


# Harnessing unconventional monomers to tailor lignin structures for lignocellulosic biomass valorization

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## Abstract

Lignin is an integral component in the plant secondary cell wall that imparts structural rigidity and integrity to plant tissues, contributes to the formation of the plant vascular system, and provides stress resilience. However, the heterogeneity and recalcitrance of lignin greatly impede the effective utilization of lignocellulosic biomass which represents the most abundant and valuable biogenic source for green and sustainable bioproducts. A customized lignin structure has the potential to alter lignin physicochemical properties and improve biomass utilization. Recently, researchers have reported that lignin polymerization can employ monomers beyond the traditional three monomers. The incorporation of these unconventional monomers can alter the physicochemical properties of lignin polymer by imparting lignin with a whole new structure. Here, we review the plant engineering efforts to alter lignin content, composition, and structure by harnessing non-conventional monomers. Rational manipulation of lignin structure with these unconventional lignin monomers may provide new strategies for minimizing the impacts of engineered lignin on plant growth and development meanwhile improving the utilization efficiency of lignocellulosic biomass.

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## Introduction

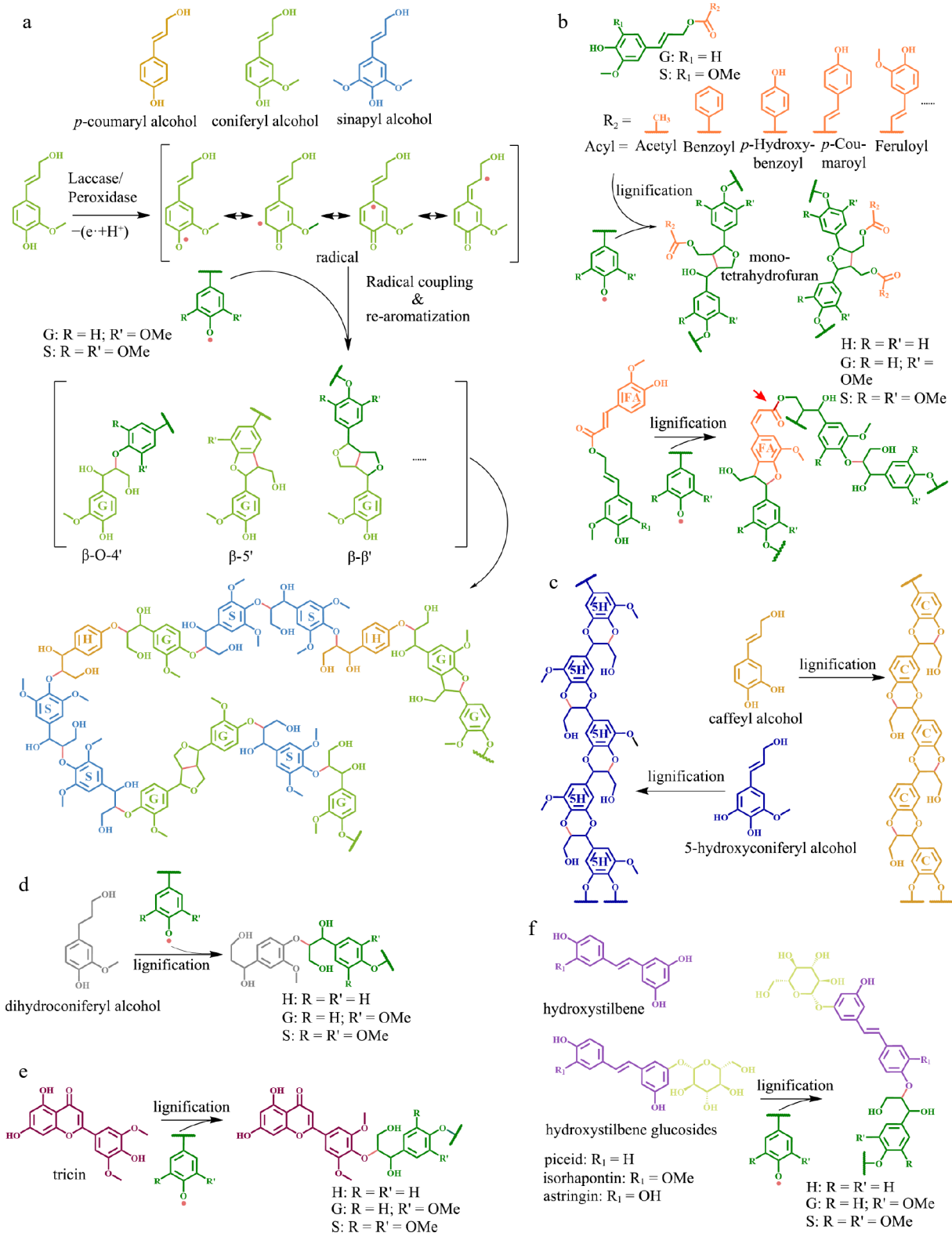
Lignin, a complex and versatile polymer derived from lignocellulosic biomass, is one of the most prevalent biopolymers on earth that serve as a huge high-quality carbon sink. Lignin has a higher carbon content than cell wall polysaccharides such as cellulose and the hemicelluloses. The polyphenolic characteristics of lignin provide plants mechanical strength, hydrophobicity, and resistance to pathogens. However, lignin drastically hampers the processing and utilization of plant biomass. For example, there were negative correlations between lignin content in plant biomass and pulp yield, forage digestibility, or polysaccharide saccharification yield<sup>[1–3]</sup>.

The backbone of lignin polymers is made up of three basic building blocks including *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin units which are derived from three *p*-hydroxycinnamyl alcohols (termed as 'monolignols'), *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively (Fig. 1a). The biosynthesis of traditional monolignols *via* the phenylpropanoid pathway is initiated by the conversion of the aromatic amino acid phenylalanine or tyrosine to cinnamic or *p*-coumaric acids *via* the phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL), respectively. The carboxylic acids are sequentially reduced to CoA-thioester, aldehyde, and alcohol by 4-coumarate coenzyme A ligase (4CL), cinnamoyl-CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD). The aromatic ring in cinnamate is hydroxylated *via* cinnamate 4-hydroxylase (C4H), a 'shikimate shunt' including *p*-hydroxycinnamoyl-CoA: quinate/shikimate hydroxycinnamoyl transferase

(HCT), *p*-coumaroyl shikimate 3'-hydroxylase (C3'H), and caffeoyl shikimate esterase (CSE), *p*-coumarate 3-hydroxylase/ascorbate peroxidase (C3H/APX), and ferulate 5-hydroxylase (F5H), then is 3-*O*-methylated and 5-*O*-methylated via caffeoyl-CoA *O*-methyltransferase (CCoAOMT) and caffeic acid *O*-methyltransferase (COMT), eventually yielding the three monolignols<sup>[4,5]</sup>.

