignification process encompasses the biosynthesis of monolignols, their transport to the cell wall, and polymerization into the final molecule. This discussion will focus on the final phase—the formation of the lignin macromolecule. Bond formation is thought to result from oxidative (radical-mediated) coupling between a monolignol and the growing oligomer/polymer. The oxidative coupling between monolignols can result in the formation of several different interunit linkages (Fig. 2). In native lignins, 8-*O*-4-linkages are the most abundant, whereas for lignins formed in vitro by mixing coniferyl alcohol, hydrogen peroxide, and peroxidase, higher percentages of 8–8- and 8–5-linkages are found (Nimz and Ludemann, 1976; Terashima et al., 1996; Chen, 1998). How is this apparent specificity in chemical bonds between lignin subunits controlled? Currently, there are two models for coupling radicals to produce a functional lignin molecule. One, the random coupling model, which emerged during early studies on the structure of lignin, centers on the hypothesis that lignin formation proceeds through coupling of individual monolignols to the growing lignin polymer in a near-random fashion (Harkin, 1967; Freudenberg and Neish, 1968; Adler, 1977). In this view, the amount and type of individual phenolics available at the lignification site and normal chemical coupling properties (Syrjanen and Brunow, 1998) regulate lignin formation.

The second model, the dirigent protein model, is more recent and suggests that lignification must be under strict regulation of specialized proteins that control the formation of individual bonds (Lewis and Davin, 1998; Davin and Lewis, 2000). This new model for lignin formation stems from the definition of dirigent proteins (Davin et al., 1997). Dirigent proteins direct the coupling of two monolignol radicals, producing a dimer with a single regio- and stereoconfiguration. These dimers are known as lignans and are commonly found in many plants. The rationale for this new model is the belief that nature would not leave the formation of such an important molecule as lignin "to chance" (Davin and Lewis, 2000). It is argued that the only way to explain the high proportion of 8-*O*-4 linkages in lignin would be through regulation by specific dirigent proteins (Davin and Lewis, 2000).

We will evaluate both models to determine how well each fits with the current state of knowledge based on experimental evidence.

## THE RANDOM COUPLING MODEL

Given the apparent discrepancy between the bond distributions in native versus in vitro lignins, it would be tempting to assume directed coupling of some type. However the typical in vitro method, despite attempts to add monolignols slowly, more accurately represents a bulk polymerization, in which the predominant reaction is dimerization of monomers. This does not accurately mimic in vivo formation of lignin, a process that is dependent upon the release of monolignols into the wall matrix followed by diffusion to the site of incorporation. The wall matrix may influence the formation of lignin. For example, synthetic lignins (dehydrogenation polymers [DHPs]) synthesized in the presence of polysaccharides have different bond distributions than simple in vitro DHPs (Terashima et al., 1995). Also, if we examine the DHP produced using isolated corn cell walls with active peroxidases, the linkage pattern is nearly the same as that observed in native

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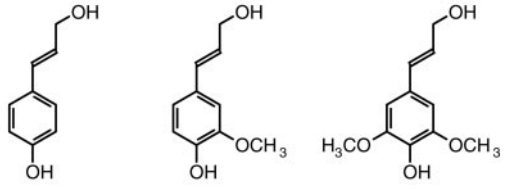


**Figure 1.** Major monolignols found in "natural" plant lignins.

lignins isolated from the stems of corn (Grabber, et al., 1996). Although one could argue that these walls were isolated from living cells containing arrays of dirigent proteins, a more compelling argument comes from a completely in vitro method employing a slow diffusion step to move the monolignols and hydrogen peroxide to a contained peroxidase. This process produced vastly different linkages than when the monolignols were simply added slowly to enzyme and hydrogen peroxide (Syrjanen and Brunow, 2000). Controlling the diffusion step actually controlled the cross coupling bonding pattern, highly favoring β-ethers (Syrjanen and Brunow, 2000). This mode of polymerization, envisioned to occur in the cell wall, was described over 20 years ago by Adler (1977).

Controlling the rate of diffusion, a potential role of the cell wall matrix, forces the formation of lignin into a stepwise addition of monolignols to the growing polymer. Lignin arises primarily from the addition of monolignols to the continually growing polymer (Adler, 1977) and not from the coupling of monolignols to produce dimers, as postulated by the dirigent protein model. Dimerization of coniferyl alcohol produces one of two easily quantifiable outcomes: either it produces an 8–8-dimer (pinoresinol) or a dimer that retains its unsaturated side chain (8–5- or 8-*O*-4-dimer; Fig. 2). In softwoods, such as pine, 8–8 units comprise only 1% to 2% of lignin (Adler, 1977); unsaturated side chains account for 2% to 4% (Adler, 1977). Therefore, approximately 95% of lignin units are not derived from dimerization reactions! Dirigent proteins that have been identified can only regulate the coupling of monolignols to produce dimers.

