Lignins

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Lignins, complex and irregular polymers present in the cell walls of vascular plants, are built from three basic monolignols. An understanding of their nature is evolving as a result of detailed structural investigations aided by improvements in analytical methodologies and the availability of mutant and transgenic plants. Oxidative phenolic coupling reactions, where monomers primarily couple endwise with the growing chain, generate the polymer. The combinatorial linkage synthesis, the random generation of new optical centres each time a monolignol couples via its sidechain, and the inclusion of monomers other than the monolignols, cascade to create polymers with enormous variation in primary structure. Lignification is a strategic process that has evolved to allow plants considerable flexibility in dealing with various environmental stresses. The malleability offers significant opportunities to engineer the structures of lignins beyond the limits explored to date.

Introduction

Lignins are abundant plant biopolymers accounting for approximately 30% of the organic carbon in the biosphere. The ability to synthesize lignins has been essential in the evolutionary adaptation of plants from an aquatic environment to land. Lignins are crucial for structural integrity of the cell wall and stiffness and strength of the stem and root. In addition, lignins waterproof the wall enabling transport of water and solutes through the vascular system, and they play a role in protecting plants against pathogens.

Because of the essential role of lignin for plant life and its relevance for a number of agro-industrial processes, the biosynthetic pathway leading to the monolignols, their polymerization and the final structure of the lignin polymer have been intensively studied over many decades. Here we summarize aspects of the current status with a focus on lignin structure.

Monolignol Biosynthesis

Lignins derive principally from three hydroxycinnamyl alcohol monomers, **M1,** differing in their degree of methoxylation, *p*–coumaryl **M1H**, coniferyl **M1G** and sinapyl **M1S** alcohols, **Figure 1** (and later in **Figure 3**); the monomer **M** labelling nomenclature is taken from Boerjan *et al.* (2003) and is explained in the **Figure 3** caption. Biosynthesis of the monolignols begins with the deamination of phenylalanine and involves successive hydroxylation reactions of the aromatic ring, phenolic *O*-methylation, and conversion of the side-chain carboxyl group to an alcohol. The metabolic grid shown in **Figure 1** (Boerjan *et al.*, 2003) includes most of the possible enzymatic conversions in the phenylpropanoid pathway that have been shown by *in vitro* experiments. Hydroxylation and methylation reactions were originally thought to occur at the level of the cinnamic acids, which were subsequently converted to the corresponding monolignols by the sequential action of 4-coumarate: CoA ligase (4CL), cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). However, *in vitro* enzymatic assays with heterologously produced enzymes, the identification of novel genes implicated in the pathway, and analyses of mutant and transgenic plants modified in monolignol biosynthesis have redefined the preferred pathways to those highlighted in **Figure 1**.

For most of the enzymes there exist multiple isoforms that are differentially expressed during development and upon environmental cues. These may have different kinetics and substrate preferences. Certain paths in the grid are, therefore, expected to be kinetically favoured in given cell types or environmental conditions, allowing for metabolic flexibility.

Transport of Monolignols

After their synthesis, the lignin precursors are transported to the cell wall where they are oxidized and polymerized. In gymnosperms and some angiosperms, monolignol 4–*O*–β–D-glucosides accumulate to high levels in the cambial tissues. It has been hypothesized that these monolignol glucosides are storage or transport forms of the monolignols and that a coniferyl alcohol glucosyl transferase, together with coniferin-β-glucosidase, may regulate storage and mobilization of monolignols. The transport route of the monolignols to the cell wall remains under investigation.

Dehydrogenation

After transport of the monolignols to the cell wall, lignin is formed through dehydrogenative polymerization of the monolignols. Monolignol dehydrogenation to radicals has