In addition to traditional monolignols, researchers have recently identified several essential unconventional phenolic compounds that can also serve as monomers participating in oxidative radical reactions and integrating into lignin polymers in certain plants. These unconventional monomers included phenolic compounds emerging from typical monolignol biosynthesis pathways (e.g., acylated monolignols, caffeoyl and 5-hydroxyconiferyl alcohols, and hydroxycinnamaldehydes) or other phenolic biosynthesis pathways, including flavonoids (e.g., tricetin and naringenin), hydroxystilbenes (e.g., resveratrol, piceatannol, and isorhapontigenin) and their *O*-glucosylated counterparts (e.g., piceid, astragalin, and isorhapontin), and ferulic acid amides (e.g., feruloyltyramine, and diferuloylputrescine)<sup>[6]</sup>. The identification of these unconventional monomers demonstrated that lignin allows compositional flexibility and structural plasticity<sup>[6,7]</sup>, opening up new avenues for engineering the lignin structure.

Monolignols are transported into the cell wall in a manner that is not fully comprehended<sup>[8]</sup>. Polymerization occurs *via* free-radical coupling mediated by peroxidases and/or laccases, generating various structures in the biopolymers<sup>[4,9,10]</sup> (Fig. 1a). Structural characteristics of lignin are primarily determined by



**Fig. 1** Different types of monomers and corresponding lignin structures. (a) Conventional monolignols and their polymerization mechanism, which is mainly carried out by an 'end-wise' process that begins with single-electron oxidation of monolignols by laccase and/or peroxidase to generate activated radical intermediates. The radicals inter-couple or couple with a radical formed on the free-phenolic ends of a growing lignin polymer to form lignin with different linkages. (b) Acylated monolignols and the resulting lignin structure. Feruloyl esters, highlighted in orange color on feruloyl monomers, can be integrated into the lignin backbone. The red arrow indicate the presence of an alkali-labile linkage in the lignin structure. (c) Caffeoyl alcohol and 5-hydroxyconiferyl alcohol (unconventional lignin monomers) and the resulting homogeneous linear lignin polymer. (d) Dihydroconiferyl alcohol and the corresponding lignin structure. (e) Tricin (a flavonoid) and the resulting lignin polymer. (f) Hydroxystilbene and hydroxystilbene glucoside, as well as their corresponding lignin structures.

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types of inter-unit linkages and functional groups attached to the monomers. There are two major types of inter-unit linkages present in lignin polymers, including ether bond linkages (e.g.,  $\beta$ -O-4 and 4-O-5) and carbon-carbon linkages (e.g.,  $\beta$ - $\beta$ ,  $\beta$ -5, 5-5, and  $\beta$ -1). The inter-unit linkages in natural lignin exhibit variation across plant species, with the  $\beta$ -O-4 linkage being the most prominent<sup>[11]</sup>. The main functional groups present in lignin include hydroxyl, methoxyl, carbonyl, carboxyl, and benzene ring groups. The proportion of each group in lignin highly depends on the types of monomers and the method of lignin processing<sup>[12]</sup>.

The molecular structures of lignin, particularly the composition, inter-unit linkages, molecular weight, and solubility have a significant impact on the physicochemical properties of lignin and the usability of lignocellulosic biomass. Unconventional monomers expand the definition of traditional lignin and provide new strategies for tailoring the structure of lignin. Recently, several review reports have discussed the occurrence and biosynthesis of unconventional monomers, as well as the structural features of unconventional lignin<sup>[6,7,13]</sup>. Here, we mainly focus on the potential utilization of those unconventional monomers, review the progress of unconventional lignin engineering, and anticipate future strategies.

## Engineering acylated lignin

The lignin in a variety of plant taxa is decorated with acylesters, including *p*-coumarate (*p*CA), ferulate (FA), *p*-hydroxybenzoate (*p*BA), benzoate (BA), vanillate (VA), and acetate<sup>[14]</sup>. The *p*CA decoration is one of the hallmarks of the commelinid monocots<sup>[15,16]</sup> and has also been found in several eudicot species such as mulberry (*Morus alba*) and kenaf (*Hibiscus cannabinus*)<sup>[17,18]</sup>. The *p*BA decoration was reported in palms, poplars, and willows<sup>[19–22]</sup>, as well as in seagrass *Posidonia oceanica*<sup>[23,24]</sup>. The acetate decoration at low levels was ubiquitous among angiosperms with substantial levels in the lignin of kenaf (*Hibiscus cannabinus*), sisal (*Agave sisalana*), abaca (*Musa textilis*), and hornbeam (*Carpinus betulus*)<sup>[7,25]</sup>. The FA decoration was also found in various angiosperms<sup>[26]</sup>. The BA decoration was reported in the lignin of some plants, including the macaúba (*Acrocomia aculeata*) palm fruit endocarp<sup>[22]</sup> and the *PtrC4H1/PtrC4H2/PtrC3H3* transgenic lines of *Populus trichocarpa*<sup>[27]</sup>. Lignin from the leaves of Canary Island date palms was decorated with *p*CA, FA, *p*BA, BA, VA, and acetate<sup>[28]</sup>. The *p*CA, *p*BA, and acetate were preferentially decorated on syringyl units<sup>[14,29,30]</sup>, whereas FA preferentially acylated guaiacyl units in eudicot lignin and syringyl units in monocot lignin<sup>[26]</sup>.

