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# Lignin polymerization: how do plants manage the chemistry so well?

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The final step of lignin biosynthesis is the polymerization of monolignols in apoplastic cell wall domains. In this process, monolignols secreted by lignifying cells, or occasionally neighboring non-lignifying and/or other lignifying cells, are activated by cell-wall-localized oxidation systems, such as laccase/O<sub>2</sub> and/or peroxidase/H<sub>2</sub>O<sub>2</sub>, for combinatorial radical coupling to make the final lignin polymers. Plants can precisely control when, where, and which types of lignin polymers are assembled at tissue and cellular levels, but do not control the polymers' exact chemical structures *per se*. Recent studies have begun to identify specific laccase and peroxidase proteins responsible for lignin polymerization in specific cell types and during different developmental stages. The coordination of polymerization machinery localization and monolignol supply is likely critical for the spatio-temporal patterning of lignin polymerization. Further advancement in this research area will continue to increase our capacity to manipulate lignin content/structure in biomass to meet our own biotechnological purposes.

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## Introduction

Lignin is an aromatic biopolymer derived from oxidative coupling of *p*-hydroxycinnamyl alcohols (monolignols) and related compounds. Lignification, that is, lignin deposition in apoplastic cell wall domains, is a pivotal biological function that early terrestrial plants acquired for land colonization. In particular, lignin is vital for the integrity of the thick secondary cell walls produced in xylem vascular tissues, such as tracheids, vessels, and fibers, where lignin is embedded with polysaccharides and confers essential mechanical properties to the cells

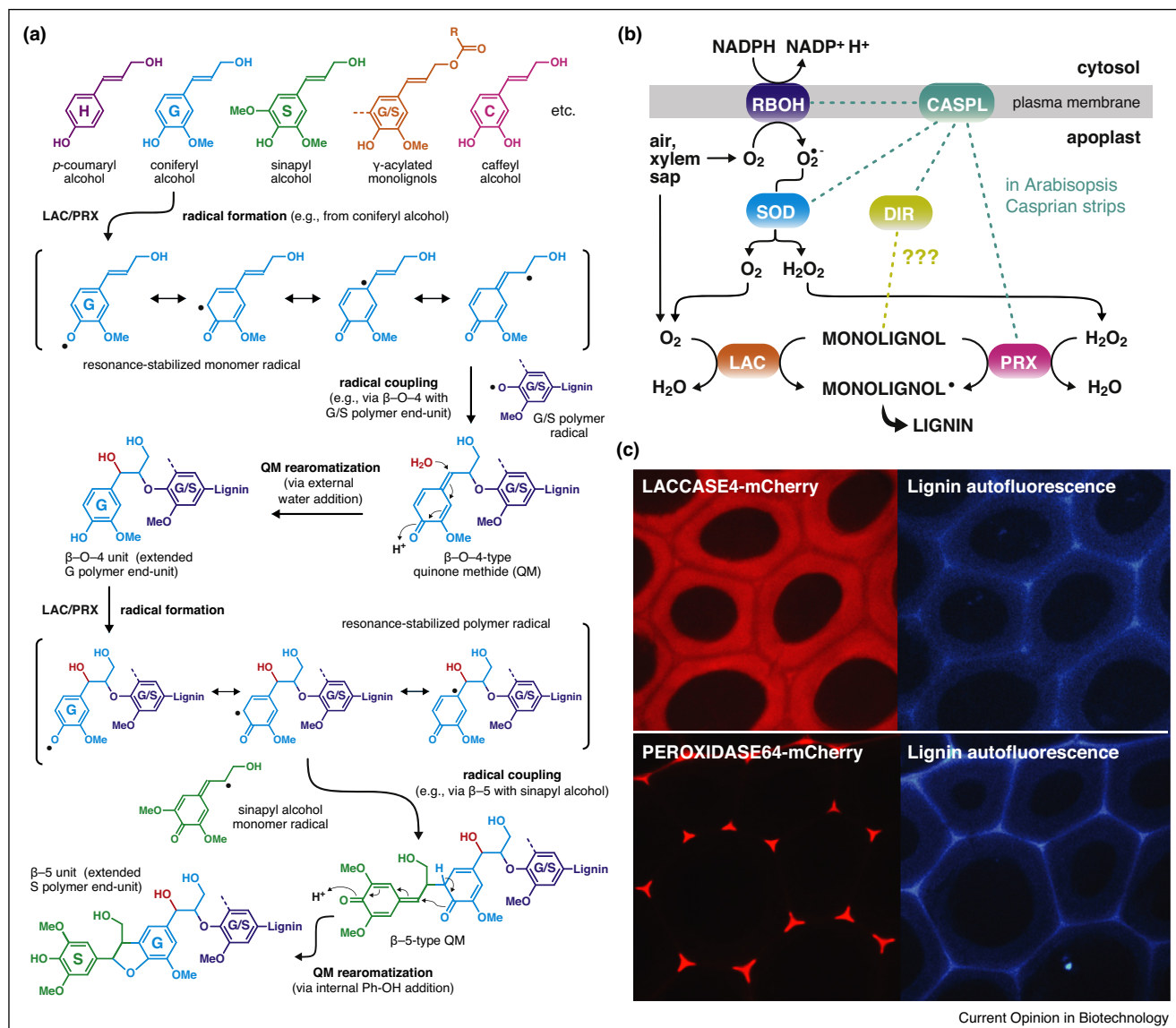
required for water transport and structural support. Furthermore, plants execute lignification in other specialized cell types, such as seedcoats, the endothecium (lining of the anther lumen), endodermal cells, and secession cells for organ removal, and also in response to pathogen attack. Typically, 20–30% of plant dry matter, that is, lignocellulosic biomass, is made up of lignins. Hence, studies on lignin biosynthesis and bioengineering are motivated not only by its fundamental importance to plant development and physiology, but also by its agro-industrial importance in the context of plant biomass utilization [1].