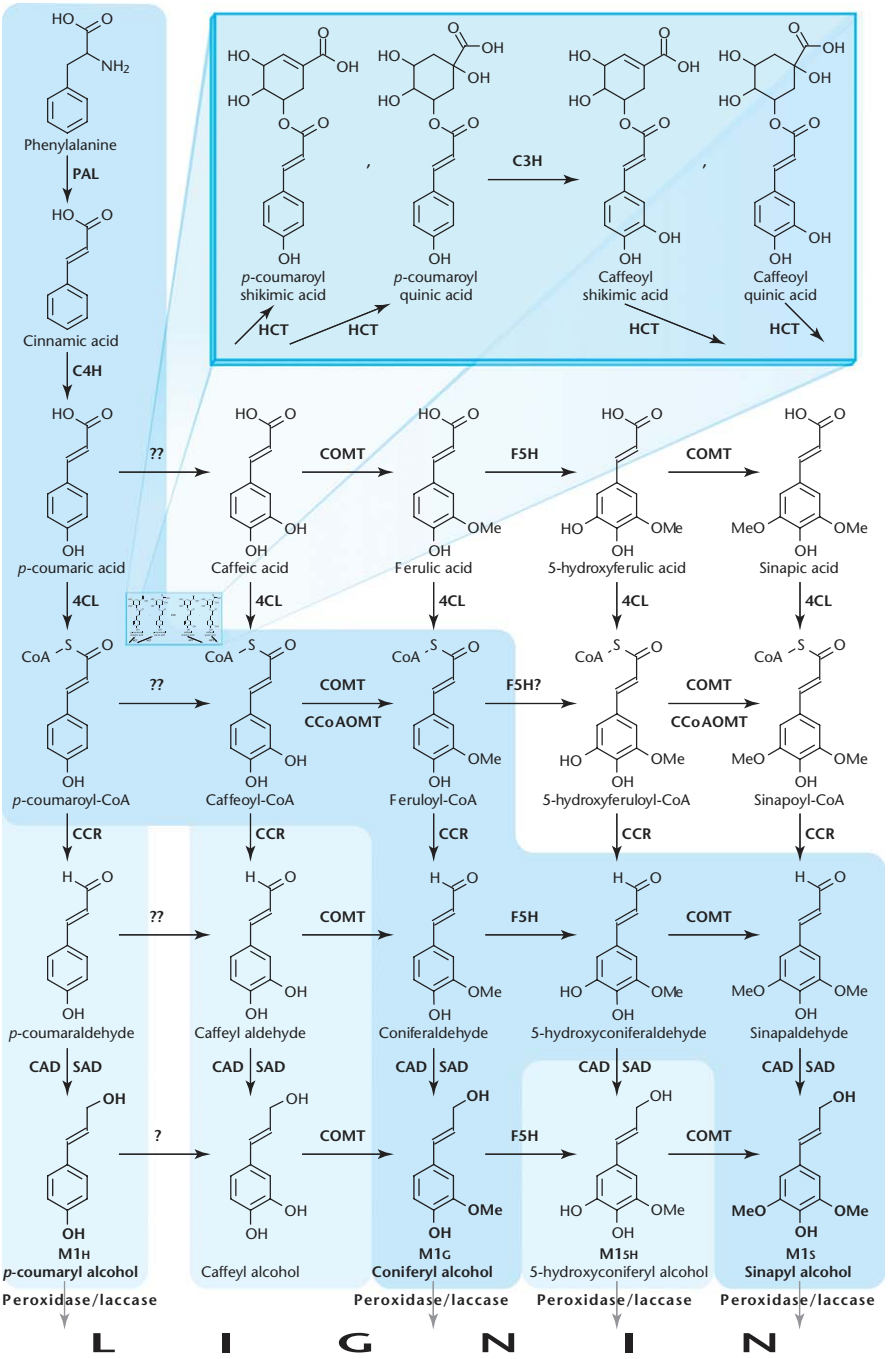


Figure 1 Phenylpropanoid and monolignol biosynthetic pathways. The blue-highlighted route towards the production of monolignols is considered to be most favoured in angiosperms. The lighter blue routes also occur, depending on the species and conditions. Nonhighlighted pathways do not play a significant role. CAD, cinnamyl alcohol dehydrogenase; 4CL, 4-coumarate:CoA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; HCT, *p*-hydroxycinnamoyl-CoA:D-quinate or shikimate *p*-hydroxycinnamoyltransferase; F5H, ferulate 5-hydroxylase; PAL, phenylalanine ammonia-lyase; SAD, sinapyl alcohol dehydrogenase. ?, conversion demonstrated; ??, direct conversion not convincingly demonstrated; F5H?, substrate not tested; others, enzymatic activity shown *in vitro*. Other pathways continue to be revealed. Modified from Boerjan (2003).

been ascribed to different classes of proteins, but peroxidases and laccases are the most highly implicated. Peroxidases use hydrogen peroxide (H_2O_2) and laccases use oxygen to oxidize their substrates. How H_2O_2 is generated in the cell wall is still a matter of debate, but evidence is emerging for a role for a nicotinamide–adenine dinucleotide phosphate (NADPH) oxidase in lignifying tissues. Several peroxidases have been colocalized in time and

space with lignification, through transcript-profiling experiments of *Zinnia elegans* cells differentiating in tracheary elements, and in developing poplar xylem. There is some evidence for the involvement of specific peroxidase isozymes in lignin polymerization. Similarly, several laccases have been colocalized with lignification and at least for one *Arabidopsis* laccase, AtLAC15, a role in lignification in the seed coat has been demonstrated by reverse genetics.

Lignification

Radical generation and radical coupling

After monolignol dehydrogenation, the phenolic radicals, which are relatively stable owing to electron delocalization that provides single-electron density to sites around the aromatic ring and to the conjugated side-chain β-position, are coupled. The most important reaction is cross-coupling