Monolignols undergo acylation prior to being incorporated into the lignin polymer<sup>[14,31]</sup>. The acylated monolignols or monolignol  $\gamma$ -carboxylate conjugates behave as authentic lignin monomer precursors incorporated into the lignin polymer by polymerization and co-polymerization with the traditional monolignols. These monolignol ester conjugates can form identical linkages as traditional monolignols except  $\beta$ - $\beta$  which is greatly influenced by the acylation (Fig. 1b)<sup>[13]</sup>. The inter-coupling of monolignols or coupling to growing polymer produces a quinone methide intermediate, which is rearomatized *via* nucleophilic attack to form various structures (Fig. 1a). Contrary to traditional monolignols  $\beta$ - $\beta$  coupling, which involves rearomatization of intermediate quinone methides *via*

free  $\gamma$ -OH groups attack to form a resinol structure, acylated monolignols preclude rearomatization *via* intramolecular cyclization and form a mono-tetrahydrofuran structure (Fig. 1b)<sup>[13]</sup>. Acylated monolignols may increase lignin polymer alkaline solubility by introducing alkaline labile ester bonds into lignin. However, different acylated monolignols exhibit distinct impacts on lignin properties. For instance, monolignol *p*-coumarate conjugate and monolignol *p*-hydroxybenzoate conjugate introduced an additional free phenolic 4'-hydroxyl group into lignin polymer in contrast to monolignol benzoate and monolignol acetate (Fig. 1b). Therefore, acylated lignin possessing different monolignol conjugates may exhibit distinct hydrophobicity since phenolic hydroxyl can increase lignin hydrophobicity<sup>[32]</sup>. Incorporation of monolignol ferulate into the backbone of lignin polymer *via* both monolignol and ferulate moieties (Fig. 1b) can produce more branched lignin<sup>[13,33]</sup>. However, some other acylated monolignols, such as monolignol *p*-coumarate and monolignol *p*-hydroxybenzoate, can only be integrated into the lignin polymer *via* their monolignol moieties. Therefore, these ester-linked phenolics can be found pendant on the lignin polymer<sup>[14]</sup>.

Monolignol acylation requires acyltransferases to conjugate the activated acyl moieties from the thioester donor to traditional monolignols. The enzymes identified for *p*-coumaroylation of monolignols in grasses included *p*-coumaroyl CoA: monolignol *p*-coumaroyltransferase (PMT) from *Oryza sativa* (OsPMT)<sup>[34]</sup>, *Branchypodium distachyon* (BdPMT)<sup>[35]</sup>, *Zea mays* (ZmpCAT)<sup>[36]</sup>, and an eudicot species *Hibiscus cannabinus* (HcPMT)<sup>[18]</sup>. The enzyme reported for feruloylation of monolignols in both monocots and eudicots included feruloyl CoA: monolignol feruloyltransferase (FMT) from *Angelina sinensis* (AsFMT)<sup>[33]</sup> and *O. sativa* (OsFMT1)<sup>[26]</sup>. Recently, an enzyme *p*-hydroxylbenzoyl CoA: *p*-hydroxylbenzoyltransferase (PHBMT1) was characterized for *p*-hydroxylbenzoylation of monolignols in poplar<sup>[37,38]</sup>. Consistent with the discovery of lignin *p*-coumaroylation and *p*-hydroxylbenzoylation primarily on syringyl units, the recombinant OsPMT, ZmpCAT, HcPMT and PHBMT1 preferentially acylated sinapyl alcohol *in vitro*<sup>[18,34,36,37]</sup>. Contrarily, the recombinant AsFMT acylated all three monolignols with a preference for coniferyl alcohol<sup>[33]</sup>. So far, all identified enzymes catalyzing monolignol acylation belong to the well-known superfamily of plant BAHD acyltransferases. It is believed that additional BAHD acyltransferases, as well as the enzymes responsible for substrate supply, are also involved in the biosynthesis of acylated monolignols.

Although it is unclear how structural changes in acylated lignin will impact its utilization, acylation has been shown to affect its physicochemical properties and biomass recalcitrance<sup>[23,33,37]</sup>. Thus, the utilization of acylated lignin holds significant potential in various applications, including lignin separation, lignocellulosic biomass saccharification, lignin valorization, and the development of durable carbon-capture biomass. For example, overexpression of AsFMT in *Arabidopsis* and hybrid poplar (*Populus alba*  $\times$  *grandidentata*) resulted in 'Zip lignin' *via* the incorporation of monolignol FA conjugates into the lignin polymer backbone<sup>[33]</sup>. The chemically labile linkages in 'Zip lignin' increased the efficiency of lignin depolymerization and the digestibility of the cell wall biomass following a mild alkaline treatment<sup>[33,39]</sup>. Additionally, heterologous expression of PMT in *Arabidopsis* and poplar resulted in the incorporation of novel *p*CA groups into the

lignin of transgenic plants<sup>[40,41]</sup>. Similarly, overexpression of *PHBMT1* in poplars increased the *pBA* amount in the lignin of transgenic plants<sup>[37,38]</sup>. The pendent *pCA* and *pBA* groups are readily clipped off from lignin upon a mild alkaline treatment, therefore, they can serve as an alternate source for producing platform chemicals. Therefore, engineering acylated monolignols can be a promising strategy for lignin valorization.

The precise biological roles of acylated lignin as well as the biosynthetic pathway of those acylated monolignols are yet unclear, impeding the progress of acylated lignin engineering. A comprehensive understanding of the biosynthesis of acylated monolignols and identifying additional key enzymes engaged in these pathways may lead us to new strategies and tools to engineer lignin with enhanced lignocellulosic biomass utilization.

## Engineering homogeneous linear lignin

Heterogeneity has long been an issue restricting the separation and utilization of lignin. Classic lignin is a complex heteropolymer of two to three different monomers with a variety of inter-unit linkages that are highly dependent on the supply of the individual monolignols at the lignification sites. Two unconventional monomers caffeoyl alcohol and 5-hydroxyconiferyl alcohol containing *O*-diphenol groups form benzodioxane structures after a rapid intramolecular trap of quinone methide intermediates during lignification to generate homogeneous and linear all-ether-linked C-lignin and 5HG-type lignin polymers, respectively (Fig. 1c)<sup>[42–44]</sup>. The aforementioned polymers have several desirable characteristics that are absent in traditional lignin, thereby rendering them suitable for effective utilization of lignin. Firstly, lignin polymers containing benzodioxane structures do not form condensed units under acidic industrial lignin fractionation<sup>[45]</sup>, thus they are more stable than traditional lignin polymers, which makes lignin separation and utilization easier. Secondly, lignin homopolymer can only be degraded into a few products (a single monomer in a high proportion) *via* hydrogenolysis<sup>[46,47]</sup>, providing a promising strategy for the valorization of lignin. Thirdly, the high-purity linear lignin itself can serve as a good raw material to produce value-added biomaterials such as high-performance carbon fibers. It is reported that C-lignin-based carbon fiber outperformed conventional Kraft lignin carbon fiber in terms of thermal stability, molecular weight, diameter, and crystallinity<sup>[48]</sup>. Additionally, the benzodioxane linkages in lignin confer distinctive thermodynamic properties during copolymerization and the linear structure facilitates the formation of different types of crystals in the co-polymer, making C-lignin a promising candidate for the development of new polymeric materials<sup>[46]</sup>. Therefore, the unique characteristics of homogeneous linear lignin make it an 'ideal lignin' to facilitate lignocellulosic biomass utilization<sup>[45]</sup>.