From the analysis of naturally occurring mutants and plants in which the expression of lignin biosynthetic genes has been down-regulated through the use of (anti)sense strategies, the biosynthesis of monolignols is becoming better understood. This approach has helped to determine the interrelationships between structure and function of lignin in plant cell walls. Plants are readily able to circumvent deficiencies in monolignol biosynthetic processes. For example, caffeic acid *O*-methyl transferase-deficient angiosperms readily incorporate 5-hydroxyconiferyl alcohol into their lignin polymers (Lapierre et al., 1988; Jouanin et al., 2000). Moreover, down-regulation of the cinnamyl alcohol dehydrogenase enzyme results in a significant shift in lignin composition; coniferyl and sinapyl aldehydes are incorporated into lignin in the place of some of the alcohols (Ralph et al., 1997; Kim et al., 2000). The latter observation is consistent with the finding that radicals of the aldehydes react in a similar manner as the alcohols (Russell et al., 1996). The incorporation of these unusual monomers has been observed in a variety of plant species using several different analytical techniques. The random model has no problem with these events; a supplied phenylpropanoid phenolic can form a radical that can be incorporated into a functional lignin molecule, depending on its chemical cross-coupling properties.

Thus, the cell wall matrix may simply control the rate of diffusion, thereby forcing the formation of lignin into a stepwise addition of monolignols to the growing polymer. The type and quantity of monolignols at the lignification site control lignin formation. The random coupling model (Fig. 3) has monolignols diffusing through the wall matrix to a peroxidase (or oxidase) along with hydrogen peroxide to form a

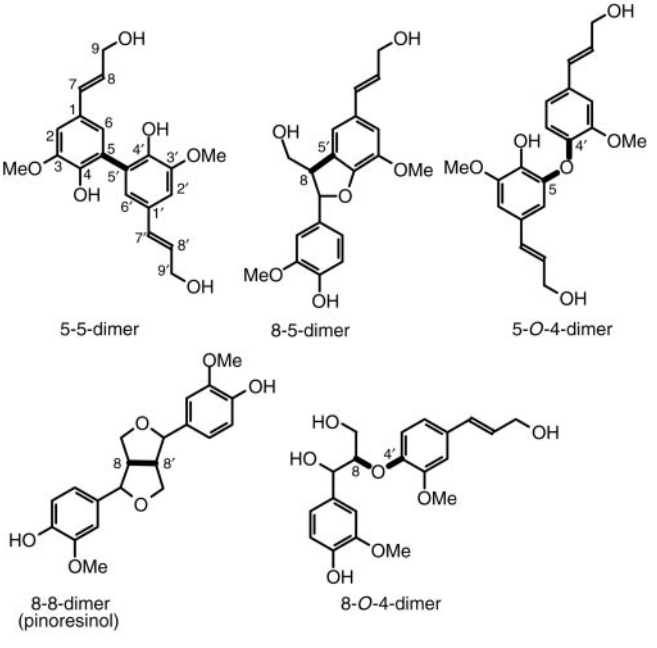


**Figure 2.** These molecules represent the potential dimers that would have to be produced by dirigent proteins in order to account for bonds typically found in different types of lignin. Monolignol carbon numbers are shown for the first dimer, 5–5, to illustrate the appropriate numbering scheme. For all the other models, only the carbons involved in the interunit bond are numbered and the bond is highlighted for easy reference. The models are based solely on coniferyl alcohol, a situation found only in gymnosperm lignins. Angiosperm lignin will contain both coniferyl and sinapyl alcohols. Both gymnosperm and angiosperm lignins will contain small amounts of *p*-coumaryl alcohol. Therefore, dirigent proteins must not only accommodate these additional monolignols, but there must also be proteins that can form specific cross products between sinapyl and coniferyl alcohols. More significantly, these dimers do not fully represent lignins, which are formed from monolignols coupling with the growing lignin oligomer/polymer.