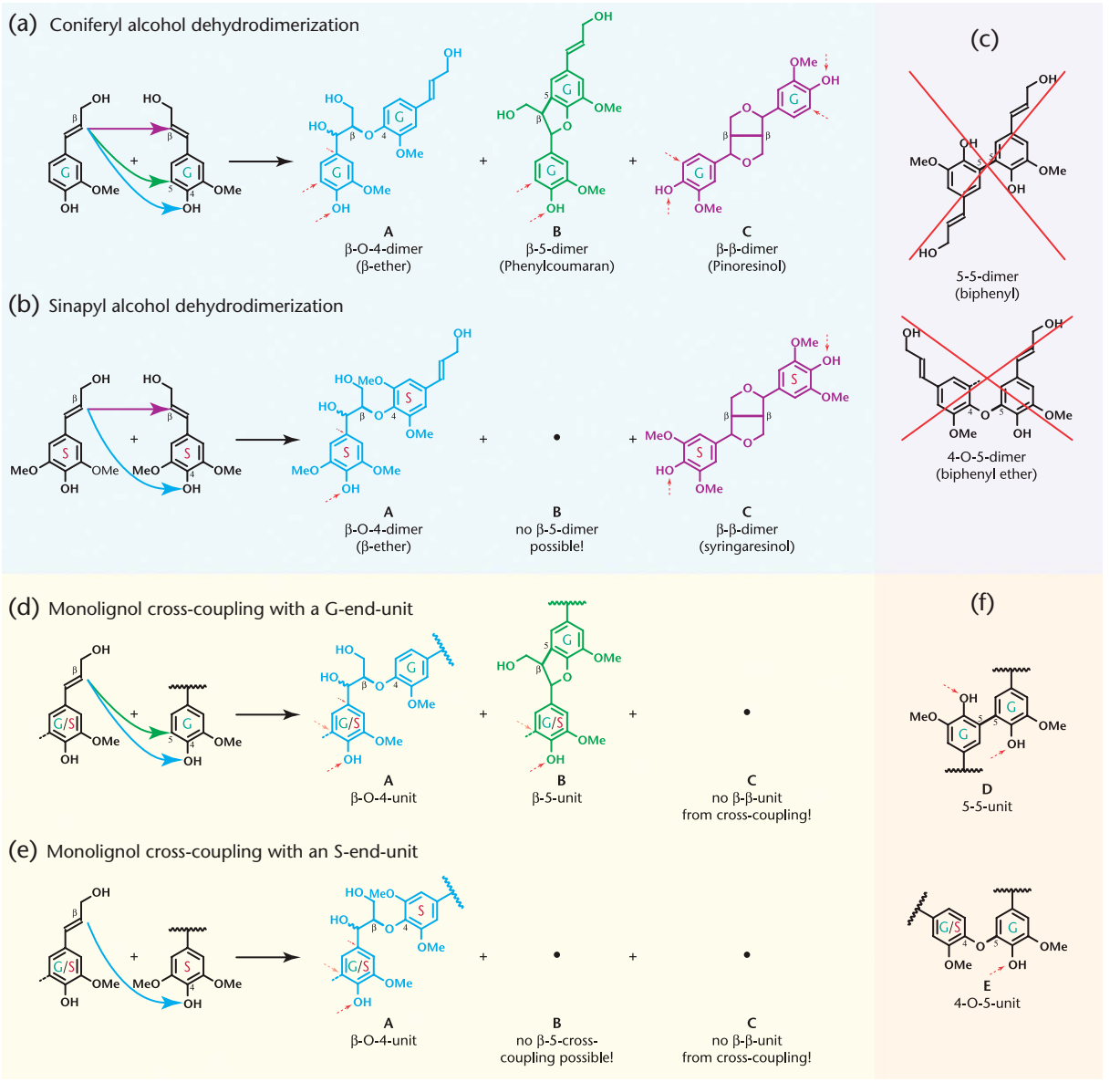
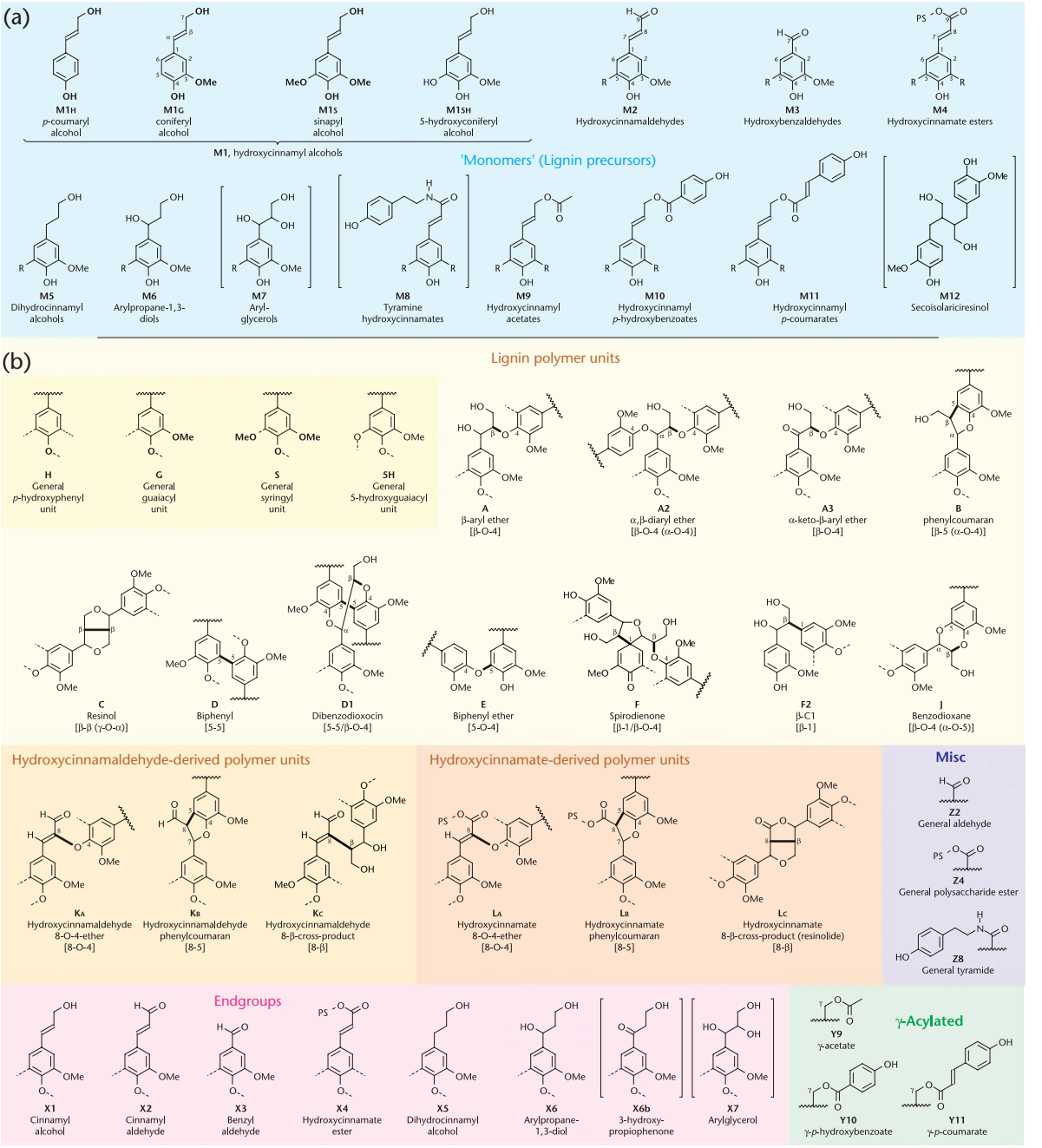


Figure 2 Lignification differs substantially from dimerization of monolignols. (a) Dehydrodimerization of coniferyl alcohol produces three dehydrodimers in comparable amounts. The new bond formed by the radical coupling reaction is drawn bolder. (b) Dehydrodimerization of sinapyl alcohol produces only two products. (c) Dehydrodimerization does not produce these structures. (d) Cross-coupling of a hydroxycinnamyl alcohol with a **G** unit gives only two main products. (e) Cross-coupling of a hydroxycinnamyl alcohol with an **S** unit leads almost exclusively to β-ether units **A**. When the polymer phenolic end unit is a β-ether, β–1-coupling may also occur to a relatively minor extent. (f) Coupling of preformed oligomers is the source of the 5–5- and 4–*O*–5 units. Red arrows indicate sites at which further radical coupling can occur during lignification; the lighter arrow to the 5-positions in (d) and (e) structures shows where coupling can occur in **G** units but not in **S** units (where the 5-position is occupied by a methoxyl group).

to the growing polymer to extend the complex three-dimensional lignin network, **Figure 2d** and **e**. But, such coupling reactions are radical quenching. Each extension of the polymer requires new radicals on each of the two coupling partners. Radicals on the growing lignin polymer may be formed directly via interaction with peroxidases. They may alternatively be generated by radical transfer from monolignols or other intermediaries. For example, sinapyl alcohol is far more readily oxidized in the presence of *p*-coumarates, which are rapidly oxidized by peroxidases. Radical transfer produces the more stable sinapyl alcohol radical. Similar radical transfer mechanisms can be envisioned between the monolignols and the growing polymer, i.e. the monolignols may act as the radical shuttles to oxidize the polymer. Alternatively, redox shuttles may be involved.

