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Enabling Lignin Valorization Through Integrated Advances in Plant Biology and Biorefining

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Abstract

Despite lignin having long been viewed as an impediment to the processing of biomass for the production of paper, biofuels, and high-value chemicals, the valorization of lignin to fuels, chemicals, and materials is now clearly recognized as a critical element for the lignocellulosic bioeconomy. However, the intended application for lignin will likely require a preferred lignin composition and form. To that end, effective lignin valorization will require the integration of plant biology, providing optimal feedstocks, with chemical process engineering, providing efficient lignin transformations. Recent advances in our understanding of lignin biosynthesis have shown that lignin structure is extremely diverse and potentially tunable, while simultaneous developments in lignin refining have resulted in the development of several processes that are more agnostic to lignin composition. Here, we review the interface between in planta lignin design and lignin processing and discuss the advances necessary for lignin valorization to become a feature of advanced biorefining.

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INTRODUCTION

Lignin valorization is the production of valuable products from lignin, an abundant but highly recalcitrant phenolic polymer of plant secondary cell walls (95). Pulp and paper mills and lignocellulosic biorefineries that produce bioethanol used in liquid transportation fuels generate large amounts of lignin as a side product. For example, the global industrial production of lignin reached approximately 100 million tonnes in 2015, with even greater capacity available (7). Presently, only a small percentage of this vast resource is used commercially; the majority of processed lignin is combusted to generate heat and power to drive biorefining processes (7). Although there is a growing awareness of lignin's potential owing to the demand for alternative fuels, chemicals, and materials, the concept of lignin valorization has been recognized for more than a century (140). The primary approaches pursued to date toward lignin valorization can be broadly categorized as (*a*) the use of lignin in a polymeric or oligomeric form; (*b*) the conversion of lignin into a valuable mixture of products, such as a biofuel blendstock or a mixture of deoxygenated aromatic compounds that can be processed in a petrochemical refinery (e.g., benzene, toluene, and xylenes); and (*c*) the conversion of lignin into a single valuable compound through so-called funneling approaches (101, 105, 113). Clearly, the ideal lignin differs for each usage, depending on the requisite chemical and physical properties. Importantly, the chemical and physical properties of lignin as it is polymerized in the plant cell wall are often changed through the various biorefining isolation processes (105). This is especially the case with processes that operate on a large scale today, such as kraft pulping, which dramatically modifies lignin's native state owing to the alkaline conditions needed for biomass delignification and lignin solubilization (17). Other common biomass fractionation methods, including sulfite pulping, soda pulping, and various pretreatments, similarly alter lignin's native state, thereby influencing its reactivity and potential for valorization (52, 73, 105). The path to lignin valorization thus requires not only recognizing lignin's potential but also understanding the transformations it undergoes during processing.

Valorization: adding value to a product (lignin) through the application of technology

Pretreatment: a chemical or enzymatic treatment of lignocellulosic biomass to facilitate subsequent cellulose and hemicellulose breakdown

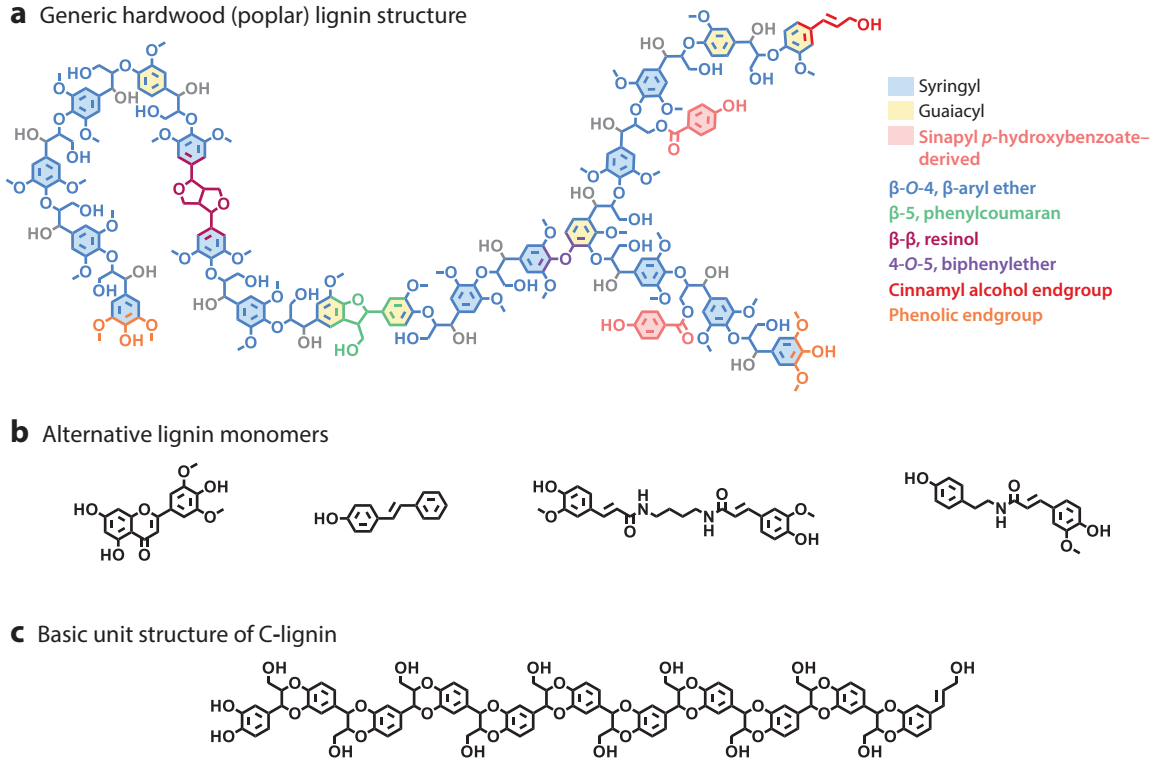


Figure 1

Structures of lignin polymers, their biosynthetic origins, and noncanonical monomers. (*a*) A generic hardwood (poplar) lignin structure, showing the major monomer types derived from sinapyl alcohol (S units) and coniferyl alcohol (G units) and the different linkage types, of which β-*O*-4 is usually the most common. C–C bonds, such as 5-5, are more common in G-rich gymnosperm lignins and are problematic for lignin deconstruction. Coumaryl alcohol (H) units are much less frequent. Panel adapted with permission from Reference 120. (*b*) Alternative lignin monomers found in some species: (*left to right*) tricin, hydroxystilbenes, diferuloylputrescine, and tyramine ferulate. (*c*) The basic unit structure of C-lignin.

Figure 1*a* shows a simplified view of a generic lignin molecule to illustrate characteristic monomers and linkage types. These features depend on the species of origin and developmental stages of the cell walls. For example, gymnosperm (softwood) lignins comprise almost wholly guaiacyl (G) units, whereas the syringyl (S)/G ratio can vary widely in angisoperms, with grasses usually having higher S/G ratios. Hydroxyphenyl (H) units generally comprise less than 5% of the total monomer units. Unlike other plant polymers, lignin is assembled via a chemically driven, radical-mediated coupling mechanism (97). The recent discovery that a wide variety of chemically enabled components can be incorporated into lignin (97) provides new opportunities for lignin engineering. Naturally occurring monolignols now include the flavanol tricin, hydroxystilbenes, diferuloylputrescine, and tyramine ferulate (97) (**Figure 1*b***), among others (23). If such molecules exhibit structures that allow β-*O*-4 coupling products (**Figure 1*a***), they can incorporate into the growing polymer, or else, like tricin, occur as end groups. Over the past 10 years, increasing interest has also been shown in C-lignin, a naturally occurring homopolymer of the noncanonical monolignol caffeyl alcohol (16) (**Figure 1*c***). C-lignin is a linear molecule that resists degradation during dilute acid pretreatment and can be chemically depolymerized through hydrogenolysis to a simple mixture of catechyl derivatives for valorization (67, 107). However, to date, it has only been

Monolignol: a hydroxycinnamyl alcohol precursor of lignin; more broadly used to describe any phenolic compound that can be incorporated into a lignin chain

found in the seed coats of a limited range of nonbiomass species, of which *Cleome hassleriana* has become a model for understanding the biosynthesis of C-lignin for translation to biomass species (147, 148).