Natural C-lignin was reported mainly in the seed coats of some orchids, cacti, *Euphorbiaceae* and *Cleome hassleriana*<sup>[42,43,47,49,50]</sup> and 5HG type lignin was detected in the seed coats of three species from *Escobaria*<sup>[43]</sup>. Recently, multiple approaches have been or are being considered to achieve a substantial yield of C-lignin in biomass crops, including poplar and alfalfa<sup>[51]</sup>.

Research on C-lignin biosynthesis in *C. hassleriana* has made great progress in providing potential targets and tools for

C-lignin genetic engineering. C-lignin started accumulating in the seed coat of *C. hassleriana* about 12–14 d after pollination<sup>[49,52]</sup>. The accumulation of C-lignin in *C. hassleriana* was accompanied by changes in the expression profiles of genes involved in the monolignol biosynthesis pathway<sup>[50]</sup>. For instance, downregulation of *CCoAOMT* and/or *COMT* inhibited the methylation of the phenolic hydroxyl group in monolignol which was long known to increase the accumulation of caffeoyl alcohol, a monolignol precursor for C-lignin<sup>[42,43]</sup>. Lignin from *Pinus radiata ccoaomt* mutant plants was reported to integrate a trace amount of caffeoyl alcohol<sup>[53]</sup>. The proportion of C-lignin in 10-day-old *comt ccoaomt* double mutant *Medicago truncatula* seedlings was more than four-fold (~1.8% of total monolignol units) higher than that in wild-type plants, however, the absolute amount of lignin in the double mutant was greatly reduced<sup>[51]</sup>. Thus, these enzymes are the primary targets for engineering C-lignin in different plants.

The concurrent down-regulation of *COMT* and *CCoAOMT* in the hairy roots of *M. truncatula* increased C-lignin accumulation in transgenic lines by ~15% of total lignin<sup>[51]</sup>. However, downregulation of *CCoAOMT* and/or *COMT* in *Arabidopsis*, alfalfa, and poplar exhibited an inability to accumulate C-lignin, impaired growth, and decreased total lignin content<sup>[54–56]</sup>, demonstrating that this strategy is not suitable for all plant species. The 5HG monomers were slightly increased in the stem of *Arabidopsis comt* mutant<sup>[56]</sup>, indicating that inhibition of *COMT* activity is critical for 5HG-type lignin biosynthesis. Nevertheless, overexpression of *F5H* in an *Arabidopsis comt* line increased the amount of 5-hydroxyconiferyl alcohol in lignin at the expense of plant fitness and total lignin content<sup>[57]</sup>.

Other key enzymes in the monolignol biosynthesis pathway that regulate caffeoyl alcohol and 5-hydroxyconiferyl alcohol *in planta* may be potential targets for homogeneous linear lignin engineering. For example, the decreased expression of *ChCAD4* and increased expression of *ChCAD5* during C-lignin deposition in the seed coat of *C. hassleriana* indicated that *ChCAD5* preferentially reduced caffealdehyde to caffeoyl alcohol while *ChCAD4* preferentially reduced coniferaldehyde to coniferyl alcohol<sup>[50]</sup>. Downregulation of *ChCAD5* reduced the C/G ratio of lignin in the *Cleome* seed coat, suggesting that *ChCAD5* was required for C-lignin biosynthesis<sup>[50]</sup>. Additionally, a seed coat-specific laccase from *C. hassleriana*, *ChLAC8*, exhibited the same temporal expression pattern as C-lignin accumulation and had a strong substrate preference for caffeoyl alcohol<sup>[58]</sup>. The increase of C-unit upon expression of *ChLAC8* in *comt* mutants of *M. truncatula* and *Arabidopsis* suggested that this enzyme promoted C-lignin formation *in planta*<sup>[58]</sup>. However, a systematic study on engineering C-lignin in *M. truncatula* hairy roots suggested that C-lignin accumulation required strong downregulation of both *COMT* and *CCoAOMT* instead of an expression of a heterologous *LAC*, *CAD*, or *CCR* with a preference for caffeoyl alcohol<sup>[51]</sup>.

Although it is promising to use genetic engineering for producing homogeneous linear polymers, existing strategies *via* interference of the key enzymes in the classic monolignol biosynthesis pathway result in low C-lignin accumulation, reduced lignin content, and stunted plant growth. Possible causes of these issues include a) an unstable supply of caffeoyl alcohol and/or 5-hydroxyconiferyl alcohol precursor which is partially due to rapid conversion of the highly reactive *O*-diphenol into other metabolites; b) blockage of the *O*-methylation

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process which may prevent the normal development of certain tissues, such as vascular tissue; and c) the disruption of metabolism homeostasis in plants arising from intricate interplay between the lignin pathway and other primary and secondary metabolic pathways. Understanding these limitations is essential for rationally designing strategies for homogeneous lignin engineering. Additionally, AI-assisted directed evolution of enzymes can be a breakthrough for the production of novel and specific tool enzymes and advances in homogeneous lignin engineering.

## Engineering low molecular weight lignin

Although there is no specific formula weight for lignin due to its heterogeneity, the average molecular weight of lignin can be estimated *via* several analytical techniques including GPC, MALDI-ToF MS, light scattering analysis, and NMR end-groups titration<sup>[59]</sup>. Additionally, the average degree of polymerization (DP) of lignin can be calculated by the monomeric composition and the relative abundance of lignin units<sup>[59]</sup>. Low molecular weight lignin is anticipated to be more easily extractable, leading to enhanced biomass processing. Moreover, the higher amount of free phenolics in lower molecular weight lignin can improve its antioxidant activity. For instance, the low molecular weight lignin showed stronger antioxidant activity when 15 different types of lignin were blended with polypropylene<sup>[60]</sup>. Another study evaluated 21 ethanol organosolv lignin samples as potential antioxidants and found that the lignin with more phenolic hydroxyl groups, fewer aliphatic hydroxyl groups, low molecular weight, and narrow polydispersity had high antioxidant activity<sup>[61]</sup>.