Nucleation sites

Lignins are first deposited in the middle lamella and the cell corners of the primary wall after the formation of the



secondary wall has started, possibly at so-called nucleation sites from which the lignin polymers can grow. The nature of these nucleation sites remains speculative. Ferulates **M4G**, **Figure 3a**, conjugated to polysaccharides, and their dehydrodimers are well established as being incorporated into grass lignins. Evidence that they may act as attachment sites for monolignols comes from the observation of 8–β-cross-coupled structures **LC**, **Figure 3b**, that arise by cross-coupling of a monolignol with ferulate. Structural cell-wall proteins rich in aromatic residues may have a similar function. Given that the middle lamella and the cell corners are rich in calcium pectate and are the first sites to be lignified, pectate-bound peroxidases may conceivably play a role in the spatial control of lignin deposition.

Dehydrodimerization

Upon dehydrogenation and radical formation, the monolignol radical will either dimerize – a relatively minor event – or will couple to the growing polymer. Dehydrodimerization results in up to three linkage types. For coniferyl alcohol, **Figure 2a**, these are via β–*O*–4-, β–5-, or β–β-coupling, producing β-ether units **A**, phenylcoumarans **B** or resinols **C**. The 5–5- and 4–*O*–5-dehydrodimers shown in many texts (and crossed out in **Figure 2c**) do not arise in any significant way from monolignol dehydrodimerization reactions; monolignol coupling involves the β-position of at least one of the monolignols. For sinapyl alcohol, **Figure 2b**, only two dehydrodimers are formed, with the resinol **C** dominating by as much as 95% under typical biomimetic conditions; β–5-coupling is not possible when the 5-position is methoxyl-substituted. Subject to simple chemical compatibility, cross-coupling between monolignols can also occur giving mixed G/S analogues (not shown). Dehydrodimerization reactions produce one of two key signatures in the polymer, a resinol unit **C** (in the case of β–β-coupling) or a cinnamyl endgroup **X1** (in the case of β–*O*–4- or β–5-dehydrodimerization).

Polymerization process

When higher order polymers are made in the lignification process, the growth of the polymer takes place by formation of bonds between monolignol radicals (coupling invariably at their β-positions) and phenoxy radicals on the polymer (coupling at their 4–*O*- or 5-positions) leading to intermediate quinone methides. These are stabilized by nucleophilic addition reactions yielding the products shown in **Figures 2d** and **e** and the lignin polymer structures in **Figure 3b**. Since the monolignol couples exclusively at its β-position, β–*O*–4-coupling with a guaiacyl or a syringyl phenolic end-unit yields β-ether structures **A** whereas β–5-coupling, only with a guaiacyl unit, can yield phenylcoumaran structures **B**. Coupling reactions of a monolignol with a syringyl end-unit, therefore, result almost solely in β-ether units **A** (**Figure 2e**). The limited range of coupling products explains why the β-ether frequency is much higher in lignins than would be predicted from examining the dehydrodimers, dispelling an argument that was recently advanced against combinatorial chemical coupling. Coupling between preformed lignin oligomers results in units linked 5–5 **D** and 4–*O*–5 **E**, both potential branch-points in the polymer chain.

Much of what is known of the radical coupling process and the parameters that determine the frequency of interunit bonds and the structure of lignin has been obtained via synthetic dehydrogenation polymers (DHPs). Lignin-like polymers can be artificially synthesized *in vitro* by dehydropolymerization of lignin precursors using peroxidase/H_2O_2 as the oxidizing agent. DHPs formed by adding lignin precursors slowly and continuously to a solution containing H_2O_2 and peroxidase create 'endwise polymers' that structurally resemble isolated plant lignins more closely than 'bulk polymers', which are formed by adding the precursors in a single