Here, we explore the interface between plant biology and genetics and biorefining with a view to identifying those features of plant feedstocks that will promote economically viable lignin valorization. We discuss first the various approaches to, and end products of, lignin valorization and then how plant biology can inform and enable these processes.

Hydrodeoxygenation (HDO): a high-pressure and moderate-temperature process in which oxygen is rejected from lignin oil by a catalytic reaction with hydrogen

Biological funneling: use of natural aromatic catabolic pathways in microbes to convert lignin degradation products to central metabolites

Clip-off molecule: a molecule present as an end group on lignin that can be easily cleaved for valorization

Lignin first: a biorefining approach that considers lignin valorization and stabilization in the design stage

Lignin second: a biorefining approach in which lignin is valorized after extraction of fermentable polysaccharides

ENABLING OPPORTUNITIES AT THE INTERSECTION OF LIGNIN ENGINEERING AND BIOREFINING

What Is Possible?

In the last 15 years, the lignin valorization field has become very active, and the chemical engineering, materials science, and lignin chemistry perspectives have been extensively reviewed (e.g., 10, 79, 98, 113). Valorization routes from lignin to materials, biofuels, and platform chemicals for polymers are summarized in **Figure 2**. While modification of lignin structure to enhance valorization has been enabled by advances in plant molecular biology, as reviewed below, substantial work on genetic modification of lignin to date has primarily targeted reducing the lignin content to enable better access to the sugar-based polymers in the cell wall for improving forage digestibility or enhancing conversion to biofuels via fermentation (11, 22). Ultimately, success in tuning the structure of lignin to enable economically viable lignin valorization requires interdisciplinary research encompassing plant biology, genetics, molecular genetics, analytical chemistry, process engineering, and chemical engineering. To date, a review of current literature highlights further need for cross-fertilization between the plant biology and chemical engineering communities in the field, with process engineering mainly utilizing commonly available lignin streams and model compounds. We are still at the early stages of directly demonstrating which lignin structures are optimal for particular lignin-derived products in the context of process and cost-efficient conversion, although empirical approaches provide a basis for this fundamental understanding.

Ideally, an understanding of lignin biology, as elaborated in this review, should make it possible to

- make lignin easier to extract and separate from polysaccharides;
- make lignin easier to depolymerize if monomers are the goal;
- tune lignin functionalization to the end product (e.g., reduce the hydrogen demand in hydrodeoxygenation (HDO) processes and tailor lignin chemistry for biological funneling);
- tailor lignin polymer chemistry and structure by altering bond types and branching if the goal is direct use as a material;
- engineer new and valuable building blocks or end products into lignin; and
- engineer clip-off molecules as a separate product stream.

Aiding Lignin Extractability from Plants

Lignin extraction is a major cost driver in existing pulp and paper biorefineries, where remaining lignin affects paper properties (e.g., color and mechanical properties) and its removal generates significant waste streams (31, 96). Regardless of the target application for lignin, its isolation from cell wall polysaccharides is often a critical step in biorefining when a lignin-derived product is a process goal. This isolation can proceed via two routes: removing lignin from the plant cell wall (lignin first) or removing carbohydrates from the plant cell wall to enrich in lignin (lignin second).

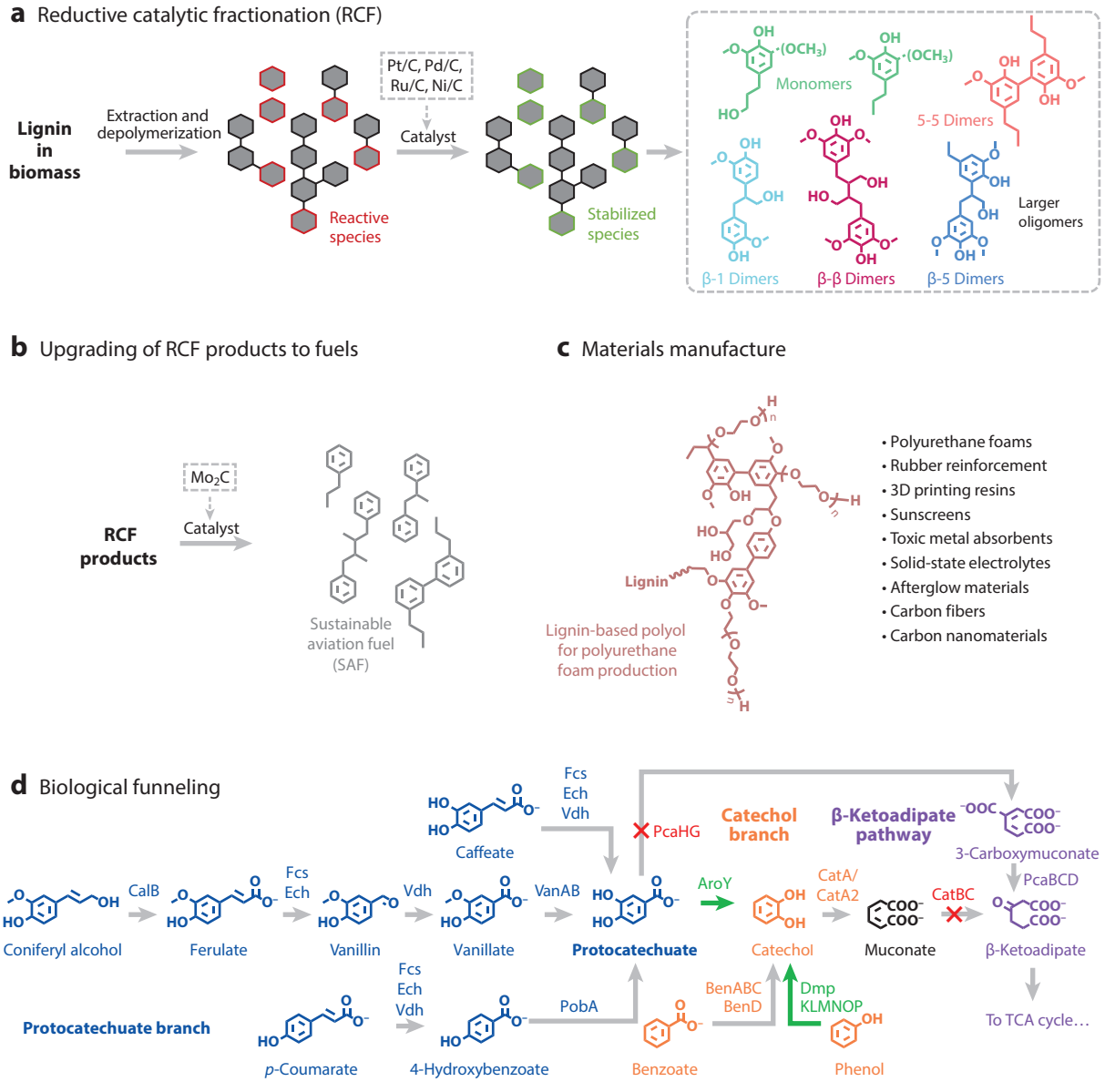


Figure 2

Processes for lignin depolymerization and valorization. The intact lignin molecule can be burned to generate electricity (not shown) or modified chemically, if necessary, for conversion to fuels, chemicals, and materials. Conversion to chemicals requires depolymerization; (*a*) RCF results in lignin oil containing monomers, a range of dimers, and larger oligomers, with the complexity of the mixtures depending on the heterogeneity of the lignin. (*b*) Further catalytic upgrading to remove oxygen results in mixed hydrocarbons suitable for sustainable aviation fuel. (*c*) Lignin can be tailored to a wide variety of applications, such as the polyurethane precursor polyol shown here. Panel adapted with permission from Reference 77. (*d*) Alternatively, lignin-derived monomers and potentially dimers can be metabolized by bacteria such as *Pseudomonas putida* via ring cleavage and funneling through central metabolism to create products such as *cis*, *cis*-muconate, a platform chemical for the manufacture of plastics. Red crosses indicate gene knockouts, and green arrows indicate introduced genes. Abbreviations: RCF, reductive catalytic fractionation; TCA, tricarboxylic acid cycle; 3D, three-dimensional.