Here we focus on the final step of lignin biosynthesis, in which monolignols secreted from cells are activated by cell-wall-localized oxidation systems, such as laccase/O<sub>2</sub> and peroxidase/H<sub>2</sub>O<sub>2</sub>. Lignin polymerization occurs via a combinatorial radical coupling process that is highly flexible in nature and allows plants to incorporate numerous lignin monomers—beyond the three classical monolignols, that is, coniferyl, sinapyl, and *p*-coumaryl alcohols—in different combinations to assemble different lignin polymers in different cell types. However, despite extensive research efforts, as recently reviewed in [2–4], many aspects of the mechanisms underlying the tight control of lignin polymerizations *in planta* remain unclear. In this review, we provide a brief overview of the latest advances in our understanding of the biochemistry and cell biology aspects of lignin polymerization.

## Lignin polymerization proceeds via simple combinatorial chemistry

Chemically, the key process of lignin polymerization involves so-called 'end-wise' polymerization in which oxidized monolignol radicals undergo cross-coupling reactions with radicals formed on the free-phenolic ends of growing lignin polymers. Permutations in the regio-chemistry of the radical coupling step and variations in the mode of the post-coupling quinone methide rearomatization step allow numerous possible inter-monomeric linkages in the final lignin polymer (Figure 1a) [1]. *In vitro* lignin polymerization experiments that produce synthetic lignin polymers or dehydrogenation polymers (DHPs) via enzymatic or chemical oxidation of monolignols have demonstrated that the propensity for lignin linkage formation primarily depends on the chemical structure of monolignols [5–9]. Overall, the composition of different monolignols available in the polymerization site is likely the most important factor controlling the