Figure 3 Lignin precursors, and structures in the polymer (Boerjan *et al.*, 2003). (a) 'Monomers' (Lignin Precursors): Lignins derive primarily from the three monolignols **M1**, namely **M1H**, **M1G** and **M1S** (where the subscripts indicate the type of aromatic nucleus, *p*-hydroxyphenyl, guaiacyl or syringyl, resulting from incorporation of the monomer). **$M1_{5H}$** is a monomer in COMT-deficient plants resulting in 5-hydroxyguaiacyl units in the form of benzodioxanes **J** in the polymer. Other precursors **M2**–**M12** incorporate into lignins in varying degrees. Bracketed compounds have not been firmly established as authentic monomers or, in the case of **M12**, are of unknown derivation. (b) Lignin Polymer Units: Units are generally denoted based on the methoxyl substitution on the aromatic ring as **H**, **G**, **S** (and **5H**); dashed bonds represent other potential attachments (via coupling reactions). The most common structures in lignins from normal and transgenic plants are shown as structures **A**–**L** with the bond formed during the radical coupling step; *p*-hydroxyphenyl units are not shown. The dashed bonds indicate substitutions by methoxyl (in syringyl components) or other attachments from coupling reactions; generic side-chains are shown truncated (zigzag lines). Most units arise from cross-coupling reactions of a monomer with the growing polymer or by polymer–polymer coupling reactions. Resinol units **C** are from monolignol–monolignol coupling (followed by further cross-coupling reactions). Most 5–5-linked units **D** are in the form of dibenzodioxocins **D1**. *bis*-Aryl ether units **A2** are rare in most lignins, but relatively prevalent in tobacco. Units **F**, β–1-structures occur mainly as spirodienones, but may partially cleave to give units **F2**. Benzodioxanes **J** result from the incorporation of 5-hydroxyconiferyl alcohol **$M1_{5H}$** monomers (see **Figure 5**). Units **K** (from coupling of hydroxycinnamaldehydes **M2**) are prevalent in CAD-deficient angiosperms. Units **L** are from ferulate incorporation in grass lignins, for example. Note that hydroxycinnamates and ferulates typically have their side-chain carbons labelled 7–9, whereas the hydroxycinnamyl alcohols are labelled α, β and γ. Endgroups arise from coupling reactions that are not at the side-chain β-position. Hydroxycinnamyl endgroups **X1** arise from dimerization reactions. Endgroups **X2**–**X6** derive from the corresponding monomers **M2**–**M6**; **X6b** may result from oxidation of **X6** units. Glycerols **X7** may be from monomers **M7** or may be produced during ball milling from β-ether units **A**. Any of the units **A**–**L** bearing a γ-OH may also bear an acyl group, partial structures **Y9**–**Y11**, and arise from the corresponding monolignols **M9**–**M11**. Finally, some other groups resulting from incorporation reactions are not accommodated by the other structures. Partial structures **Z8** are from incorporation of monomer **M8**; general aldehydes **Z2** are from hydroxycinnamaldehyde **M2** or hydroxybenzaldehyde monomers **M3** and include structures **K**; general esters **Z4** result from the incorporation of hydroxycinnamates **M4** and their dehydrodimers and include structures **L**. Modified from Boerjan (2003).

batch. Such DHP experiments have indicated that lignin structure depends on the supply rate of the monomers, the rate of radical generation, the presence of polysaccharides in the DHP mix and the presence of the growing lignin polymer.

As originally presented by Karl Freudenberg (Freudenberg and Neish, 1968), the actual process of lignification occurs without the biochemical controls seen in the biosynthesis of the precursor monolignols, giving rise to a class of racemic polymers with no defined primary sequence. Alternative schemes, based on ideas of controlled coupling by proteins harbouring arrays of dirigent sites and template replication, are not supported by any experimental evidence. Polymer models have been developed (Boerjan *et al*., 2003; Brunow, 2001) that attempt to account for average structural features in various lignins. For example, models of a softwood and a hardwood lignin are shown in **Figure 4**. It is important to appreciate that such models in no way attempt to define any primary structure or sequence since lignins themselves have no such primary structure. Each of these models, as drawn, has enormous numbers of possible isomers. The softwood model in **Figure 4a** has 46 optical centres. However, the relative stereochemistries of pairs of centres in ring structures (phenylcoumarans **B**, resinols **C** and dibenzodioxocins **D**) are fixed. We, therefore, insinuate that there are 2^{34} (over 17 billion) physically distinct isomers for the structure shown. For the poplar model in **Figure 4b**, we calculate that there are 2^{33} (over 8.5 billion) physically distinct isomers. Since lignins are not optically active, the optical centres must be generated randomly, i.e. under simple chemical control. Variations in the sequences of units produce many more possible isomers.

Lignin Composition and Structure

General aspects

The amount and composition of lignins vary among taxa, cell types and individual cell-wall layers and are influenced by developmental and environmental cues. Although exceptions exist, dicotyledonous angiosperm (hardwood) lignins consist principally of **G** and **S** units and traces of **H** units, **Figure 3b**, whereas gymnosperm (softwood) lignins are composed mostly of **G** units with low levels of **H** units. Lignins from grasses (monocots) incorporate **G** and **S** units at comparable levels, and more **H** units than dicots. **H** units, derived from the incorporation of the monolignol *p*-coumaryl alcohol **M1H** into lignins, should not be confused with *p*-coumarate esters **Y11,** which appear as pendant groups acylating grass lignins.

As explained above, the most frequent lignin inter-unit linkage is the β–*O*–4- (β-aryl ether) linkage **A**, **Figure 3b**, as can also be seen in the nuclear magnetic resonance (NMR) spectra of poplar lignins, **Figure 5a**. It is also the one most easily cleaved chemically, providing a basis for industrial processes and several analytical methods. The other linkages **B**–**F**, **Figure 3b**, are all more resistant to chemical degradation. The relative abundance of the different linkages depends largely on the relative contribution of a particular monomer to the polymerization process. For example, because of the availability of the C_5 position for coupling, lignins composed mainly of **G** units contain more resistant (β–5 **B**, 5–5 **D**, and 4–*O*–5 **E**) linkages than lignins incorporating **S** units. Lignins containing more significant levels of **H** units (from *p*-coumaryl alcohol **M1H**) are found in softwood compression wood zones and various plants deficient in C3H and shikimate *p*-hydroxycinnamoyltransferase (HCT) have elevated H unit levels (Ralph *et al*., 2006).

A broader definition of lignins

Lignins may be derived from several other monomers besides just the three monolignols **M1** (bold in **Figure 3a**). Many plants contain lignins substantially derived from other monomers, and all lignins contain traces of units from apparently incomplete monolignol biosynthesis and other pathway reactions. Many of these units have been recently identified by their more substantial incorporation into lignins in transgenic and mutant plants that are perturbed in the monolignol pathway.

Variously acylated lignin units **Y** can be found across the plant kingdom. Acetates **Y9** in palms and kenaf, as well as the low levels in many hardwoods, *p*-hydroxybenzoates **Y10** in poplars, palms and willows, and *p*-coumarates **Y11** in all grasses all derived from acylated monolignols **M9**–**M11**. Because acylated components can comprise a significant fraction of the polymer (over 50% of kenaf bast fibre lignin units are acetylated, for example), acylated monolignols should be considered to be authentic lignin precursors.