Whatever the process, the effects on lignin structure and the composition of chemical treatments must be carefully considered as they relate to the target product.

In the case of lignin extraction from the intact plant cell wall, many processes that involve either acidic or basic conditions have long been studied for removing lignin in a solubilized form from intact polysaccharides. As reviewed extensively in the literature, the typical chemistry of lignin in these reaction conditions involves aryl-ether bond cleavage and deleterious reactions (with a mechanism that depends on the reaction conditions) that lead to condensation products that exhibit a higher fraction of carbon–carbon (C–C) linkages (1, 101) (**Figure 3**). Conversely, in the last 10 to 15 years, the advent of lignin-first biorefining approaches has enabled the development of multiple stabilization strategies that are able to selectively extract lignin from the plant cell wall and use some form of stabilization chemistry to either convert reactive intermediates into stable products or prevent aryl-ether bond cleavage through aldehyde or diol-based capping of the common motif found in the β-*O*-4 linkage (1, 94). Depending on the approach taken, the lignin can be either an intact polymer or isolated as an oil with monomers and C–C linked oligomers as desired products (1, 98). Based on a techno-economic analysis (TEA) of a particularly well-studied lignin-first biorefining method known as reductive catalytic fractionation (RCF) (12) (**Figure 2*a***), multiple variables are important for delignification, all of which could be potentially tuned through improved understanding of lignin in planta, including the rate and extent of lignin extraction as well as the reaction conditions (mainly reactor pressure) and solvent required to achieve rapid and effective lignin extraction.

How both lignin chemistry and its interaction with other plant cell wall components affect lignin extractability is only partly understood. Studies conducted on genetically modified alfalfa suggested that an increase in the H unit fraction within lignin is correlated with a decrease in lignin molecular weight and improved extractability (149). The idea that lower molecular weight lignin polymers are more efficiently extracted was also suggested by the results of the RCF of poplar biomass using a flow-through configuration in which lignin was subjected to extraction, depolymerization, and catalytic stabilization to yield monomers (5). In this case, the overall S/G ratio of the products increased with lignin extraction time, which was interpreted, based on monomer coupling models and demonstration of high-molecular-weight S-rich polymers appearing at later flow-through times, as indicating that lower-molecular-weight and/or more compact polymers can more easily diffuse within the internal pores of the wood particles (5). Transport models of lignin extraction from the plant cell wall have been developed (114), and additional insights from the molecular level to mesoscale in terms of lignin distribution and interaction with other cell wall polymers, including across reaction conditions, will be key to further refining our collective understanding of how to remove lignin most efficiently from the cell wall.

In contrast to extracting lignin from an intact cell wall, optimizing lignin valorization processes to isolate residual solid lignin following carbohydrate solubilization necessitates a comprehensive strategy. This strategy should exceed merely processing the remaining lignin fraction after the required polysaccharide portion is obtained. In the initial studies on biomass pretreatment for fermentable sugar release, the composition and structure of the residual lignin were frequently overlooked (82). This becomes particularly problematic when reactions leading to lignin condensation are possible. Other methods take advantage of the complete solubilization of biomass in the presence of more sophisticated catalysts that allow one-pot transformations of sugars and lignin (67), usually to produce mixtures of compounds for fuel applications. An interesting alternative strategy, co-treatment, circumvents chemical pretreatment entirely, instead focusing on the effectiveness of mechanical shearing to disrupt lignocellulose. This technique, akin to cud chewing in cows, has been applied to one-pot fermentation systems with substrates such as

Techno-economic analysis (TEA): a method of analyzing the technical and economic performance of a process or product using mathematical modeling

Reductive catalytic fractionation (RCF): a lignin-first biorefining strategy yielding close to theoretical amounts of lignin monomers using reductive catalysts to generate stabilized products

Co-treatment: a combination of physical disruption of biomass with its simultaneous saccharification

Lignocellulose: plant dry matter (biomass)

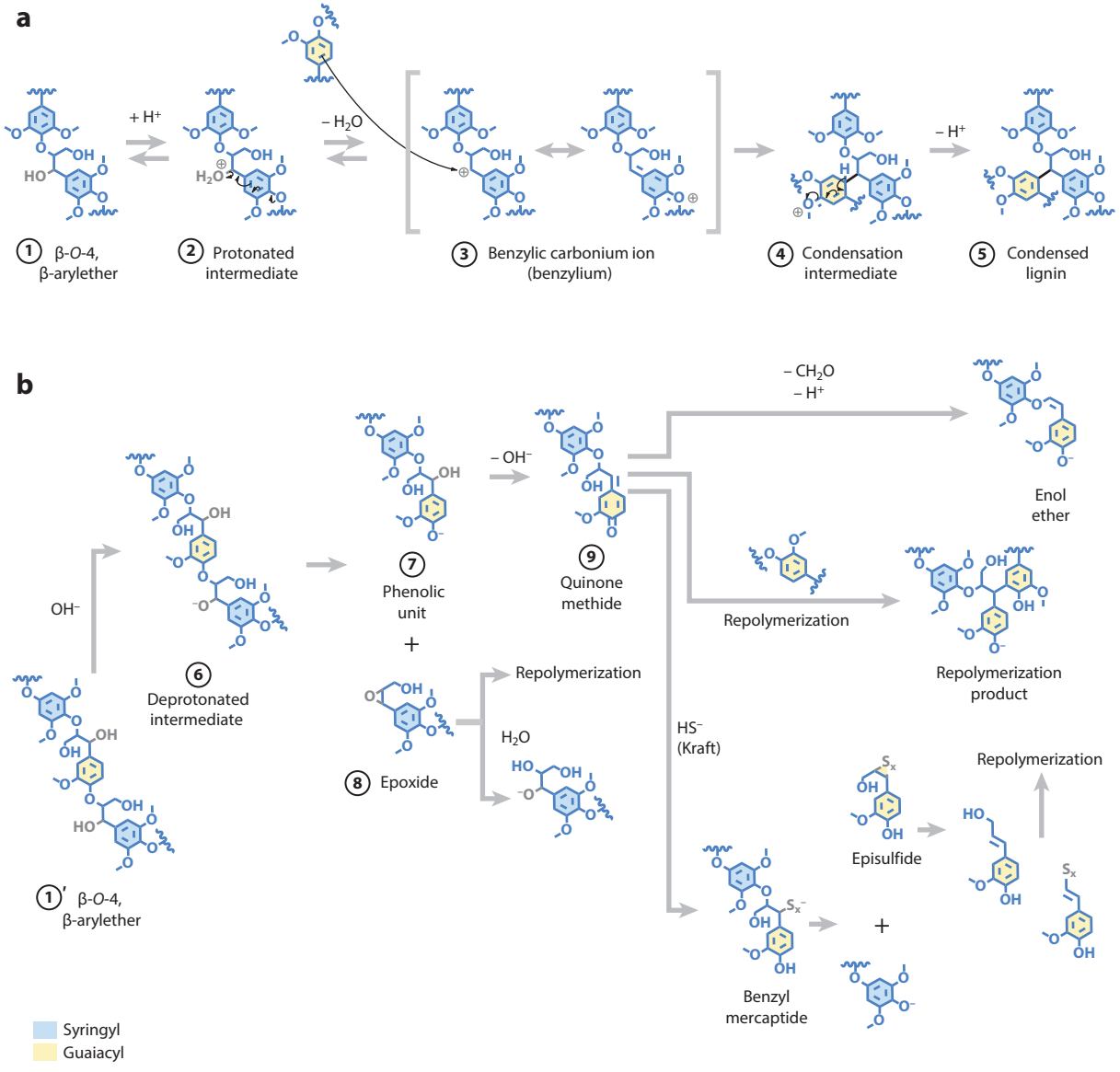


Figure 3

Modification of β-*O*-4 substructures in (*a*) acidic and (*b*) basic media. After protonation (②) and dehydration, a typical lignin β-aryl ether unit (①) forms the resonance-stabilized benzylic carbonium ion (benzylium ion; ③), which is prone to nucleophilic attack from a lignin G unit to form a carbon–carbon bond, producing an intermediate (④) that after rearomatization yields a condensed lignin structure (⑤). Panel adapted from Reference 1 (CC BY 3.0). (*b*) A typical lignin substructure having lignin β-aryl ether units (①′) deprotonates in basic medium (⑥) and then transforms into a phenolic unit (⑦) and an epoxide (⑧) from which multiple reaction channels are opened, some of which end in condensation products. Importantly, the phenolic unit (⑦) reacts via the central intermediate quinone methide (⑨). Panel adapted from Reference 105 with permission from the Royal Society of Chemistry.