Lignin monomers with fewer radical coupling sites may inhibit lignin elongation during active lignin polymerization, resulting in short lignin. Several unconventional monomers including hydroxybenzaldehydes from engineered *Arabidopsis*<sup>[62]</sup>, dihydroconiferyl alcohol (Fig. 1d) and guaiacylpropane-1,3-diol in gymnosperms<sup>[63,64]</sup>, as well as tricin (Fig. 1e) in grass<sup>[65]</sup> are capable of only a single coupling reaction *via* their hydroxyl group located on fourth position in the benzene ring, suggesting that they may only serve as lignin initiators. Incorporation of such monomers into lignin can reduce its length and molecular weight. For instance, expression of the bacterial *hydroxycinnamoyl-CoA hydratase-lyase (HCHL)* gene, which could cleave the propanoid side-chain of a hydroxycinnamoyl-CoA lignin precursor to generate the corresponding hydroxybenzaldehydes, in the lignifying tissues of *Arabidopsis* inflorescence stem resulted in the accumulation of side-chain-shortened monomer, decreased lignin chain length, and increased saccharification yield of biomass<sup>[62]</sup>. Engineering low molecular-weight lignin is a promising strategy to increase lignocellulosic biomass processing efficiency as well as valorizing lignin. Therefore, the identification of additional potential genes will make a worthwhile contribution to engineering low molecular-weight lignin in the future.

## Engineering hydrophilic/hydrophobic lignin

Diverse functional groups with varying polarity impart hydrophobic or hydrophilic characteristics to complex lignin polymer. Hydrophobic lignin is essential for transporting water and nutrients in the plant vascular system. However, the solubility of lignin is largely affected by its hydrophobicity.

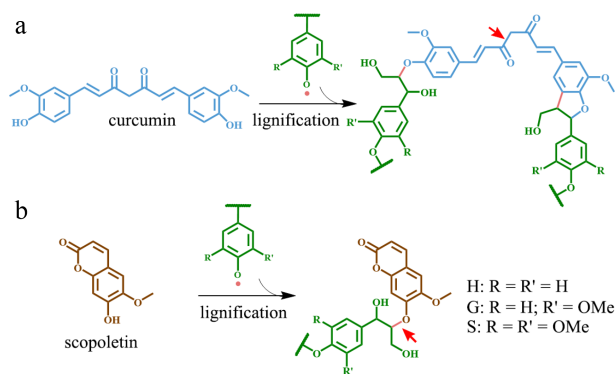
Increasing the hydrophilicity of lignin can render lignin more water soluble and reduce hydrophobic interactions between lignin and hemicelluloses, which may improve biomass saccharification yield<sup>[44]</sup>. Contrarily, increasing the hydrophobicity of lignin can inhibit the enzymatic hydrolysis of cellulose<sup>[66]</sup>, thereby enhancing the durability of lignocellulosic biomass during storage.

The number of hydroxyl and carboxylic groups in lignin significantly affects its hydrophobicity. Unlike aliphatic hydroxyl, phenolic hydroxyl can increase lignin hydrophobicity<sup>[32]</sup>. The addition of certain unconventional monomers into lignin can significantly alter the quantity and type of hydroxyl groups in lignin polymer. For example, monomers substituted with sugar moieties such as hydroxystilbene glycosides (Fig. 1f) found in the lignin of Norway spruce bark increased the aliphatic hydroxyl group<sup>[67,68]</sup>. In contrast, the esterification of the aliphatic hydroxyl group at the  $\gamma$ -position of traditional monolignols, including monolignol *p*-hydroxybenzoate conjugate and monolignol *p*-coumarate conjugate, introduced an additional free phenolic 4'-hydroxyl group into lignin. Therefore, incorporating these acylated monomers into the lignin polymer can boost its hydrophobicity. Interestingly, it was reported that the higher quantity of monolignol *p*-hydroxybenzoate conjugate in seagrass *Posidonia oceanica* might be associated with the higher organic carbon storage capacity of its ecosystem<sup>[23]</sup>.

Since the hydrophobicity of lignin is crucial for the normal growth of plants, future genetic engineering efforts to reduce the hydrophobicity of lignin must take full advantage of the natural unconventional monomers and focus on fine-tuning lignification in plants.

## Engineering alternative lignin

The artificially lignified maize cell walls system offered the potential to utilize a variety of phenolic compounds, including flavonoid derivatives, guaiacyl butenol, rosmarinic acid, caffeic acid derivatives, ferulic acid derivatives, coniferyl and sinapyl glucosides, and sinapoyl glucose as alternative monomers to form novel lignin *in vitro*<sup>[69–71]</sup>. Thus, it is conceivable to tailor the lignin structure *in planta* by substituting some fraction of the traditional monolignols with those alternative monomers through metabolic engineering to enhance lignocellulosic biomass utilization. Some chemically labile linkages can be introduced into lignin *via* alternative monomers, thereby significantly increasing the saccharification efficiency of engineered biomass. For instance, curcumin (diferuloylmethane) (Fig. 2a) was successfully incorporated into lignin polymer of *Arabidopsis* through simultaneous expression of *Curcuma longa*'s *Diketide-CoA synthase (DCS)* and *curcumin synthase 2 (CURS2)* driven by the secondary cell wall *CELLULOSE SYNTHASE A4 (CesA4)* promoter<sup>[72]</sup>. The engineered plants showed normal growth and their lignocellulosic biomass incorporating curcumin into lignin had a 14%–24% higher saccharification efficiency following alkaline pretreatment as compared with the wild type<sup>[72]</sup>. Likewise, a coumarin compound called 'scopoletin' (Fig. 2b) was also incorporated into the lignin polymer of *Arabidopsis* *via* simultaneous expression of *FERULOYL-CoA 6'-HYDROXYLASE 1 (F6'H1)* and *COUMARIN SYNTHASE (COSY)* driven by the *CesA4* promoter<sup>[73]</sup>. The transgenic events improved the saccharification efficiency of engineered lignocellulosic biomass by 40%



**Fig. 2** Phenolic metabolites functioning as alternative monomers in lignin polymer. (a) Curcumin and the resulting lignin structure. (b) Scopoletin and corresponding lignin structure. The red arrow indicates the alkali-labile linkage on lignin.

following alkaline pretreatment without affecting plant growth as compared to wild type plants<sup>[73]</sup>.