Figure 1



**(a)** Monolignols and their polymerization. Lignin polymerization proceeds mainly via the 'end-wise' polymerization process in which an oxidized monolignol radical cross-couples with a radical formed on the free-phenolic ends of a growing lignin polymer. As an example, sequential polymer chain extension reactions via  $\beta$ -O-4 and  $\beta$ -5 radical coupling reactions with coniferyl and sinapyl alcohols are illustrated. **(b)** Proteins comprising the lignin polymerization machinery. Laccase (LAC) and peroxidase (PRX) oxidative enzymes catalyze the phenoxy radical formations from monolignols. Apoplastic hydrogen peroxide, which is required in PRX-catalyzed oxidation, may be produced from apoplastic oxygen via two-step enzymatic reactions by NADPH oxidase, or Respiratory Burst Oxidase homolog (RBOH) protein, and superoxide dismutase (SOD). In the Casparian strip of *Arabidopsis* root endodermal cells, Casparian strip membrane domain proteins (CASPLs) are hypothesized to assemble the lignin polymerization proteins and guide where the Casparian strip forms. In addition, dirigent-domain-containing proteins (DIRs) are also implicated to function in directing the correct lignin depositions in the Casparian strips of *Arabidopsis*. **(c)** Localization of native-promoter-driven, mCherry-tagged LAC4 and PRX64 in cross sectional views of *Arabidopsis* interfascicular fibers. LAC4 and PRX64 display distinct localizations in the secondary cell wall and middle lamella cell wall domains, respectively, thus suggesting their functions are spatial separated during fiber lignification [56\*\*].

structural organization of the final lignin polymer. This notion has been corroborated by theoretical studies using advanced computational methods [10–13] and generally supported by structural comparisons with various natural and genetically altered lignins incorporating different

lignin monomer mixtures (citation for the COiB lignin review about 'Structure and Bioengineering?'). Additionally, numerous physio-chemical factors, such as monomer supply rate, pH, ionic strength, and concentration of polymerization enzymes and their capability to oxidize

different monomeric/polymeric substrates, influence the formation of DHPs [5,14,15,16\*,17]. Notably, DHP formation can be affected by the presence of various cell-wall-associated polysaccharides [18–21]. Given that lignin polymerization *in planta* typically occurs after the deposition of polysaccharides in developing cell walls, pre-existing polysaccharide matrices may act as scaffolds for lignin polymerization and provide specific local environments within which monolignols undergo combinatorial radical coupling [20–22]. In fact, some polysaccharide-modified transgenic and mutant Arabidopsis lines display altered lignin deposition [23–25], while the impact of polysaccharide modifications on lignin chemical structures *in planta* is an active area of research.

### Undeciphered role of dirigent proteins in lignification

The structural features of natural lignin polymers and their variability in mutant and transgenic plants may be addressed elsewhere in this issue of Current Opinion in Biotechnology (citation for the COiB lignin review about ‘Structure and bioengineering’?). All the lignin structural data accumulated to date are in accordance with the view that the *in planta* lignin polymerization step occurs via simple combinatorial chemistry solely under chemical and physical control, that is, none of the proteins implicated in lignification exert a direct influence on the chemistry of lignin polymerization beyond the formation of phenoxy radicals [26]. The alternative hypothesis that a class of dirigent-domain-containing proteins (DIRs) control the primary structure of lignin [27], similarly to how DIRs control the regio/stereo-chemistries of analogous radical coupling reactions in the biosynthesis of lignans [28,29], still lacks sufficient biochemical and genetic evidence at this time. Nevertheless, some recent studies have implicated an intriguing function for DIRs in directing the spatial patterning of lignin deposition in specific cells [29–31]. In particular, the putative function of DIR10/ESB1 in the correct formation of the lignin-based Casparian strip in Arabidopsis roots is intriguing (Figure 1b) [30] (+citation for the COiB lignin review about ‘Casparian strips’?). It is possible that, despite its inability to mediate the regio/stereo-selectivity of coniferyl alcohol coupling *in vitro* [32], DIR10/ESB1 may play a role in initiating lignin polymerization or localizing lignin pre-polymers in the Casparian strip, although whether DIR10/ESB1 can bind monolignols, lignin polymers, and/or their radicals is yet to be determined.

### Respective roles of laccases and peroxidases in lignification

The combinatorial radical coupling of monolignols in cell walls may be initiated by the action of oxidative enzymes such as O<sub>2</sub>-dependent laccases and H<sub>2</sub>O<sub>2</sub>-dependent peroxidases (Figure 1b). The catalytic promiscuity and large gene families of both enzyme types have long made it difficult to establish their functions *in planta* but recent

studies have started to uncover the roles of specific laccases and peroxidases in lignin polymerization.