corn stover, switchgrass, and poplar biomass, utilizing *Clostridium thermocellum* and *Saccharomyces cerevisiae* for polysaccharide degradation and sugar fermentation, respectively (8). This method has resulted in high sugar fermentation yields, raising interest in whether this physical disruption technique can be developed into an efficient lignin-second approach if energy input for

mechanical shearing could be minimized. The goal would be to deliver lignin that retains native-like properties, enriched for downstream valorization (76).

Lignin-derived oil: oil produced from the chemical depolymerization of lignin (also termed bio-oil)

Tuning Lignin Chemistry for Materials

The search for uses of high-volume lignin from biorefining waste has driven the development of lignin as a renewable feedstock for materials manufacture (30, 63, 100). This includes the use of lignin in fibers, polymers, foams, resins, adhesives, binders, and reinforcing agents, among others, and finding applications in diverse areas such as environmental remediation, energy storage, packaging, targeted drug delivery, adsorption, catalysis, construction, and numerous others. However, many uses of lignin in materials require it to first be chemically modified. For example, oxypropylation converts lignin to a polyol (**Figure 2*c***) to replace the sucrose or glycerol polyols commonly used in foam preparations, and the resulting foams have improved compressive properties (66). Polymer strength, for example, in hydrogels, can be enhanced by the presence of so-called sacrificial bonds that rupture and then reform, effectively dissipating mechanical energy under high loading (104). In a related application, the incorporation of alkali-pulped wheat straw lignin as a partial substitute for carbon black, the most prevalent reinforcing agent in the rubber industry, improved rubber properties within a dual cross-linking network of sulfur covalent bonds and zinc-based coordination (sacrificial) bonds (125). The coordination bonds involve the phenolic and aliphatic hydroxyl groups of the lignin and the carboxyl groups of the lignin-associated coumarates and ferulates. Wheat straw lignin has a high S/G ratio of around 2:1, prompting future studies to investigate the effectiveness of lignins with adjustable monomer ratios and acylation patterns as rubber reinforcing agents, thereby facilitating the design of lignins for high-volume applications in the rubber industry.

In an alternative strategy, lignins derived from pulping can be processed for applications in three-dimensional (3D) printing. Using what is usually considered low-quality lignin (also called technical lignin) derived from acid-pretreated wheat straw, O'Dea and coworkers (88) demonstrated a catalytic depolymerization and stabilization method in the presence of glycerin working at ambient pressure (processes similar in part to those happening in RCF). Such a process yields lignin-derived oil useful for high-performance additive manufacturing via stereolithography 3D printing by incorporation of acrylated lignin-derived oil into a photocurable 3D printing resin (88). Another interesting valorization route for lignin via additive manufacturing for 3D printing is shown in the replacement of the commonly used acrylonitrile butadiene styrene by a lignin-modified nylon composite containing 40–60% sinapyl alcohol–rich lignin, which provides increased stiffness and tensile strength at room temperature but reduced viscosity in the melt and can be further improved by the incorporation of low amounts of discontinuous carbon fibers, potentially also derivable from lignin (86).

After oxidation of the G and S units of lignin to form acidic chromophores and encapsulation within a fatty acid matrix, lignin exhibits room temperature phosphorescence (RTP) properties. These properties make it a suitable candidate for the manufacture of afterglow structural materials (123). Similar oxidation can be performed directly with wood in situ. Because the lifetime of RTP depends on the wood source (123), it is possible that lignin structure and composition in the wood may be tunable for imparting RTP properties.

Nearly all carbon fibers for use in lightweight materials manufacture are made from polyacrylonitrile (PAN), which is sourced from fossil carbon–derived acrylonitrile. Carbon fibers can be spun from kraft lignin (industrial lignin obtained from kraft pulping), but their properties are poor because of structural heterogeneity (65, 85). Fractionation of kraft lignin to increase molecular weight and decrease polydispersity improves the elastic modulus of fibers that are cospun

with PAN (65). Because of its homogeneity, C-lignin appears to be ideal for the generation of carbon fibers, providing high graphitic structure and ion conductivity in the absence of supplementation with PAN (85), but is unfortunately not yet available in the quantities necessary for materials manufacture, highlighting the potential benefits of a genetic engineering approach.

Lignin has shown significant potential as a raw material for the fabrication of carbon nanomaterials across diverse applications (115). Kraft lignin can be transformed into nanoparticles through dissolution in tetrahydrofuran followed by the slow introduction of water via dialysis. The nanoparticles can be incorporated into transparent nanocomposite films by mixture with poly(vinyl alcohol), leading to materials with improved mechanical and thermal performance as a result of interfacial adhesion with the matrix through hydrogen bond formation and exhibiting good ultraviolet (UV) shielding and antioxidant functionalities (115). Pretreatment of wild-type and genetically engineered poplar biomass with deep eutectic solvents (DESs) under microwaves resulted in higher saccharification efficiency in the engineered lignin, which contained an increased amount of H monomers and *p*-hydroxybenzoate units, and the remaining lignin fraction was present as small and homogeneous lignin nanoparticles (135).

Most studies to date on lignin conversion to materials have focused on using lignins sourced from available waste streams, without much attention given to lignins generated by in planta engineering. It is now crucial to extend this research to determine the specific structural, chemical, and physical characteristics of lignins that are best suited for particular manufacturing applications, offering performance-advantaged attributes.

Deep eutectic solvent (DES): a homogeneous mixture of Lewis or Bronsted acids or bases with a lower melting point than those of its constituents

Saccharification efficiency: the percentage of total available cell wall sugars released as monomers after treatment with cellulolytic enzymes

Sustainable aviation fuel (SAF): aviation fuel derived from bio-based materials

Tuning Lignin Chemistry for Depolymerization and Conversion to Fuel Blendstocks or Valuable Mixtures of Products

The conversion of lignin to fuels has been pursued since at least the early 1900s, driven by the need to produce transportation fuels from biogenic waste feedstocks, including lignocellulose (140). For the aviation sector in particular, biofuels are vital to achieve the industry's goal of net carbon neutrality by 2050 (41). Aviation fuels consist of a blend of aromatic, cycloalkane, isoalkane, and linear alkane hydrocarbons. Cyclic molecules serve as an indispensable element in aviation fuels, particularly in improving jet engine seal swelling characteristics. However, the current absence of robust, large-scale processes to generate renewable cyclic species is a significant roadblock to reaching 100% sustainable aviation fuel (SAF) blends (59). Furthermore, the higher densities of cycloalkanes and aromatics compared to iso- and n-alkanes contribute to increased fuel density.