Understanding alternative monomers biosynthesis and metabolism in plants as well as careful design are prerequisites for the successful engineering of non-native lignin. Following the successful integration into *Arabidopsis* lignin polymers, curcumin was also produced and incorporated into poplar lignin *via* heterologous expression of both *DCS* and *CURS2* driven by the secondary cell wall *CELLULOSE SYNTHASE A8-B* promoter (*ProCesA8-B*). The transgenic trees exhibited shoot-tip necrosis and yield penalties without improving the saccharification yield<sup>[74]</sup>, demonstrating that phenolic metabolism differs among species and that the strategy and knowledge acquired in model plants must be re-evaluated before being applied to crops. Additionally, some non-native lignin may be discovered to exist as authentic lignin in some tissues of certain plants in the future. Therefore, deep insights into unconventional lignin biosynthesis and regulation *in planta* may guide us to rationally design and engineer alternative lignin in different crops.

## Conclusions and perspectives

Plant lignin structural engineering research is still in its infancy and often encounters multiple challenges. Perturbances in the structure and content of lignin often inhibit plant growth and development. Despite experimental success stories, there can be possible unexpected results for the production of engineered lignin in field-grown trees on a commercial scale. Additionally, chemical modifications in lignin structure can exert toxic effects on plant health and the ecosystem. Some other challenges associated with engineered lignin may include the limited biodegradability in soil by microorganisms, the unpredictable lifecycle of resulting bioproducts, and environment-related regulatory standards. Therefore, careful designing of lignin monomers and a precise fine-tuning of lignin structure are necessary to minimize its adverse effects on plant growth as well as the environment.

The plant biologists pursued novel approaches for tailoring lignin to improve lignocellulosic biomass utilization without drastically altering lignin functioning in plants. The type and quantity of lignin monomers define lignin structure and are critical factors for redesigning lignin. Despite the remarkable flexibility of the lignification process for diverse phenolics *in vitro*, only some of them were suitable for lignin modification *in*

*planta*<sup>[71]</sup>. Probably, we can let the plant's innate lignification mechanism choose the most suitable monomers for lignin engineering. Intriguingly, a vast number of phenolic compounds have been shown to behave as unconventional monomers that can integrate into lignin *via* radical coupling reactions. These native unconventional lignin monomers reshape the structure of lignin by offering a variety of functional groups and inter-unit linkages to lignin polymer. In the future, targeting these unconventional monomers may provide novel strategies to minimize the impacts of engineered lignin on plant development while improving the utilization efficiency of lignocellulosic biomass.

## Author contributions

The authors confirm their contribution to the paper as follows: study conception and design: Zhao Y; data collection: Zhao Y, Abid M, Xie X, Fu Y, Huang Y, Cai Z, Lin H; draft manuscript preparation: Zhao Y, Abid M, Xie X, Fu Y, Huang Y, Cai Z, Lin H. All authors reviewed the results and approved the final version of the manuscript.

## Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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## References

1. Studer MH, DeMartini JD, Davis MF, Sykes RW, Davison B, et al. 2011. Lignin content in natural *Populus* variants affects sugar release. *Proceedings of the National Academy of Sciences of the United States of America* 108:6300–5
2. Chen F, Dixon RA. 2007. Lignin modification improves fermentable sugar yields for biofuel production. *Nature Biotechnology* 25:759–61
3. Baucher M, Halpin C, Petit-Conil M, Boerjan W. 2003. Lignin: genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology* 38:305–50
4. Dixon RA, Barros J. 2019. Lignin biosynthesis: old roads revisited and new roads explored. *Open Biology* 9:190215
5. Vanholme R, De Meester B, Ralph J, Boerjan W. 2019. Lignin biosynthesis and its integration into metabolism. *Current Opinion in Biotechnology* 56:230–39