Polysaccharide ferulate esters **M4G** (and their dehydrodimers that derive from radical coupling reactions resulting in important polysaccharide–polysaccharide cross-linking) are also incorporated into lignins, particularly in grasses where they appear to function as nucleation sites for lignin polymerization. Cross-coupled structures **L** (**Figure 3b**) result in lignins. The wound-response product tyramine ferulate **M8G** (and possibly other hydroxycinnamate analogues) is integrally polymerized into polymer fractions in normal tobacco. Its incorporation level is particularly enhanced in CCR-deficient transgenic tobacco.

Dihydroconiferyl **X5G** and guaiacylpropane-1,3-diol **X6G** units are readily detected in gymnosperm lignins, suggesting that the monomer **M5G** (DHCA) is always produced with coniferyl alcohol **M1G** in gymnosperms; the monomer guaiacylpropane-1,3-diol **M6G** is produced from DHCA under peroxidase/H_2O_2 conditions. DHCA-derived units **X5G** and **X6G** are major components of the lignin in a CAD-deficient pine mutant where about half of the DHCA units are involved in 5–5-coupled structures **D**. Another benzyl-reduced structure in lignins has been shown to derive from incorporation of preformed

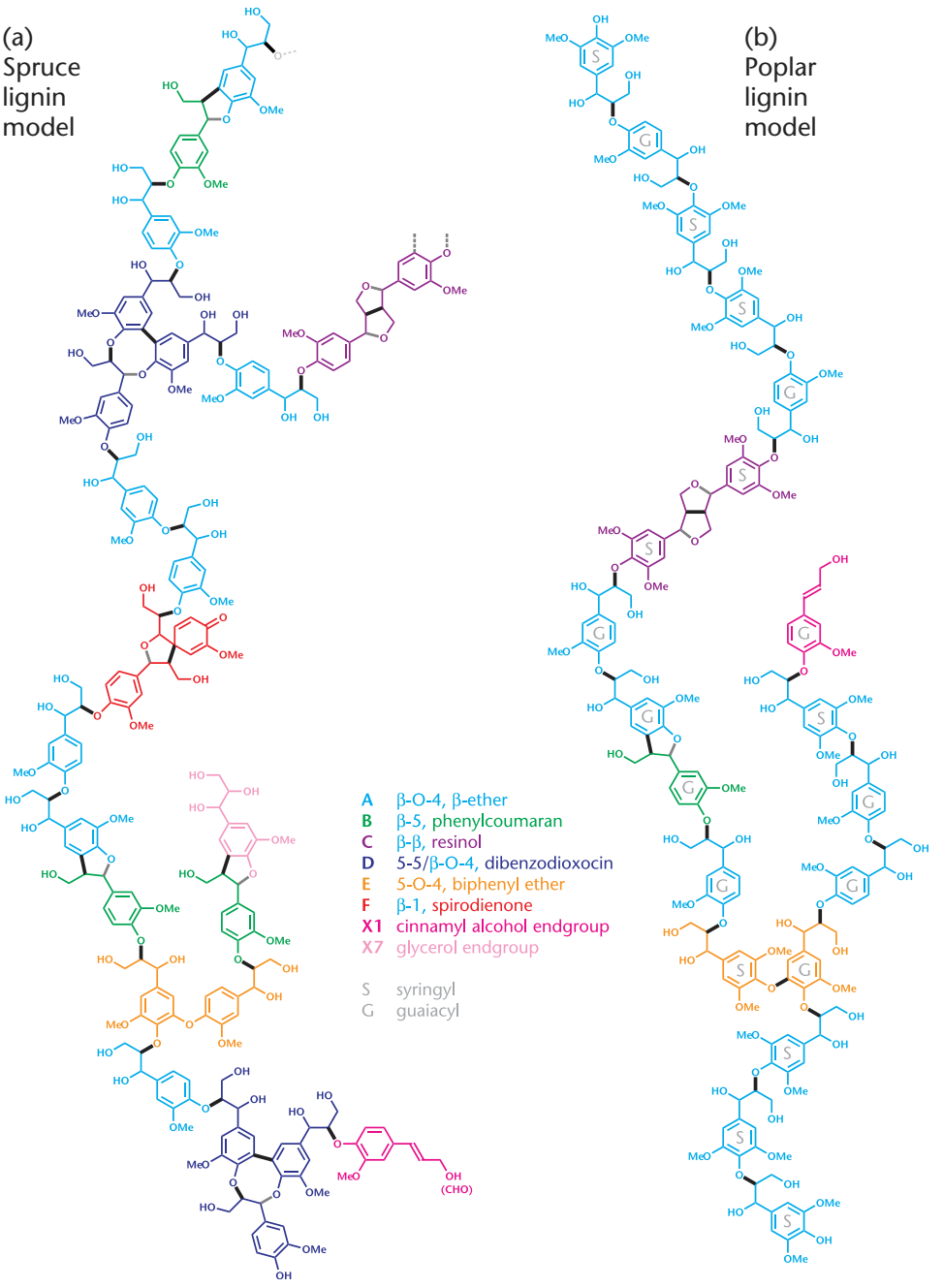


Figure 4 Lignin Polymer Models for (a) a softwood (spruce) lignin with 25 units, redrawn from (Brunow, 2001), and (b) a hardwood (poplar) lignin with 20 units, redrawn from (Boerjan *et al.*, 2003). Colour coding is uniform across the two models. Bold, black bonds indicate the bonds formed by radical coupling during lignification; lighter (grey) bonds result from postcoupling internal rearomatization reactions; α-OH groups from nucleophilically added water assume the colours of their parent structure. The softwood lignin is more branched and contains a lower proportion of β-ether units **A**. The branch points (4–O–5-units **E**, orange; dibenzodioxocin units **D**, dark blue) are differentiated by unique colouring, even though such units may also be β-ethers, for example. Note that each of these structures represents only one of billions of isomers (Ralph *et al.*, 2004). Caution: these are ONLY MODELS! They do not imply any primary structure or sequencing in the lignins themselves but attempt to accommodate the main linkage types and their approximate relative frequencies. Modified from Brunow G (2001) and Ralph *et al.* (2004).

secoisolariciresinol **M12** in softwoods. Secoisolariciresinol presumably is derived from β–β-coupling of coniferyl alcohol but the pathway by which it becomes reduced for lignification remains speculative. It is problematic to class secoisolariciresinol, an indirect result of monolignol dimerization, as a lignin 'monomer' but the evidence is compelling that it is indeed incorporated into lignin as such and is, therefore, a lignin precursor.