Lignin-derived aromatic compounds have demonstrated superior performance attributes compared to traditional aromatic blends employed in aviation fuel (108). These advantages include increasing the low values of dielectric constants commonly seen in aliphatic-based blends and a reduction in postcombustion particulate formation. The aromatic and cycloalkane components are particularly promising derivatives from lignin. The conversion of lignin into a SAF blendstock necessitates HDO processes that selectively retain the aromatic and/or cycloalkane constituents (**Figure 2*a***). A large body of work has described catalysts that can bring about the reductive depolymerization of lignin that results in lignin oils, but oxygen contents were generally still above 25% (reviewed in 62). Recent improvements included employing a ruthenium/aluminum oxide (Ru/Al_2O_3) catalyst to yield an SAF component that successfully tested as a 10% blend (137). A breakthrough was recently reported via a continuous, two-stage catalytic process utilizing molybdenum carbide (Mo_2C) as an abundantly available catalyst (**Figure 2*b***). This process deoxygenated poplar lignin into 87.5% aromatic hydrocarbons at 86% theoretical carbon recovery with less than 0.5 weight % oxygen residue (108), resulting in an SAF with fuel system compatibility at high-blend ratios with conventional fuel. This significant advancement was based

on the development of a flow-through reductive catalytic process, which effectively decoupled solvolysis from hydrogenolysis, enabling the study of extraction intermediates (4). This offered insights into the optimal lignin structure and composition for input. Perhaps surprisingly, the S/G ratio of lignin did not seem to influence the yield of monomer intermediates in this process (5).

The intrinsic molecular structure of lignin in planta governs the optimization trajectory of a lignin-centric biorefinery targeting SAF blendstocks. Accordingly, in order to maximize the product yield within the jet fuel range, feedstocks that can be processed with relative ease should be prioritized over those with energy-dense, C–C-coupled lignins, thereby circumventing the need for the development of selective C–C cleavage technologies. In this context, C-lignins exhibit noteworthy potential, given their theoretical depolymerizability and high energy density. However, before such technology can be deemed feasible for industrial-scale implementation, comprehensive incorporation into high-yielding biomasses must first be achieved (see the section titled Lignin Engineering In Planta).

Tuning Lignin Chemistry for Depolymerization and Conversion to Single Products

The conversion of a heterogeneous lignin substrate to a single product or purified products can be achieved through various chemo-catalytic and biocatalytic funneling strategies. The production of high yields of single small-molecule products from lignin, however, often relies on lignin depolymerization catalysis to create aromatic monomers. Today, C–O bond cleavage is accessible via many different chemistries to yield aromatic monomers, but, in many lignin depolymerization processes, aromatic dimers and oligomers linked by C–C bonds remain intact, which thereby creates a theoretical limit on aromatic monomer yields that depends on the content of β-*O*-4 linkages (105). This immediately suggests that engineering higher intermonomer C–O bond content in lignin is a useful strategy for funneling approaches. Despite the wealth of C–O bond cleavage strategies, C–C bond cleavage is a major challenge for the biorefining community, with only a few reports in the literature showing definitive C–C bond cleavage (111). Solutions include the development of enzymes capable of cleaving C–C-linked compounds to monomers (60, 69, 93) or potentially in planta lignin engineering to reduce or eliminate the C–C bonds, as more S-lignin favors β-*O*-4 bond formation at the expense of C–C bond formation (78).

Following depolymerization, lignin-derived products can be either separated into different streams for further chemical processing or fed to microbial cultures for conversion to central metabolites through microbial aromatic metabolism (an example of biological funneling) (129, 131) (**Figure 2*d***). Centrifugal partition chromatography has been successfully employed to separate vanillin, syringic acid, syringaldehyde, vanillic acid, and *p*-hydroxybenzoic acid (pHBA) from the products of lignin subjected to alkaline aerobic oxidation (3). pHBA is an important chemical feedstock, used in the production of liquid crystal polymers, and represents a potential lignin clip-off product that can be generated by genetic engineering (83) (see the section titled Lignin Engineering In Planta).

For biological funneling, the functionality of the lignin-derived monomers influences how they are used and what products can be made. Among multiple other studies, Linger et al. (70) showed that *Pseudomonas putida* KT2440 can convert model compounds and heterogeneous lignin-enriched streams to polyhydroxyalkanoates as plastic precursors through funneling via aromatic ring cleavage followed by conversion to central carbon metabolism via β-ketoadipate. To broaden the repertoire of end products, and improve metabolic conversion rates, subsequent studies have examined engineering around bottlenecks in the metabolism of *P. putida* to generate strains capable of accumulating *cis*, *cis*-muconate (**Figure 2*d***), a platform chemical for plastic production (57, 69). Other microorganisms such as *Rhodococcus opacus* PD630 can also use lignin-derived aromatics for

cell growth and have been engineered for the production of gallate from G- or S-lignin-derived aromatics through the expression of different *O*-demethylase systems (14). In most microbes studied to date, biological funneling with mixtures of H-, G-, and S-type compounds leads to different accessible products from aromatic catabolism than those produced from H- and G-type compounds (49, 87, 91), such that product choice and lignin substrate chemistry must be carefully considered, and—ideally—co-optimized for maximum product yields.

PLANT BIOLOGICAL INTERVENTIONS FOR LIGNIN VALORIZATION

Enzymatic Control of Lignin Content and Composition

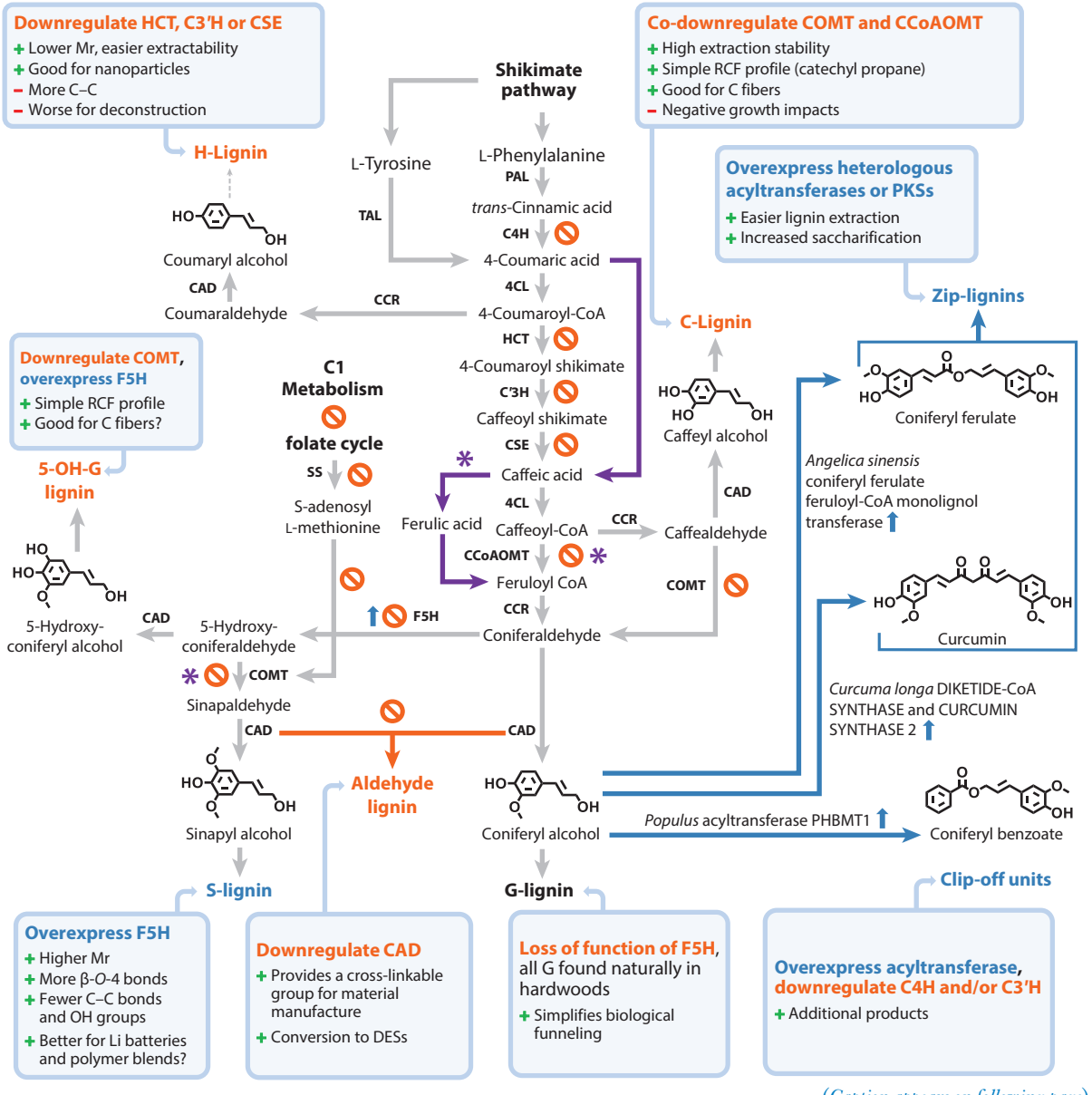
To formulate effective biological interventions for lignin valorization, a full understanding of the molecular regulation of lignin content and composition is necessary. The enzymatic pathways leading to the building blocks of lignin have been extensively reviewed (24, 44, 120). The model in **Figure 4** highlights the key decision points for entry into G- and subsequently S-lignin formation. Importantly, there are differences between the pathways in dicots and grasses, such as the key role of tyrosine ammonia-lyases and the greater involvement of interconversions at the acids level in the grasses (9, 10). Despite its complexity, the model in **Figure 4** is still an oversimplification, partly because of built-in redundancies in some steps (10, 84).