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6. del Río JC, Rencoret J, Gutiérrez A, Elder T, Kim H, et al. 2020. Lignin monomers from beyond the canonical monolignol biosynthetic pathway: Another brick in the wall. *ACS Sustainable Chemistry & Engineering* 8:4997–5012
7. del Río JC, Rencoret J, Gutiérrez A, Kim H, Ralph J. 2022. Chapter One - Unconventional lignin monomers—Extension of the lignin paradigm. *Advances in Botanical Research* 104:1–39
8. Perkins M, Smith RA, Samuels L. 2019. The transport of monomers during lignification in plants: anything goes but how? *Current Opinion in Biotechnology* 56:69–74
9. Boerjan W, Ralph J, Baucher M. 2003. Lignin biosynthesis. *Annual Review of Plant Biology* 54:519–46
10. Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W. 2010. Lignin biosynthesis and structure. *Plant Physiology* 153:895–905
11. Lewis NG, Yamamoto E. 1990. Lignin: occurrence, biogenesis and biodegradation. *Annual Review of Plant Physiology and Plant Molecular Biology* 41:455–96
12. Ma R, Zhang X, Wang Y, Zhang X. 2018. New insights toward quantitative relationships between lignin reactivity to monomers and their structural characteristics. *ChemSusChem* 11:2146–55
13. Ralph J, Lapierre C, Boerjan W. 2019. Lignin structure and its engineering. *Current Opinion in Biotechnology* 56:240–49
14. Ralph J. 2010. Hydroxycinnamates in lignification. *Phytochemistry Reviews* 9:65–83
15. Karlen SD, Free HCA, Padmakshan D, Smith BG, Ralph J, et al. 2018. Commelinid monocotyledon lignins are acylated by *p*-coumarate. *Plant Physiology* 177:513–21
16. Smith DCC. 1955. Ester groups in lignin. *Nature* 176:267–68
17. Yamamoto M, Tomiyama H, Koyama A, Okuizumi H, Liu S, et al. 2020. A century-old mystery unveiled: Sekizaisou is a natural lignin mutant. *Plant Physiology* 182:1821–28
18. Mottiar Y, Smith RA, Karlen SD, Ralph J, Mansfield SD. 2023. Evolution of *p*-coumaroylated lignin in eudicots provides new tools for cell wall engineering. *New Phytologist* 237:251–64
19. Smith DCC. 1955. *p*-Hydroxybenzoate groups in the lignin of Aspen (*Populus tremula*). *Journal of the Chemical Society* 3:2347–51
20. Landucci LL, Deka GC, Roy DN. 1992. A<sup>13</sup>C NMR study of milled wood lignins from hybrid *Salix* clones. *Holzforschung* 46:505–11
21. Lu FC, Ralph J, Morreel K, Messens E, Boerjan W. 2004. Preparation and relevance of a cross-coupling product between sinapyl alcohol and sinapyl *p*-hydroxybenzoate. *Organic & Biomolecular Chemistry* 2:2888–90
22. Rencoret J, Kim H, Evaristo AB, Gutiérrez A, Ralph J, et al. 2018. Variability in lignin composition and structure in cell walls of different parts of macaúba (*Acrocomia aculeata*) palm fruit. *Journal of Agricultural and Food Chemistry* 66:138–53
23. Kaal J, Serrano O, del Río JC, Rencoret J. 2018. Radically different lignin composition in *Posidonia* species may link to differences in organic carbon sequestration capacity. *Organic Geochemistry* 124:247–56
24. Rencoret J, Marques G, Serrano O, Kaal J, Martínez AT, et al. 2020. Deciphering the unique structure and acylation pattern of *Posidonia oceanica* lignin. *ACS Sustainable Chemistry & Engineering* 8:12521–33
25. Ralph J. 1996. An unusual lignin from kenaf. *Journal of Natural Products* 59:341–42
26. Karlen SD, Zhang C, Peck ML, Smith RA, Padmakshan D, et al. 2016. Monolignol ferulate conjugates are naturally incorporated into plant lignins. *Science Advances* 2:e1600393
27. Kim H, Li Q, Karlen SD, Smith RA, Shi R, et al. 2020. Monolignol benzoates incorporate into the lignin of transgenic *Populus trichocarpa* depleted in C3H and C4H. *ACS Sustainable Chemistry & Engineering* 8:3644–54
28. Karlen SD, Smith RA, Kim H, Padmakshan D, Bartuce A, et al. 2017. Highly decorated lignins in leaf tissues of the Canary Island date palm *Phoenix canariensis*. *Plant Physiology* 175:1058–67
29. del Río JC, Rencoret J, Marques G, Gutiérrez A, Ibarra D, et al. 2008. Highly acylated (acetylated and/or *p*-coumaroylated) native lignins from diverse herbaceous plants. *Journal of Agricultural and Food Chemistry* 56:9525–34
30. del Río JC, Rencoret J, Prinsen P, Martínez ÁT, Ralph J, et al. 2012. Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. *Journal of Agricultural and Food Chemistry* 60:5922–35
31. Hatfield RD, Marita JM, Frost K, Grabber J, Ralph J, et al. 2009. Grass lignin acylation: *p*-coumaroyl transferase activity and cell wall characteristics of C3 and C4 grasses. *Planta* 229:1253–67
32. Li M, Pu Y, Ragauskas AJ. 2016. Current understanding of the correlation of lignin structure with biomass recalcitrance. *Frontiers in Chemistry* 4:45
33. Wilkerson CG, Mansfield SD, Lu F, Withers S, Park JY, et al. 2014. Monolignol ferulate transferase introduces chemically labile linkages into the lignin backbone. *Science* 344:90–93
34. Withers S, Lu F, Kim H, Zhu Y, Ralph J, et al. 2012. Identification of grass-specific enzyme that acylates monolignols with *p*-coumarate. *Journal of Biological Chemistry* 287:8347–55
35. Petrik DL, Karlen SD, Cass CL, Padmakshan D, Lu F, et al. 2014. *p*-coumaroyl-CoA: monolignol transferase (PMT) acts specifically in the lignin biosynthetic pathway in *Brachypodium distachyon*. *The Plant Journal* 77:713–26
36. Marita JM, Hatfield RD, Rancour DM, Frost KE. 2014. Identification and suppression of the *p*-coumaroyl CoA:hydroxycinnamyl alcohol transferase in *Zea mays* L. *The Plant Journal* 78:850–64
37. Zhao Y, Yu X, Lam PY, Zhang K, Tobimatsu Y, et al. 2021. Monolignol acyltransferase for lignin *p*-hydroxybenzoylation in *Populus*. *Nature Plants* 7:1288–300
38. de Vries L, MacKay HA, Smith RA, Mottiar Y, Karlen SD, et al. 2022. pHBM1, a BAHD-family monolignol acyltransferase, mediates lignin acylation in poplar. *Plant Physiology* 188:1014–27
39. Kim KH, Dutta T, Ralph J, Mansfield SD, Simmons BA, et al. 2017. Impact of lignin polymer backbone esters on ionic liquid pretreatment of poplar. *Biotechnology for Biofuels* 10:101
40. Smith RA, Gonzales-Vigil E, Karlen SD, Park JY, Lu F, et al. 2015. Engineering monolignol *p*-coumarate conjugates into poplar and Arabidopsis lignins. *Plant Physiology* 169:2992–3001
41. Sibout R, Le Bris P, Legée F, Cézard L, Renault H, et al. 2016. Structural redesigning Arabidopsis lignins into alkali-soluble lignins through the expression of *p*-coumaroyl-CoA:Monolignol Transferase PMT. *Plant Physiology* 170:1358–66
42. Chen F, Tobimatsu Y, Havkin-Frenkel D, Dixon RA, Ralph J. 2012. A polymer of caffeyl alcohol in plant seeds. *Proceedings of the National Academy of Sciences of the United States of America* 109:1772–77
43. Chen F, Tobimatsu Y, Jackson L, Nakashima J, Ralph J, et al. 2013. Novel seed coat lignins in the Cactaceae: structure, distribution and implications for the evolution of lignin diversity. *The Plant Journal* 73:201–11
44. Mottiar Y, Vanholme R, Boerjan W, Ralph J, Mansfield SD. 2016. Designer lignins: harnessing the plasticity of lignification. *Current Opinion in Biotechnology* 37:190–200
45. Li Y, Li S, Kim H, Motagamwala AH, Mobley JK, et al. 2018. An "ideal lignin" facilitates full biomass utilization. *Science Advances* 4:eaa2968
46. Li Y, Meng X, Meng R, Cai T, Pu Y, et al. 2023. Valorization of homogeneous linear catechyl lignin: opportunities and challenges. *RSC Advances* 13:12750–59
47. Wang S, Zhang K, Li H, Xiao L, Song G. 2021. Selective hydrogenolysis of catechyl lignin into propenylcatechol over an atomically dispersed ruthenium catalyst. *Nature Communications* 12:416
48. Nar M, Rizvi HR, Dixon RA, Chen F, Kovalcik A, et al. 2016. Superior plant based carbon fibers from electrospun poly-(caffeyl alcohol) lignin. *Carbon* 103:372–83