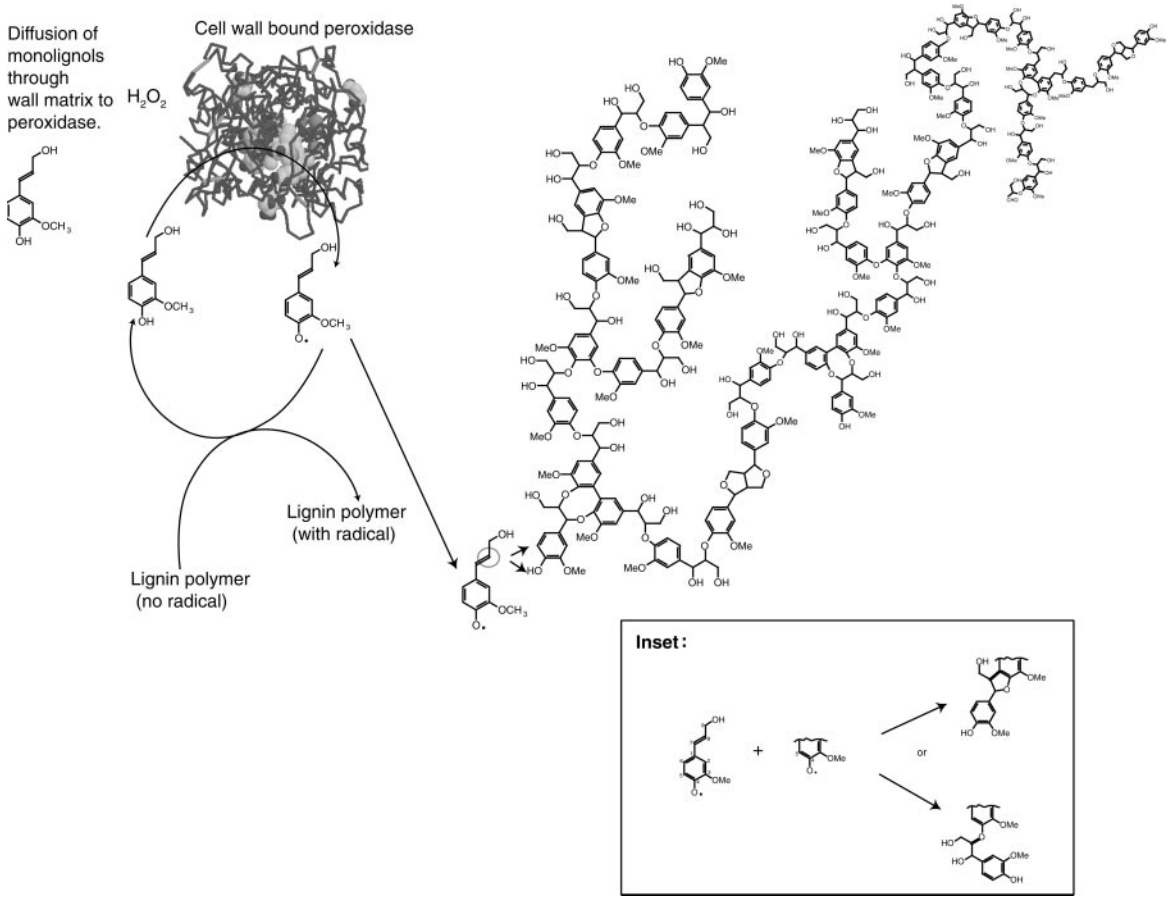


**Figure 3.** A representation of the random model for lignin formation. Formation of monolignol radicals occurs by interaction with wall-bound peroxidases and hydrogen peroxide. Newly formed radicals diffuse to the lignin polymer and can undergo one of two possible fates: (a) if the lignin polymer is at its base oxidative state, the higher oxidation state on the monolignol can be transferred to the lignin molecule, returning the monolignol to ground state; or (b) if the lignin polymer is at a higher oxidation state, the monolignol radical can undergo an oxidative coupling reaction to from a covalent bond. Transfer of the monolignol radical to lignin polymer results in a ground state monolignol that diffuses back to the peroxidase to form a monolignol radical again. The lignin molecular structure presented here is based on the work of Brunow (1998). Inset, Monolignol radical coupling possibilities with the radical that is formed on the lignin polymer. The preference is always for the monolignol to couple at its 8-position.

monolignol radical. The newly formed radical will diffuse to the lignin polymer where it will couple with a radical on the polymer. If there is no radical on the lignin polymer, the newly formed monolignol radical will transfer its higher oxidation state to the polymer and freely diffuse back to the peroxidase to undergo oxidation a second time and complete the formation of a new lignin bond. Therefore, the type and quantity of monolignols at the lignification site control lignin formation.