The biosynthesis of C-lignin serves as a distinct case where methylation at the 3-*O* position is suppressed. The ornamental plant *Cleome hassleriana* is a model species for studying C-lignin biosynthesis due to the shift in lignin composition in the seed coat from all G-lignin to all C-lignin, approximately 14 days postpollination. This switch coincides with the suppression of two key enzymes in G- and S-monomer synthesis, the caffeic acid and caffeoyl CoA *O*-methyltransferases (OMTs), along with the appearance of laccase and cinnamyl alcohol dehydrogenase forms with substrate preferences for the corresponding nonmethylated lignin monomer precursors (128, 147). The requirement for loss of function of both caffeic acid/5-hydroxyconiferaldehyde 3/5-*O*-methyltransferase (COMT) and caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) indicates the enzymatic redundancy for methylation at the 3-*O*-position of monolignol precursors.

In contrast to the well-understood pathways of monolignol biosynthesis, our understanding of monolignol transport from the cytosol to the apoplast, as well as the initiation and extension mechanisms of lignin polymers within the cell wall, is limited. Despite repeated attempts to discover monolignol transporters, no transporter for G or S monolignols has yet been identified at the molecular level. Rather, biophysical considerations suggest that passive diffusion is sufficient to account for the passage of monolignols across the plasma membrane (121), and this could be facilitated by polymerization of the monolignols in the apoplastic space (92, 148).

It is unclear how monolignol polymerization is initiated within the apoplast, although the process appears to start at the cell corners. Tricin has been suggested to act as an initiator for lignification in grass cell walls because it can only be incorporated at the start of the chains, but direct evidence for an initiator function is inconclusive, although genetically reducing tricin levels in grasses can reduce lignin content (61). Monolignol polymerization proceeds by a free-radical reaction initiated by the activities of laccases (LACs) or peroxidases. Both are encoded by large gene families in plants, making it difficult to ascribe specific functions to them because of redundancy. In *Arabidopsis*, LAC4 and LAC17 are the major laccases associated with lignification, and the simultaneous knockout of LAC4, LAC17, and LAC11 results in plants with lignin essentially only in the Casparian strip of the root (144), where its polymerization appears to be largely under the control of peroxidases (64). Downregulation of LAC8 reduces the proportion of C-lignin in the seed coat of *C. hassleriana*, and ectopic expression of *C. hassleriana* LAC8 facilitates C-lignin biosynthesis in *Arabidopsis* stems fed with caffeyl alcohol (128).

Recent reports have linked specific laccases or peroxidases to lignification through genetic analysis of other species (e.g., 141), although it is less clear whether these enzymes will prove useful in lignin engineering. Their potential roles may be hard to discern without complex genetic analysis to address redundancy, since their substrate preferences in vitro may not predict their in vivo roles (39). In *C. hassleriana*, caffeyl alcohol, the preferred substrate of LAC8, is a powerful inhibitor of the oxidation of coniferyl alcohol by other cell wall laccases, explaining the abrupt switch from G- to C-lignin biosynthesis during seed coat development (148). The large pool of coniferyl alcohol in the *C. hassleriana* seed coat during the stages of C-lignin biosynthesis may reflect reduced



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Tuning lignin biosynthesis for valorization. The model shows the monolignol biosynthetic pathways indicating steps that have been up- or downregulated by genetic manipulation to direct the pathway toward the preferential production of H, G, S, 5-OH-G, or C monolignols or, by expression of heterologous enzymes, zip-lignins or clip-off products. Light blue boxes indicate the results of the various modifications and their implications for lignin valorization. The acids route to G-monolignol biosynthesis is shown in purple, and *O*-methylation steps are marked with purple asterisks. Overexpression is in blue, downregulation and loss of function are in orange, positive outcomes are marked with a green plus sign, and negative outcomes are marked with a red minus sign. Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CCoAOMT, caffeoyl CoA 3-*O*-methyltransferase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid/5-hydroxyconiferaldehyde 3/5-*O*-methyltransferase; CSE, caffeoyl shikimate esterase; C3′H, coumaroyl shikimate 3′-hydroxylase; C4H, cinnamic acid 4-hydroxylase; DES, deep eutectic solvent; F5H, ferulic acid/coniferaldehyde 5-hydroxylase; HCT, hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase; Li, lithium; Mr, relative molecular mass; PAL, L-phenylalanine ammonia-lyase; PKS, polyketide synthase; RCF, reductive catalytic fractionation; SS, S-adenosyl L-methionine synthase; TAL, L-tyrosine ammonia-lyase; 4CL, 4-hydroxycinnamate CoA ligase.

passive diffusion of coniferyl alcohol to the apoplast because of this block in its polymerization (148). These results suggest that C-lignin engineering may not require the complete inhibition of coniferyl alcohol formation.

Lignin Engineering In Planta

Based on the examples above and others, **Figure 4** summarizes what might constitute optimal lignins for various downstream uses. Such lignins may occur naturally or may be generated through genetic engineering. The latter approach to date has been primarily targeted toward reducing lignin content or altering lignin composition to lessen the recalcitrance of woody biomass for paper/pulp or biofuel production or of forage crops for enhanced digestibility. This research has been extensively reviewed (e.g., 11, 15, 97, 138) and is based on many years of work by multiple groups to unravel the pathways of monolignol biosynthesis. Subsequently, the genes encoding many of the enzymes in the monolignol pathway have provided a basic tool kit (through mutation, RNA interference, gene editing, or overexpression) to alter lignin composition by modifying flux into specific monolignols. The major conclusions drawn from this work are that lignin content can be reduced, with or without alterations in composition, by targeting any one of a number of enzymatic steps for downregulation (**Figure 4**). These include L-phenylalanine ammonia-lyase (PAL), L-tyrosine ammonia-lyase (TAL), cinnamic acid 4-hydroxylase (C4H), 4-hydroxycinnamate CoA ligase (4CL), COMT, CCoAOMT, cinnamoyl CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), ferulic acid/coniferaldehyde 5-hydroxylase (F5H), and LAC within the monolignol pathway and polyfolylglutamate synthase and SAM synthetase in the C1 pathway. Downregulation of both COMT and CCoAOMT blocks both G- and S-lignin for C-lignin formation, loss of function of F5H blocks S-lignin formation, and strong F5H overexpression results in lignin comprised almost solely of S units (78, 147). Overexpression of a bacterial shikimate kinase inhibited shikimate recycling in the cytosol, thereby blocking formation of coumaroyl shikimate (the substrate of C3′H) and reducing G- and S-lignin; total lignin, though H unit–rich, was unaffected (42). To generate additional H-lignin, any one of the enzymes that work in series to introduce the 3-hydroxy function of monolignols (HCT, C3′H, and CSE) can be downregulated (e.g., 149). Downregulation of COMT with simultaneous overexpression of F5H leads to a lignin composed primarily of 5-hydroxyguaiacyl units (130) (**Figure 4**) linked through benzodioxane units as in C-lignin. In rice, the OMT OsCAldOMT1 is involved in the biosynthesis of both classical monolignol and tricin precursors, allowing alterations to both initiator and main chain units simultaneously (61).

