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Phenolic cross-links: Building and de-constructing the plant cell wall

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1 Introduction

A cell wall built mainly from polysaccharides is a distinctive feature of Streptophyta, the branch of the green plant lineage that includes terrestrial plants. Microfibrillar cellulose forms a composite in combination with a very complex set of other polysaccharides, providing a matrix for the embedding of insoluble microfibrils superimposed on a covalent network of phenolic polymers. The polysaccharide composite is a dynamic structure, subject to turnover while the cell is expanding, whereas the plant cell has very limited ability to modify the phenolic network. Deposition of the phenolic network is a phenomenon that occurs during wall maturation – when cell expansion is complete – culminating in some cell types with deposition of a secondary wall on the inside of the primary wall. In cells that differentiate into vessel elements, deposition of the phenolic network may in fact run to completion post-mortem[1], i.e. lignification of the wall progresses after the cell

nucleus has disintegrated relying only on H_2O_2 and monolignols from living neighborings cells.

Lignin is the major constituent of the phenolic network and is composed entirely of phenolics. Lignin polymerization could comprise and may even be nucleated by phenolic substituents on polysaccharides and tyrosine residues of a class of cell wall structural glycoproteins known as extensins, as shall be discussed in this review. The occurrence of phenolic esters on plant polysaccharides was recently reviewed by Harris and Trethewey[2].

Polymerization of phenolics in the wall is mediated by reactive oxygen species in the apoplast. The enzymes involved, the types of reactions and the chemical bonds formed are the unifying principle for which we use the term *cross-link* when the result truly is cross-linking of two polymeric entities and *phenolic inter-unit linkages* when a similar chemical bond is formed between phenolic entities in intra-chain links and as part of monolignol polymerization, *in vivo* or *in vitro.*

The fact that some reactive oxygen species play a role in wall loosening during growth while others mediate cross-linking, and hence retard cell expansion, complicates the unraveling of the biochemical control of these processes[3]. In addition, the expansive gene families encoding peroxidases[4] and laccases, which catalyze cross-linking, pose a challenge when aiming to assign specific roles to individual enzymes.

The oxidative regime in the apoplast is brought about by several paths involving, for example, amine oxidases, oxalate oxidase, and NAD(P)H oxidase. The importance

of different enzyme classes differs between plant families and it is possible that different oxidative systems arose via convergent evolution[5]. Generalizations and inferences from one plant species to the next should thus be made with caution. The present review focuses on the phenolic linkages within the plant cell wall, their biological function, and how to make and break them. With respect to the application of this knowledge, phenolic cross-links and inter-unit linkages may be both a blessing and a nuisance. Enzyme-mediated cross-linking may be used to bio-engineer new composites and other “green” materials. Near the end of this review, we will cover the use of cross-linking enzymes for the manufacture of fiber cell composites, nanocellulose materials and hydrogels for use in biomedical materials for example. On the other hand, lignification of secondary cell walls is the major reason for the recalcitrance of biomass to disassembly. Nature-inspired approaches to facilitate cleavage of phenolic cross-links may allow the most challenging impediments to a global move towards a bio-based society based on second generation biofuels to be overcome. This review is concluded with a perspective on these matters.

2 Occurrence and physiological function of polymers involved in phenolic cross-linking

2.1 Occurrence and physiological function of lignin

Lignin is a cell wall polymer of randomly linked hydroxycinnamyl alcohols (or monolignols), which are principally *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. The polymerization of these C6-C3 alcohols results in the formation of the H, G, and S units of lignin, respectively[6] (Figure 1). Lignin is found in the cell

walls of all vascular plants (tracheophytes) where it plays an essential role in growth and development. Lignin contributed to the emergence of tracheophytes by imparting the water-conductive vascular elements with structural rigidity and hydrophobicity. The hydrophobic nature of lignin is essential for it to confer strength to secondary walls[7] while the unwettable property of lignin is indispensable for the transport and distribution of water in tracheary elements of terrestrial plants[8]. As a key structural element of the cell wall, lignin also constitutes a defense barrier against herbivores and in the root endodermis it is a barrier to soil-borne pathogens[9]. These features enabled vascular plants to reach much larger physical dimensions than their non-lignified bryophyte ancestors[10]. Material strength has been investigated in transgenic trees that have a modified lignin content or structure[11,12]. In addition, the role of lignin in lodging resistance in cereals has been debated for more than a century[13,14]. What is probably less obvious is that lignin influences the microbial populations in both the rhizosphere and the endosphere, as observed in studies of transformants modified in lignin biosynthesis[15,16].

Figure 1: Lignin molecule constructed from three 4-hydroxyphenylpropanoids known as monolignols, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Once incorporated into a lignin polymer, they are represented as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) moieties. The structure depicts types of phenolic inter-unit linkages and possible branch points but does not represent a real lignin as stochiometry has not been considered.