## THE DIRIGENT PROTEIN MODEL

Let us consider the dirigent protein model to see how the data fit the hypothesis of a protein-directed bonding pattern in lignin. According to this model, bond formation to form a lignin polymer is under strict control of dirigent proteins. There is no doubt that the recently discovered dirigent proteins, with no enzymatic activity of their own, direct specific bond formation in *Forsythia suspensa*, resulting in optically pure dimers (Davin et al., 1997). To date numerous genes encoding these proteins have been putatively identified in a range of plants (Davin and Lewis, 2000). Only one such protein has been characterized sufficiently to reveal that its activity results in the preferential production of an 8–8-linked coniferyl alcohol dimer (pinoresinol). However, we are unaware of a dirigent protein directing the formation of the most common lignin bond, 8-*O*-4-linkages between monolignols, despite the occurrence of lignans containing the 8-*O*-4-linkage (Wallis, 1998); nor are we aware of any evidence for dirigent proteins that result in the formation of lignin. Furthermore, although a polyclonal antibody raised against the dirigent protein reacted with epitopes in vascular tissue of *F. suspensa* (Davin and Lewis, 2000) is an interesting observation, it is not proof for an actual involve-

ment of dirigent proteins in the formation of lignin. Alternatively, they could have a role as initiation sites for lignin formation (Davin and Lewis, 2000), helping direct the initial monolignols to specific sites within the wall matrix, or they may have no relationship to lignification at all. The fact that other species contain DNA sequences homologous to the gene encoding the *F. suspensa* dirigent protein does not prove the existence of functional lignin forming dirigent proteins in these other species.

If lignification involves truly regulated coupling of radicals, every bond formed between two monolignols or between a monolignol and a growing lignin polymer must be represented by a specific dirigent protein (Fig. 4). This would require some 50 proteins to account for all the bonds observed in lignin involving the monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols). Furthermore, it has been proposed that once an initial polymer of lignin is formed, it acts as a template for the subsequent formation of additional lignin molecules with a specific pattern of bonds (Guan et al., 1997; Sarkanen, 1998). This implies that individual molecules of lignin would be identical because the types of monolignols and the bonds among lignin molecules are the same. It is important to remember that although the lignin models that can be drawn on paper seem planar (see Figs. 3 and 4), the molecules are three dimensional, making it difficult to see how monolignol radicals are going to diffuse to precise spots on the first molecule (template) and bond in a predetermined fashion. Lignin formation occurs within the wall matrix, filling in spaces between wall polysaccharides (i.e. xylans and cellulose microfibrils), making it difficult to have a precise surface upon which to build the next lignin polymer. The hypothesis that an initial polymer of