Downregulation of CAD in some species can result in lignin composed almost entirely of monolignol aldehydes (145); such reactive aldehyde groups in lignin could be useful for materials manufacture, and phenolic aldehydes derived from such lignins have been converted to DESs for

Recalcitrance: the resistance of lignocellulosic biomass to enzymatic sugar release

Zip-lignin: a lignin engineered to contain easily cleavable bonds

further lignin pretreatment in a closed-loop biorefining process (55). The effectiveness of aldehyde generation through CAD engineering depends on the extent of redundancy of the *CAD* gene family in the particular species targeted. For example, in *Medicago truncatula*, the loss of function of a single *CAD* gene resulted in massive aldehyde incorporation (145), whereas, in *Arabidopsis thaliana* engineered to produce H-lignin by loss of function of coumaroyl shikimate 3′-hydroxyalse, concomitant loss of function of four *CAD* genes failed to generate any H-aldehydes in the lignin (84).

The recently realized flexibility of lignin structure has opened up a new paradigm for lignin engineering beyond changes to proportions of the major H, G, and S components. To facilitate lignin extractability, labile bonds were introduced through the random incorporation of monolignol ferulate esters into poplar lignin (132) (**Figure 4**). The so-called zip-lignins were readily degradable to smaller, and therefore more extractable, lignin chains. Similarly, the diferuloyl methane curcumin from turmeric (*Curcuma longa*), which is structurally similar to monolignol ferulates, was introduced into the lignin of *A. thaliana* through the expression of two genes from *C. longa*, resulting in a lignin that is easier to degrade under alkaline conditions (90). The incorporation of the coumarin scopoletin to account for over 3% of the lignin in *A. thaliana* gave similar results (40), as did the incorporation of 3,4-dihydroxybenzoate, synthesized by the product of a bacterial 3-dehydroshikimate dehydratase, in hybrid poplar (119). In the latter case, the levels of lignin were correspondingly reduced by the diversion of flux from the shikimate pathway. Monolignol benzoates can likewise be incorporated into lignin through the expression of a monolignol acyltransferase or the downregulation of the cytochrome P450 enzymes C4H and C3′H to generate clip-off products that could be valorized separately from the lignin (54).

In view of its favorable properties for materials and products, attempts have been made to engineer C-lignin in plants. Downregulation of CCoAOMT in pine led to the incorporation of a low level of C units into the plant's G unit–rich lignin (122). By contrast, C-lignin was engineered to a level of around 15% of the total lignin in CCoAOMT downregulated hairy roots of the *Medicago truncatula comt* mutant, and preliminary fractionation studies suggested that the C-lignin was physically separate from the H-/G-lignin (35). However, seedlings of the *comt ccoaomt* double mutant failed to progress beyond the first two weeks of development, suggesting that it will be necessary to fine-tune expression of the loss of function of the two OMTs using gene editing approaches that target lignin modification to fibers but not to xylem (21, 139).

The outcomes of the above studies have mostly been evaluated in terms of the resulting saccharification efficiency of biomass, not their usefulness for lignin valorization per se. However, the results suggest that "any aromatic compound that is made in lignifying cells, that meets the minimum requirement of having a phenolic function, and that has the right chemical properties to diffuse through a lipid bilayer, may incorporate into the lignin polymer and can therefore be considered as a candidate alternative lignin monomer" (40). Thus, in the future, new monolignols can be engineered to introduce new functional groups into lignin that will favor uses for materials or chemicals.

It is also possible to envisage designer crops in which lignin is overproduced as a precursor for materials and chemicals. Lignin overaccumulation is seldom achieved through the overexpression of single enzymes found in these pathways but rather through the ectopic expression of transcription factors (TFs), although a recent report of increased lignification following the overexpression of LACs (136) is consistent with the idea that the initiation of lignin polymerization rather than monomer supply per se is key in determining lignin amount. Intensive study over the past 10 to 15 years has defined a hierarchy of TFs, both activators and repressors, that controls the extent and location of lignification during plant development (89, 146). Lignin biosynthesis is also under the regulation of a number of microRNAs (miRNAs) (142, 143). The overexpression of miR828 in poplar downregulated genes for lignin biosynthesis by targeting the positive MYB171

and MYB011 TFs that activate PAL and CCR transcription, resulting in reduced lignin content in cell walls. Conversely, the suppression of miR828 elevated the expression of lignin biosynthetic genes and increased lignin levels (127). Other miRNAs appear to specifically target the expression of LACs (74). miRNA engineering appears to be a promising new approach for engineering increased or ectopic lignin production.

Loss of function of the negative regulator WRKY12 leads to dramatic ectopic lignification in the stem pith, associated with enhanced stem biomass, in both dicot and monocot plants (29, 124). Transient expression of MYB85 has proven to strongly enhance flux toward the monolignol pathway in *Nicotiana benthamiana* (56).

Lignification is also controlled by posttranslational modification of both enzymes and TFs. This includes phosphorylation of enzymes such as PAL and COMT and the TF NST-1, ubiquitination of PAL, glycosylation of LACs and peroxidases, and *S*-nitrosylation of the TF VND7 (71, 112, 142). Targeting these steps could provide alternative routes to lignin engineering in the future.

Impediments to In Planta Lignin Engineering

Despite uncertainties about the control of monolignol supply to the apoplast and how lignin polymerization is initiated, our current knowledge of lignin biosynthesis suggests that we already possess a robust genetic tool box for lignin modification. However, using the tools available to date, many attempts to modify lignin, either reducing its levels to promote saccharification or altering its composition toward valorization, have encountered problems with negative growth impacts (e.g., 13, 36, 37), the underlying causes of which have often remained unresolved.

Ectopic activation of defense responses is commonly observed following lignin modification, suggesting that growth–defense trade-offs are responsible for altered growth. This model has received some support but clearly does not apply in all cases, as suppressor mutations of modified lignin-induced growth reduction can still permit ectopic defense gene expression (reviewed in 37). In a study of 13 selected phenylpropanoid mutants in *Arabidopsis*, the mutants fell into five different subgroups reflecting system-wide effects on multiple biological processes (126). Even if ectopic defenses are not the cause of reduced growth, understanding the mechanisms underlying their activation is important for the development of sustainable sources of bioengineered lignins.

It is logical to view the wall exterior to the cell as the preferred place to deposit engineered polymers such as lignin, whose polymerization system is naturally localized to the apoplast. However, the cell wall is not a static barrier of polymers but rather a flexible and highly dynamic compartment that, in addition to controlling structure and turgor, also contains surveillance mechanisms that coordinate stimuli both inside and outside of the cell (133). A number of receptor kinase–like proteins have been implicated in cell wall signaling across the apoplast, of which the most studied is FERONIA, which transduces signals regulating cellular morphogenesis, shoot and cell wall integrity, and male–female gametophyte interactions (47).

Altered lignification can result in the release of pectic oligosaccharides that activate defense response genes (27). In the *Arabidopsis ccr1* mutant, this process involves FERONIA-dependent transcriptional activation of a set of wall-modifying enzymes, including the endopolygalacturonase ADPG1. Ectopic expression of ADPG1 appears to catalyze the processing of released pectins, leading to the generation of elicitors, followed by their recognition by wall-associated kinase (WAK) receptors and subsequent activation of induced defenses (27, 72). Blocking S-lignin by loss of function of ferulate/coniferaldehyde 5-hydroxylase in *Arabidopsis* results in the activation of a different set of defense genes than those in the *ccr1* mutant (28), likely involving nonpectic elicitors and yet-to-be-determined receptors. Clearly, we still have a lot to learn about the role of lignin in plant cell signaling.