49. Tobimatsu Y, Chen F, Nakashima J, Escamilla-Treviño LL, Jackson L, et al. 2013. Coexistence but independent biosynthesis of catechyl and guaiacyl/syringyl lignin polymers in seed coats. *The Plant Cell* 25:2587–600
50. Zhuo C, Rao X, Azad R, Pandey R, Xiao X, et al. 2019. Enzymatic basis for C-lignin monomer biosynthesis in the seed coat of *Cleome hassleriana*. *The Plant Journal* 99:506–20
51. Ha C, Escamilla-Trevino L, Zhuo C, Pu Y, Bryant N, et al. 2023. Systematic approaches to C-lignin engineering in *Medicago truncatula*. *Biotechnology for Biofuels and Bioproducts* 16:100
52. Zhuo C, Wang X, Docampo-Palacios M, Sanders BC, Engle NL, et al. 2022. Developmental changes in lignin composition are driven by both monolignol supply and laccase specificity. *Science Advances* 8:abm8145
53. Wagner A, Tobimatsu Y, Phillips L, Flint H, Torr K, et al. 2011. CCoAOMT suppression modifies lignin composition in *Pinus radiata*. *The Plant Journal* 67:119–29
54. Meyermans H, Morreel K, Lapierre C, Pollet B, De Bruyn A, et al. 2000. Modifications in lignin and accumulation of phenolic glucosides in poplar xylem upon down-regulation of caffeoyl-coenzyme A O-methyltransferase, an enzyme involved in lignin biosynthesis. *Journal of Biological Chemistry* 275:36899–909
55. Marita JM, Ralph J, Hatfield RD, Guo D, Chen F, et al. 2003. Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3-O-methyltransferase and caffeoyl coenzyme A 3-O-methyltransferase. *Phytochemistry* 62:53–65
56. Do CT, Pollet B, Thévenin J, Sibout R, Denoue D, et al. 2007. Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in *Arabidopsis*. *Planta* 226:1117–29
57. Weng J, Mo H, Chapple C. 2010. Over-expression of F5H in COMT-deficient *Arabidopsis* leads to enrichment of an unusual lignin and disruption of pollen wall formation. *The Plant Journal* 64:898–911
58. Wang X, Zhuo C, Xiao X, Wang X, Docampo-Palacios M, et al. 2020. Substrate specificity of LACCASE8 facilitates polymerization of caffeoyl alcohol for C-lignin biosynthesis in the seed coat of *Cleome hassleriana*. *The Plant Cell* 32:3825–45
59. Crestini C, Melone F, Sette M, Saladino R. 2011. Milled wood lignin: a linear oligomer. *Biomacromolecules* 12:3928–35
60. Pouteau C, Dole P, Cathala B, Averous L, Boquillon N. 2003. Antioxidant properties of lignin in polypropylene. *Polymer Degradation and Stability* 81:9–18
61. Pan X, Kadla JF, Ehara K, Gilkes N, Saddler JN. 2006. Organosolv ethanol lignin from hybrid poplar as a radical scavenger: relationship between lignin structure, extraction conditions, and antioxidant activity. *Journal of Agricultural and Food Chemistry* 54:5806–13
62. Eudes A, George A, Mukerjee P, Kim JS, Pollet B, et al. 2012. Biosynthesis and incorporation of side-chain-truncated lignin monomers to reduce lignin polymerization and enhance saccharification. *Plant Biotechnology Journal* 10:609–20
63. Ralph J, MacKay JJ, Hatfield RD, O'Malley DM, Whetten RW, et al. 1997. Abnormal lignin in a loblolly pine mutant. *Science* 277:235–39
64. Ralph J, Lapierre C, Marita JM, Kim H, Lu F, et al. 2001. Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochemistry* 57:993–1003
65. Lan W, Lu F, Regner M, Zhu Y, Rencoret J, et al. 2015. Tricin, a flavonoid monomer in monocot lignification. *Plant Physiology* 167:1284–65
66. Yang Q, Pan X. 2016. Correlation between lignin physicochemical properties and inhibition to enzymatic hydrolysis of cellulose. *Biotechnology and bioengineering* 113:1213–24
67. Neiva DM, Rencoret J, Marques G, Gutierrez A, Gominho J, et al. 2020. Lignin from tree barks: chemical structure and valorization. *ChemSusChem* 13:4537–47
68. Rencoret J, Neiva D, Marques G, Gutiérrez A, Kim H, et al. 2019. Hydroxystilbene glucosides are incorporated into Norway spruce bark lignin. *Plant Physiology* 180:1310–21
69. Grabber JH, Schatz PF, Kim H, Lu F, Ralph J. 2010. Identifying new lignin bioengineering targets: 1. Monolignol-substitute impacts on lignin formation and cell wall fermentability. *BMC Plant Biology* 10:114
70. Grabber JH, Ress D, Ralph J. 2012. Identifying new lignin bioengineering targets: impact of epicatechin, quercetin glycoside, and gallate derivatives on the lignification and fermentation of maize cell walls. *Journal of Agricultural and Food Chemistry* 60:5152–60
71. Grabber JH, Davidson C, Tobimatsu Y, Kim H, Lu F, et al. 2019. Structural features of alternative lignin monomers associated with improved digestibility of artificially lignified maize cell walls. *Plant Science* 287:110070
72. Oyarce P, De Meester B, Fonseca F, de Vries L, Goeminne G, et al. 2019. Introducing curcumin biosynthesis in *Arabidopsis* enhances lignocellulosic biomass processing. *Nature Plants* 5:225–37
73. Hoengenaert L, Wouters M, Kim H, De Meester B, Morreel K, et al. 2022. Overexpression of the scopoletin biosynthetic pathway enhances lignocellulosic biomass processing. *Science Advances* 8:eabo5738
74. De Meester B, Oyarce P, Vanholme R, Van Acker R, Tsuji Y, et al. 2022. Engineering curcumin biosynthesis in poplar affects lignification and biomass yield. *Frontiers in Plant Science* 13:943349



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