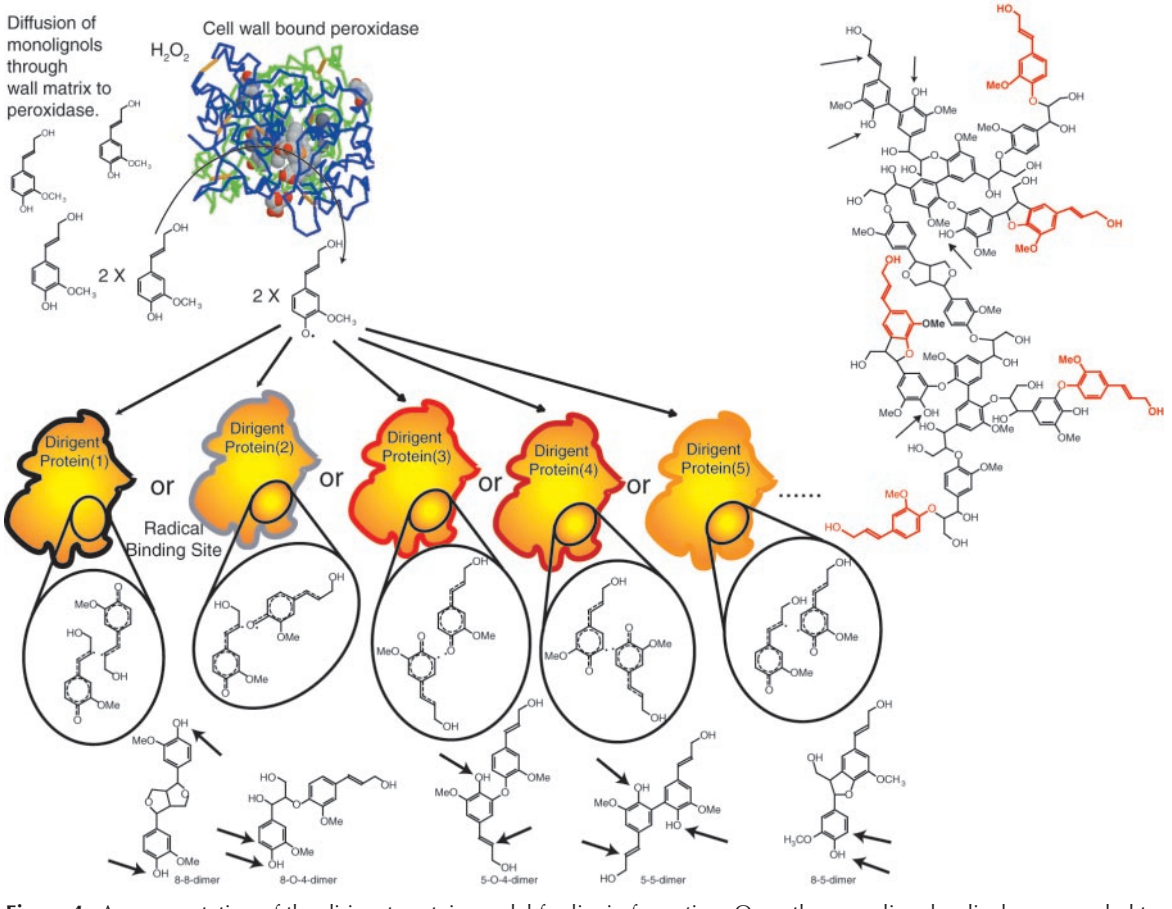


**Figure 4.** A representation of the dirigent protein model for lignin formation. Once the monolignol radicals are coupled to form regio- and stereospecific dimers, the individual dimers would have to be coupled together to form the lignin polymer. Although it is theoretically possible to couple dimers together, it is difficult to produce the bond frequencies that are known to exist in lignin. The above segment of "lignin" was formed by coupling preformed dimers together. The bold red residues of the lignin molecule represent unsaturated cinnamyl alcohol terminal end groups that could not undergo any further oxidative coupling reactions. The frequency of these groups formed from dimer coupling is approximately 33% of the total residues. The frequency of end groups in normal G-type lignins (coniferyl alcohol) is on the order of 1% to 2%. Producing angiosperm lignins (containing both sinapyl and coniferyl alcohols) requires additional dirigent proteins to accommodate all sinapyl and coniferyl alcohol dimers. Arrows on the figure indicate the potential sites for cross coupling with new monolignols to form the lignin molecule.

lignin acts as a template for the exact replication of additional lignin molecules can be tested using a near homopolymer, such as the high *erythro*-8-*O*-4 polymer (Landucci, 2000). If the template hypothesis is correct, adding a homopolymer to an in vitro system should result in a DHP composed only of a single monolignol and with a single type of bond, or at the very least a regular polymer with short repeat units. Adding a mixture of monolignols to the system should not matter because the template is expected to select only the monolignol used to produce the original homopolymer.

Let us now consider the potential formation of 8-*O*-4-linkages directed by a dirigent protein. With the correct dirigent protein, two monolignol radicals would be held in the proper position to produce the linkage which, as with the pinoresinol structure, would be optically pure (Fig. 2). The resulting dimer can only add to another monolignol via the phenolic moiety. The second moiety in the dimer (in which the phenol is now etherified) will be blocked from any further reactions, resulting in its unsaturated side chain remaining in the polymer. If this scenario produces the 8-*O*-4-linkages in lignin in vivo, there should be a high proportion of terminal alcohol residues in lignin (Fig. 4), a feature that is not observed (Adler, 1977). The monolignol dimers that are formed from dirigent proteins cannot readily cross couple to form a growing lignin polymer. If dirigent proteins are involved in the formation of lignin bonds, there must be not only proteins that bind monolignols (to form dimers), but also proteins to bind dimers and lignin polymers to control adequately the growing lignin polymer. Dirigent proteins identified to date result simply in the formation of dimers or lignans and are quite specific. The dirigent protein for the formation of pinoresinol does not accept sinapyl or *p*-coumaryl alcohols as substrates (Davin et al., 1997). In the light of the results obtained from the analysis of mutant and transgenic plants that incorporate nontraditional phenolics into lignin, additional dirigent proteins that can accommodate these unusual monomers would have to be present within the wall matrix.