Genome-wide association study (GWAS): the study of a genome-wide set of genetic variants in different individuals to determine if any variant associates with a trait

The growth defects of *ccr1* mutant *Arabidopsis* plants are not restored by simultaneous loss of function of FERONIA (72). This is consistent with their cause possibly being the result of loss of xylem integrity, and this is supported by the growth restoration following vessel-specific reintroduction of functional CCR1 in a *ccr1* mutant or CRISPR-Cas9-disrupted lines (20, 21, 139). Avoiding lignin modification in xylem vessels therefore presents an approach to countering negative growth effects. Strategies will differ depending on whether gene knockout or overexpression are required. In the latter case, it has been shown that fiber-specific expression of a transcription factor that blocks the expression of lignin biosynthetic genes results in altered lignin amounts but with maintenance of growth in hybrid poplar (32). Finally, it may be important to avoid the disruption of lignification in the Casparian strip of the root, which is responsible for the control of water and solute balance and possesses a mechanism to sense its integrity and respond with additional lignification (99).

New Approaches to Gene Discovery for Lignin Valorization

In addition to the potential negative growth impacts described above, impediments to in planta lignin engineering include the likely requirement for multigene transformation and regulatory issues around genetically engineered forests, where forest certification systems can block innovation (109), or grassland crops, where gene flow to wild populations is a concern (80). If valorization were independent of lignin composition, then biomass yield and extractability would be the most important considerations, and yield improvement is highly amenable to natural genetic selection.

Natural variation has already been exploited for commercial varieties of corn, sorghum, and other grass species with altered lignin properties. For example, the well-known brown-midrib (*bmr*) mutants possess mutations in genes of monolignol biosynthesis (103) or related input pathways from primary metabolism (2). Such mutants can sometimes show pleiotropic effects on growth and/or disease resistance (58), although the direction of these effects can depend on genetic background (34), suggesting that *bmr* lines could be developed as feedstocks for bioprocessing. However, broader genome-wide association study (GWAS)-based selections can provide a whole-genome appreciation of beneficial alleles for feedstock selection, and so better inform purpose-driven accelerated domestication.

Since the publication of the *Populus trichocarpa* genome (117), more than 1,000 individual lines covering the natural distribution of the species have been selected, resequenced, and grown in common gardens at multiple locations. Genetic analysis has revealed over 28 million single-nucleotide polymorphisms (SNPs) within this population. Phenotyping of these populations for compositional traits has led to the association of SNPs with natural variation in lignin composition and levels of lignin precursors and related phenolic compounds (118). However, many of the genes that appear to control lignin content or composition in GWASs are not directly associated with the lignin biosynthesis pathway itself, and their functions have yet to be determined. One interesting example is a case where SNPs that were associated with lignin content across the *Populus* species range and in plants grown under different environmental conditions were shown to reside in a *5-enolpyruvylshikimate-3-phosphate* (*EPSP*) *synthase-like* gene (134). EPSP synthase catalyzes a critical step in the shikimate pathway that generates L-phenylalanine as substrate for phenylpropanoid biosynthesis. However, the rare *EPSP synthase* alleles were not associated with enzymatic properties that could influence substrate supply but rather with structural changes in the protein that introduced a helix-turn-helix motif in the N terminus, converting the enzyme to a TF that acted as a repressor of lignin biosynthesis (134) and therefore a potential new tool for in planta lignin engineering.

Lignin optimal for valorization should have a defined structure determined by the genotype of the plant that is not affected by the pretreatment or extraction process (118). To this end, it is useful to include the response of the lignin to processing as a phenotypic screen in GWAS analysis. This can be labor intensive but is highly informative. For example, studies had demonstrated positive relationships between high S/G ratio and lignin processability in *Populus* (110), but differences in S/G ratio among natural variants of *Populus* failed to predict catalytic depolymerization monomer yields (5). GWASs can also be expanded by the incorporation of multiomic (transcriptomic and metabolomic) phenotyping, and this could become a key new approach in the molecular breeding of trees for improved wood properties (25, 50, 118).

TECHNO-ECONOMIC ANALYSIS AND LIFE CYCLE ASSESSMENT OF LIGNIN VALORIZATION

The concept of lignin valorization was born mainly from economic arguments. When developing a process for a large-scale application, even though it must be technically sound, it is essential to acquire further insights into its feasibility by assessing both economic and environmental aspects. For this, TEA is a useful approach for conducting process design, informed by experimental data, to estimate costs (e.g., capital and operating costs) alongside mass balances and energy balances (106). In addition, environmental impacts can be evaluated by a life cycle assessment (LCA), which is a standardized instrument to model the entire process (from feedstocks to waste management), together with an assessment of product uses in specific markets (81).

As lignin valorization strategies mature, many groups have begun to apply TEA and LCA to their and others' processes, but the studies differ in the processes and end products considered, and the conclusions are therefore hard to compare. Some studies only address lignin fractionation (48) or technically evaluate a process that produces lignin with a low degree of structural modification but focus the economic analysis on other products (75), others compare the economics from isolated lignin to different end products (e.g., 33, 51, 102) or to the same end product using different conversion technologies (e.g., 88), whereas other models consider conversion efficiency, unit conversion cost, product selling price, and energy requirements for lignin valorization in the context of bioethanol production (43). The lignin-first RCF approach has been examined in multiple studies (12, 116). Although the RCF process still has considerable technical hurdles to overcome before it can be an industrial reality (19), it has now been demonstrated to be feedstock agnostic (46) and to work at different scales (18). To progress, the main cost and environmental drivers must be addressed, including solvent consumption (6, 45) and reactor pressure (26, 53, 88). Moreover, its integration with other processes, such as the production of phenol, propylene, pulp amenable to ethanol production, and phenolic oligomers useful for ink production, must be considered (68).

Few of these studies have incorporated an understanding of the agronomic effects of targeted alterations to the feedstock, although a study considering the economics of ethanol production from a large population of 1,089 individual poplar trees that covered a wide range of lignin contents (from 0.192 to 0.266 mg per mg biomass^{-1}) and compositions (S/G ratio from 1.8 to 2.5) concluded that minimal fuel selling price was largely determined by tree size (trunk diameter) and carbohydrate content (38). This study, which incorporated a simulation model of the whole poplar supply chain, did not include lignin valorization.

CONCLUDING REMARKS

We have described the potential routes to tailor lignin for use to produce fuels, chemicals, and materials. A major question for the future is, Because we can engineer lignin, do we need to? TEA will

help decide which combinations of innovations in plant genetics, agronomy, process engineering, catalysis, separations, and/or funneling technology are best for lignin valorization. For example, would the development of biological systems for cleavage of C–C bonds and equally efficient funneling of differently substituted monomers make engineering lignin composition unimportant? If the answer is yes, then lignin (plant) yield and possibly extractability become more important than composition. Closer integration of plant biology and biorefining is crucial to address these questions as we move to reduce humankind's carbon footprint.

SUMMARY POINTS

1. Lignin is currently an undervalued component in the processing of lignocellulosic biomass and has the potential to serve as a source of materials, fuels, chemicals, and polymers.
2. Lignin structure and composition are tunable through genetic engineering or breeding to incorporate natural variation.
3. Developments in chemical and biological catalysis are increasing the carbon efficiency of lignin conversion to platform molecules for chemicals manufacture.
4. In planta lignin engineering often introduces growth penalties that may arise from multiple causes, including disruption to innate cell wall–sensing mechanisms.
5. Breeding through genetic selection, with high-throughput deconstruction as the target trait, has the potential to accelerate lignocellulosic feedstocks for lignin valorization in species with large-scale genetic/genomic resources, such as poplar and sorghum.

FUTURE ISSUES

1. In spite of the advances in in planta lignin engineering, research on catalysis and downstream processing has tended to use existing high-volume lignin streams from the pulp and paper industry. Techno-economic analysis and life cycle assessment should be applied with multiple feedstocks (natural and engineered) and processing approaches to determine optimal lignin valorization strategies.
2. More work is needed on understanding growth impacts of cell wall engineering and how these may be overcome through tissue-specific lignin modification. Can designer lignins, including engineered C-lignin, replace natural lignins in ways that fully maintain plant function?
3. Understanding the most atom- and energy-efficient, cost-effective, and greenhouse gas emissions–advantaged valorization pathways for lignin will require the close collaboration of the plant biology and biorefining communities.

DISCLOSURE STATEMENT

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