The essential role of laccases in the lignification of major xylem tissues in Arabidopsis was demonstrated through the characterization of multiple knockout mutants [3,33–35]. The *lac4 lac17* double mutants deficient in LACCASE4 (LAC4) and LAC17 functions showed hypolignified fibers and collapsed xylem vessel phenotypes in their inflorescence stems [33,34]. Additional loss of function of LAC11, in the *lac4 lac17 lac11* triple mutant, led to severe growth defects and failure to detect lignin in either stems or roots by histochemical analysis [35]. Overall, these laccase mutant studies have suggested that laccase activities are essential for lignin formation in the major xylem tissues of Arabidopsis and further analysis of this gene family and multiple mutant alleles will expand our understanding of laccase function in lignification. The tissue and cell type-specific expression patterns of laccase genes are post-transcriptionally regulated by microRNAs [36–38,39\*]. For instance, overexpressing microRNA397 (miR397) in poplar [36] and Arabidopsis [37] and overexpressing miR528 in maize [39\*] resulted in substantial reductions in lignin content along with reductions in transcript abundances of a subset of laccase genes. Recent bioengineering studies have highlighted laccase and laccase-specific microRNA genes as useful targets in biomass engineering for desirable lignin content and/or composition [39\*,40–42].

Like laccases, peroxidases have been also suggested to have an active role in cell wall lignification in many plant species [3,43,44\*]. In Arabidopsis, the lignification in the Casparian strip of root endodermal cells is largely mediated by PEROXIDASE64 (PRX64) because it is precisely localized to the Casparian strip and its microRNA-mediated cell-specific downregulation in root endodermis led to loss of Casparian strip function which depends on lignin deposition [45] (+citation for the COiB lignin review about ‘Casparian strips’?). The same study also identified an NADPH oxidase or Respiratory Burst Oxidase homolog (RBOH) protein, RBOH-F, which produces the H<sub>2</sub>O<sub>2</sub> required for PRX64-catalyzed lignin polymerization (Figure 1b). Furthermore, Casparian strip membrane domain proteins (CASPLs) are hypothesized to assemble other lignin polymerization proteins and thus guide where the Casparian strip forms (Figure 1b) [45]. Lignin accumulation in the floral abscission zone of Arabidopsis likely occurs via as yet unidentified peroxidases because mutations in RBOH-D/F or pharmacological inhibition of H<sub>2</sub>O<sub>2</sub>-dependent peroxidase function prevented lignin accumulation in secession cells developed in the abscission zone [46\*\*].

Peroxidase function, however, is insufficient to rescue the severe hypolignification phenotypes observed in the xylem and fiber tissues of the *lac4 lac11 lac17* mutant

of Arabidopsis [35]. Nevertheless, single or double knock-out mutants in several peroxidase genes exhibited typically minor but noticeable reductions in lignin content and/or altered lignin composition in Arabidopsis inflorescence stems [3,47–54]; notably, the *prx72* mutant showed ~35% lignin reduction and also sometimes displayed collapsed xylem vessel phenotypes with reduced stem heights [48]. These data suggest that, besides laccases, at least some of these peroxidases play a role in the lignification of xylem/fiber tissues in Arabidopsis.

A probable hypothesis for the respective functions of laccases and peroxidases during xylem lignification is that their functions are spatially and/or developmentally separated. Amongst many other laccases and peroxidases, *LAC4* and *PRX64* are the most highly expressed oxidative genes in Arabidopsis inflorescence stems along the lignification developmental gradient [55]. A recent subcellular localization analysis of native-promoter-driven, fluorescently-tagged *LAC4* and *PRX64* demonstrated that *LAC4* is specifically localized throughout the secondary cell wall layers in fibers and vessels, whereas *PRX64* localization is confined to the middle lamella and cell corners in fibers (Figure 1c) [56\*\*]. These localization data suggest that *LAC4* mediates lignin polymerization throughout the thick secondary cell wall layers whereas *PRX64* functions primarily within the middle lamella in fibers. Considering the bulk of the biomass of Arabidopsis inflorescence stems is derived from secondary cell walls, this notion is in line with the dramatic loss of lignins in the *lac4 lac11 lac17* triple mutant in contrast to the limited lignin depletion in the peroxidase mutants analyzed to date [35]. However, no *prx64* mutants have yet been described and thus the function of *PRX64* in the lignification of xylem/fiber tissues in Arabidopsis remains unclear. It is also noteworthy that ZPO-C, a Zinnia *PRX64/66/72* homolog peroxidase, was shown to be localized to the secondary cell walls of xylem vessels and was also specifically expressed in developing xylem vessels in Zinnia [57]. This contrasts the fiber-specific expression of *PRX64* in Arabidopsis, although both proteins appeared to be localized to primary cell wall domains in tricellular junctions [56\*\*,57]. Overall, future studies should further closely investigate the spatio-temporal localization of the diverse laccase and peroxidase proteins implicated in lignification.