Glycerols **X7** are being increasingly authenticated. Originally thought to derive from β-ethers during the milling steps in lignin isolation, they are also found in biomimetically synthesized polymers which have not been subject to such physical treatment. The glycerols **M7** may, therefore, also be authentic lignin monomers produced from hydroxycinnamyl alcohols under peroxidase/H_2O_2 conditions.

Cinnamaldehydes **X2** and benzaldehydes **X3** are readily detected in lignins. Postlignification oxidation reactions could produce them from cinnamyl alcohol endgroups **X1**. Direct incorporation of the aldehyde monomers into the polymer is highly implicated by the observations of increased levels in CAD-deficient plants. Products **K** of endwise hydroxycinnamaldehyde incorporation into the growing polymer are revealed by NMR. Hydroxycinnamaldehyde-8–*O*–4-linked units **K**$_A$ are particularly prominent in CAD-deficient angiosperms as evidenced by the release of specific marker compounds derived from them during thioacidolysis (Lapierre *et al.*, 2004), a popular ether-cleaving analysis method for lignins. Incorporation profiles of hydroxycinnamaldehydes **M2** provide further evidence that lignification reactions are under simple chemical control. In tobacco, sinapaldehyde **M2s** is found 8–*O*–4-coupled (in structures **K**$_A$) to both **G** and **S** units, whereas coniferaldehyde **M2G** is only found cross-coupled to **S** units. Coniferaldehyde has not been successfully cross-coupled to guaiacyl models *in vitro* either. An important corollary is that hydroxycinnamaldehydes are incorporated integrally into angiosperm **G**/**S**-lignins but only poorly into gymnosperm **G**-lignins.

The most striking example of lignins incorporating substantial quantities of a monomer produced as a result of truncated monolignol biosynthesis is in caffeic acid *O*-methyl transferase (COMT)-deficient angiosperms, **Figure 5**. Plants depleted in COMT produce less sinapyl alcohol **M1s** but essentially substitute it with a monomer derived from its unmethylated precursor, 5-hydroxyconiferyl alcohol **M1**$_{5H}$. The incorporation is typically endwise, but coupling with the *o*-diphenol results in novel cyclic structures, benzodioxanes **J**, in the lignin. As with other products, such units can be found at very low levels in normal plants.

Variability and Topochemistry

Lignin deposition is one of the final stages of xylem cell differentiation and mainly takes place during secondary thickening of the cell wall. Microautoradiography and UV-microspectrometry have shown that the three monolignols are incorporated at different stages of cell-wall formation (Terashima *et al.*, 1993). Typically, **H** units are deposited first, followed by **G** units and **S** units still later in angiosperms. Lignin in vessels is generally enriched in **G** units, whereas lignin in fibres is typically enriched in **S** units.

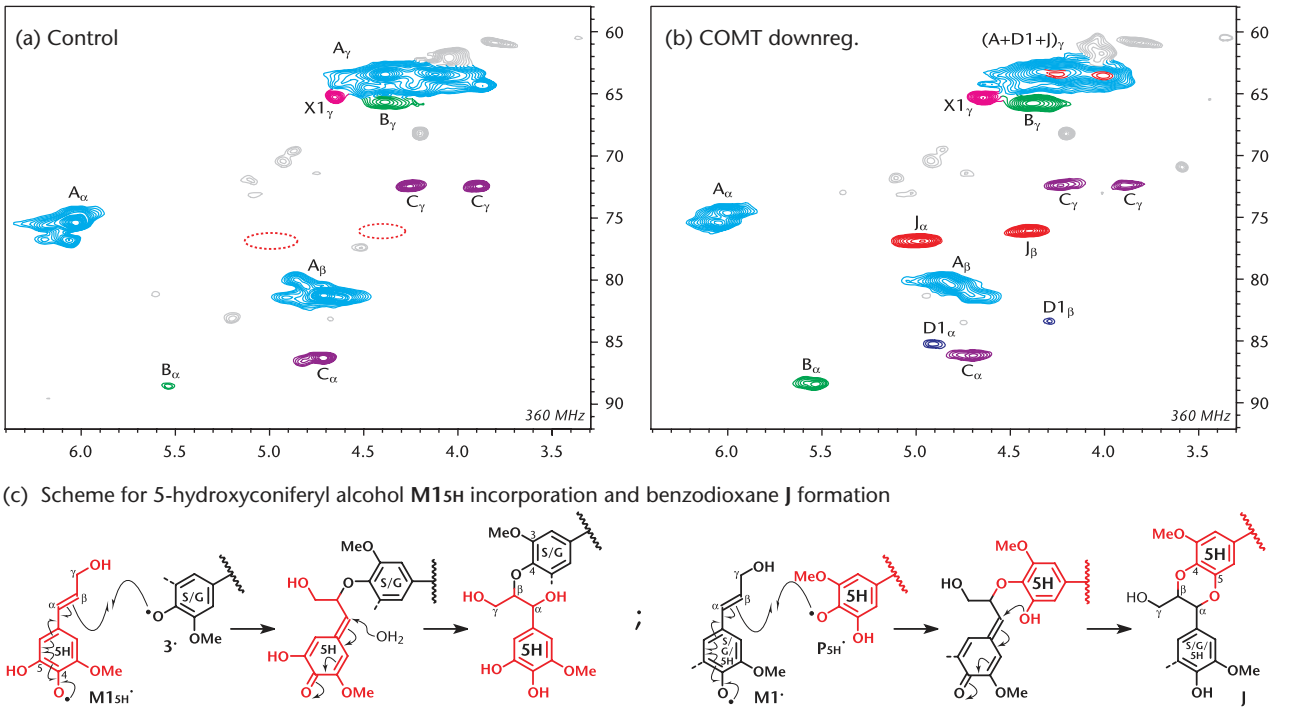


Figure 5 Partial poplar lignin NMR HMQC spectra (at 360 MHz) showing major lignin peaks and highlighting new peaks for benzodioxane units **J** in COMT-downregulated plants. Acetylated lignins were from (a) a control poplar, (b) a COMT-downregulated poplar transgenic. (c) Scheme for production of benzodioxanes **J** in lignins via incorporation of 5-hydroxyconiferyl alcohol **M1**$_{5H}$ into a guaiacyl lignin. Lignin unit designations are the same as in **Figure 2**–**4**. **Modified from Ralph J *et al.* (2001) Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR.** *Phytochemistry* **57: 993–1003.**