Another consideration is that lignin production via dirigent proteins is a highly stereo- and regiocontrolled synthesis producing optically active units. Various fragments carefully excised from lignins show no detectable optical activity—they are racemic (Freudenberg et al., 1965; Ralph et al., 1999; Akiyama et al., 2000). To explain this observation, one must envision a second array of dirigent proteins with the opposite stereo- and regiospecificity to generate a racemic mixture of coupling products in the lignin molecule, a notion proposed by the dirigent protein advocates (Lewis and Davin, 1998; Davin and Lewis, 2000). Thus, there would be a need for twice as many dirigent proteins to account for all the bonds and to produce opposite optical activities. An alternative is that with the template hypothesis, the next polymer formed would result in exactly the opposite optical activity, producing a racemic mixture (Lewis and Davin, 1998).

Finally, it is interesting to note that there appears to be no protein-mediated control, only chemical control, over the nucleophilic addition of water to quinone methide intermediates following 8-*O*-4 radical coupling. Native lignin (in vivo) and DHPs (in vitro) form a 50:50 *erythro*:*threo* isomer mixture for β-guaiacyl units, and an approximately 80:20 *erythro*:*threo* mixture for β-syringyl units (Brunow et al., 1993). These isomers are formed from the addition of water to re-aromatize the quinone methide intermediate-coupling product. It raises the question of why the plant would so carefully control the lignin structure through dirigent proteins yet leave this stereochemistry under simple chemical control? The type of isomer formed affects the shape and properties of the lignin molecule.

The immunolocalization and in situ hybridization data (Davin and Lewis, 2000) and the fact that several other species contain sequences that are homologous to the *F. suspensa* dirigent protein gene do warrant further investigation into the role of dirigent proteins, which may reach beyond the mere formation of lignans. Additional issues need to be addressed, however, before the dirigent protein hypothesis can be extended to control of bond formation in lignin. Other dirigent proteins (producing specific bond patterns) must be identified and shown to function during lignification. Assembling the dimers formed by dirigent proteins into a lignin polymer requires additional dirigent proteins that bind the lignin polymer and control the specific addition of the dimer or at least a monolignol. Such proteins should also be found in the lignifying wall matrix. Their involvement in lignin biosynthesis can be demonstrated via genetic approaches, now that it is possible to isolate genes encoding dirigent proteins. The application of transgenic approaches can be applied to down-regulate their expression. Changing the expression of one or more of these genes should drastically alter lignin composition and structure or limit the amount of lignin formed.

## CONCLUSIONS

The issue of bond specificity in lignins is complex. Recent in vitro experiments are able to reproduce the bond distribution of native lignin adequately, whereas the results from the analysis of mutants and transgenic plants indicate metabolic plasticity in lignin biosynthesis. This plasticity and the ability to form lignin through random coupling may actually be an advantage in the defense against pathogens. The lack of regularity poses a problem to the evolution of hydrolytic enzymes in fungi or insects (Denton, 1998), thereby protecting the plant from invasion. The ran-

dom model for lignification reflects the actual process, i.e. the polymerization of lignin resulting from coupling between monolignol radicals and a radical form of the growing lignin molecule (Fig. 3). To date there are no observations that demand absolute structural control over lignin formation, i.e. the types of monolignols supplied and the rate at which they reach the individual sites of lignin formation readily explain structural features of lignin. Metabolic control over the process is exerted at the level of monolignol synthesis and transport to the wall matrix. Therefore, the random model for lignification is not invalidated, despite such claims (Lewis and Davin, 1998; Davin and Lewis, 2000). What does need to be addressed in more detail is how different tissues within the plant are able to achieve variation in lignin composition. For example, is this the result of differential expression of lignin biosynthetic genes, control over monolignol transport through the cytoplasm, or control over the chemical environment in the cell wall? At this time the dirigent protein model is an interesting hypothesis that requires key experimental evidence to substantiate its involvement in the actual formation of lignin polymers. Claims that this model is the obvious correct one and replaces the random coupling model are therefore premature. The plant has apparently evolved a particularly elegant process for producing key lignin polymers, one without direct structural control beyond careful regulation of monolignol supply to lignification sites.

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