### Localization of the polymerization machinery directs the spatial control of lignification

The mechanisms controlling the spatio-temporal patterning of lignin polymerization remain largely elusive. During protoxylem tracheary element (TE) formation, for example, lignin is precisely deposited in the spiral secondary cell walls while the intervening primary cell walls remain lignin free. In Arabidopsis, laccase genes, such as *LAC4*, *LAC11*, and *LAC17*, are specifically expressed during the onset of secondary cell wall formation [55]

and *LAC4* and *LAC17* have been shown to be precisely localized to secondary cell wall domains during protoxylem TE formation [58]. Furthermore, *LAC4* is highly immobile post secretion to secondary cell walls as recently determined by a fluorescence recovery after photobleaching (FRAP) analysis [56\*\*], suggesting that *LAC4* is specifically secreted and tightly anchored to polysaccharide-rich secondary cell wall domains [56\*\*,58]. In contrast, recent imaging analyses of lignin polymerization *in planta* using chemical probes, such as fluorescently-tagged [58,59] and chemical-reporter-tagged monolignols [60,61,62\*], have suggested that monolignols are highly mobile once exported to the apoplast.

Collectively, the precise localization of oxidative enzymes in specific cell wall domains is likely the key determinant of the spatial patterning of lignin deposition (Figure 1c) [56\*\*,58]. This model is in good agreement with the model proposed for lignin deposition in the Casparian strip in root endodermis cells, where CASPLs position the lignin polymerization machinery, including *PRX64*, *RBOH-F* and *DIR10/EFB1*, by forming a protein scaffold to capture and polymerize monolignols at the site where the Casparian strip forms [45] (+citation for the COiB lignin review about ‘Casparian strips’?). In turn, the spatial variability in the deposition of different lignin polymers derived from different monolignol precursor mixtures [2–4], is not necessarily directed by the monomer specificity of the polymerization machinery but is also (more likely) controlled by the local supply of monolignols, that is, by the controlled expression of genes responsible for monolignol synthesis and/or transportation, in lignifying cells and/or in neighboring cells that supply monolignols (citation for the COiB lignin review about ‘Transportation’?).

### Conclusions

Although the actual polymerization process of monolignols proceeds via simple combinatorial chemistry, the processes in which different monolignols are assembled and activated for lignin polymerization are under tight biological control. The emerging view is that specific laccase and/or peroxidase proteins are secreted to facilitate the spatial control of lignin deposition in distinct cell wall domains. However, to what degree their substrate specificities influence the incorporation of different monolignol types into lignin polymers remains unknown; although several *in vitro* peroxidase/laccase catalytic assay and DHP preparation studies reported to date used recombinant enzymes expressed in bacterial systems (e.g., with *Escherichia coli*), it is also important to test their functions by using enzymes expressed with eukaryotic systems that facilitate the *N*-glycosylations essential for these extracellular glycoproteins [63,64]. Besides, it is still to be determined how other possible protein members comprising the polymerization machinery (e.g., *RBOHs*,



superoxide dismutase, DIRs and/or CASPLs), reactive oxygen species, polysaccharide matrices and any other molecules present in the lignin polymerization site contribute to the assembly and polymerization of monolignols in different cell types. There is a need for further high-resolution analysis of gene expression in specific cell types as well as of protein localization and lignin chemical structure down to individual cell wall layers. By developing a unified view of how plants elaborately execute this last act in cell wall lignification, we may be able to more precisely manipulate cell wall structures for sustainable production of biofuels and biomass-based materials.

### Conflict of interest statement

Nothing declared.

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