A large proportion of **S** units is also found in secondary walls of ray parenchyma. In gymnosperms, the lignin deposited in compression wood is enriched in **H** units. In gramineous monocotyledons, lignin incorporates significant amounts of hydroxycinnamate esters. Ferulate-polysaccharide esters **M4G** (and minor amounts of the *p*-coumarate analogues **M4H**) are rapidly deposited at the early stages of lignification, whereas *p*-coumarates **Y11**, acylating lignin sidechains mainly on **S** units, are deposited throughout lignification. The difference in timing of monolignol deposition is associated with variations in lignin unit distribution in the individual cell-wall layers, as shown by immunocytochemistry with antibodies raised against pure **H**, pure **G** or mixed **GS** synthetic lignins.

The chemical nature of the carbohydrate matrix and the orientation of the cellulose microfibrils logically influence lignin deposition. In the middle lamella and the primary wall, lignin forms spherical structures (Donaldson, 1994), whereas in the secondary wall, lignin forms lamellae that follow the orientation of the microfibrils (Atalla and Agarwal, 1985). During deposition, lignin may form chemical bonds with the hemicellulose component in the wall as it gradually eliminates water forming a hydrophobic environment.

From these data, one can conclude that lignin deposition, and the relative incorporation of the different monolignols into the polymer, are spatially and temporally regulated. The mechanisms controlling this process are not yet fully resolved but are likely governed by the interplay between the spatio-temporal expression of monolignol biosynthetic genes, the kinetics of monolignol delivery to the cell wall and the chemistry of monolignol coupling to the growing polymer in the complex macromolecular environment of the cell wall.

Biotechnology

Lignin biosynthesis is an active field of research partly because of its economic relevance. For the production of high-quality paper, lignin needs to be extracted from the pulp by expensive and environmentally hazardous processes requiring large amounts of energy and chemicals. Lignins also inhibit saccharification in processes aimed at producing simple sugars for fermentation to ethanol. Producing plants with less lignin has been successfully demonstrated by downregulation of 4CL. Such plants offer potential advantages for the pulp and paper industry and as forage for ruminant animals. However, the first attempts to reduce lignin content resulted, instead, primarily in changes in lignin composition and structure. Large reductions in CAD activity only slightly reduced lignin content in angiosperms because the plants were able to circumvent the block in CAD activity by shipping the CAD substrates, the cinnamaldehydes, to the cell wall for polymerization. The altered lignin structure did not affect overall plant growth and development. These data show how adaptable plants are in building their cell wall. Chemical pulping experiments with wood harvested from transgenic poplars downregulated for CAD have demonstrated that the modifications in lignin structure result in an altered chemical reactivity, which reduces the consumption of chemicals needed to remove lignin from the pulp. The pulp yield was simultaneously enhanced. These data also show that significant improvements in pulping efficiency can be achieved without strong reductions in lignin content.

Downregulation of COMT primarily affected the biosynthesis of **S** units. In these plants, a novel unit $\mathbf{M1_{5H}}$, was copolymerized into the polymer and resulted in new types of chemical bonds **J**, **Figure 5**, again demonstrating the extraordinary adaptability of the lignification process. These plants were more difficult to pulp, presumably because of the reduced level of **S** units and the presence of benzodioxane units **J**, derived from 5-hydroxyconiferyl alcohol $\mathbf{M1_{5H}}$, which are not cleavable in base. The results again demonstrated that lignin composition plays an important role in lignin extractability and that it can be modified without affecting plant viability. Although COMT-downregulated plants have no obvious benefit for the chemical pulp industry, they have improved digestibility in ruminants. A possible explanation lies in reduced lignin-polysaccharide cross-linking when 5-hydroxyconiferyl alcohol replaces sinapyl alcohol. The quinone methide resulting from monomer addition to a 5-hydroxyguaiacyl endgroup is rapidly internally trapped by the 5-phenol, **Figure 5c**, thwarting a pathway by which polysaccharides trap quinone methides resulting in cross-linking. Downregulation of COMT, PAL, CCoAOMT, C3H and HCT have all resulted in improved digestibility in alfalfa (Reddy *et al*., 2005).

A lignin polymer enriched in **S** units is less branched and has a higher β-ether content (for reasons obvious from **Figure 2**) than lignins rich in **G** units. Overexpression of F5H in poplar and aspen has successfully generated plants with extremely high syringyl levels and consequently improved pulping characteristics (Huntley *et al*., 2003). An additionally appealing objective is to engineer **S** lignins into gymnosperms, which typically have no syringyl component. Through the combined expression of angiosperm genes (*f5h*, *comt* and perhaps the required *cad*), it might be possible to divert coniferaldehyde and coniferyl alcohol toward the synthesis of **S** units resulting in more facile lignin extractability.

Conclusions

Lignification is an extraordinarily flexible process producing complex racemic aromatic heteropolymers – lignins. Simple chemical coupling propensities, however, constrain the synthesis to limited structural diversity. Control over the process is exerted by the cell, primarily in the delivery of monomers to the lignifying zone and the concentration of substrates required for radical generation, but all evidence points to the polymerization process itself being independent of protein/enzyme control. Lignification can, therefore,

respond efficiently to perturbations caused by natural processes or by our interference with pathway genes. To date, all of the genes in the monolignol pathway have been up- or downregulated providing new insight into normal and perturbed lignification, and sometimes delivering plant materials with improved conversion efficiencies in processes such as chemical pulping, biomass saccharification and fermentation and ruminant digestibility.

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