Lignans are also derived from coupling of phenylpropanoid units to produce dimers or oligomers linked via C-C and/or C-O interunit bonds. However, unlike lignin, lignans do not have any known essential structural role in vascular tissues[17] The

occurrence and immense diversity of lignan structures have recently been reviewed[18] and shall not be covered here. The diversification of lignan structures may reflect an underlying arms race as the prime function of lignans is as defense compounds directed towards plant pathogens[19]. Defense reactions of plants include wall repair involving ectopic lignification drawing upon glycosylated monolignols stored in the vacuole and possibly also the lignans released as defense compounds[20,21]. Discriminating experimentally between the involvement in defense of lignin and lignans respectively has been complicated by the discovery of lignin oligomers formed in the cytoplasm and stored in the vacuole**[22]**, presumably to take part in wall repair during pathogen invasion.

2.2 Occurrence and physiological function of ferulate cross-links

Ferulic and *p*-coumaric acid are major hydroxycinnamic acid derivatives that are incorporated into the cell wall and play an important role in cell wall extensibility and biodegradability. Feruloylated polysaccharides are characteristic of two major groups of angiosperms: commelinid monocotyledons (Poales, Commelinales, Zingiberales, Arecales) and the 'core' Caryophyllales[23,]2. In commelinid monocotyledons, ferulic acid is esterified to the glucuronoarabinoxylan (GAX)[24]; while in Caryophyllales, it is esterified to arabinan and galactan side-chains of rhamnogalacturonan-I (RGI)[25,]23[,26].

Ferulic acid and its oligomers act as important components of the plant cell wall, forming cross-links between polysaccharide chains and lignin, between polysaccharides[27], and between polysaccharides and proteins[28,29,30]. Oligomerization of the feruloylated polysaccharides tethers these cell wall

constituents together with implications for the physiological functions of the cell wall with respect to extensibility[31,32,33], growth cessation[34], and increasing recalcitrance towards enzymatic degradation and pathogen invasion[35,36,37]. The function of feruloylated structures has been studied in two closely related Chenopodiaceae species, sugarbeet and beetroot. It has been noted that in sugarbeet 20% of the feruloyl moieties were incorporated into dimers in contrast to only 10% in beetroot. Sugarbeet can sustain its structure at temperatures of 100°C for several hours, whereas beetroot softened within 20 – 30 min, indicating a role for feruloylation in cell-cell adhesion[38].

Ferulate-based cross-links are thus critically important. Plant families that do not feature feruloylated polysaccharides may have evolved functionally equivalent cell wall structures in an attempt to overcome this deficiency.

2.3 Occurrence and physiological function of extensins

Although cell wall proteins are quantitatively minor components of the cell wall, they are integral to cell wall assembly and architecture. Of the diverse classes of cell wall structural proteins, the role of one subgroup – extensins – is most well understood[39,40]. However, there are other types of cell wall protein cross-links that we will not discuss in detail here, including the cysteine domain (CD) crosslink found in the C-terminal of some glycine rich proteins (AtGRP3 At2g05520, AtGRP3s At2g05380 and At2g05520)[41]. AtGRP3 has been shown to become part of a supramolcular complex with pectin and Wall Associated Kinase 1[42,43]. The far-reaching implications of an arabinogalactan protein, APAP, which was

demonstrated to carry covalently attached arabinoxylan and pectin[44] also fall outside the scope of this review.

Extensins are particularly relevant here because of their ability to self-assemble. Non-covalent association has been demonstrated between extensins and pectic polysaccharides, in addition to interactions with other cell wall constituents[45,46]. The cell walls of root hairs are vulnerable structures and easily accessible for investigation and, in several extensin mutants, the root hair structure is compromised, see below. Extensin sequence motifs are not only found in structural wall proteins. Leucine Rich Repeat-Extensins (LRX) are extracellular proteins involved in signalling and wall-sensing[47,48]. Genetic lesions in these proteins also result in root hair phenotypes[49].

The ability of extensins to form covalent cross-links via their tyrosine (Tyr) residues leading to cross-linked structures that superficially are analogous to the ferulic acid cross-links is interesting. At the functional level, the similarity is probably not superficial, as demonstrated in grasses, which lack extensins with cross-link motifs. It is, therefore, tempting to speculate that ferulate cross-links on GAX have made extensin cross-links superfluous in grasses[50]. The cross-linking motifs missing in structural cell wall proteins in grasses are present in their LRXs.

Ringli[51] has shown that, in Arabidopsis, Tyr residues in the extracellular domain of, LRX1, have an important function in wall-sensing. However, a carefully prepared truncated series of the apoplastic domain, which included replacing Tyr residues with Phe, led to the conclusion that other mechanisms of anchoring to the wall than

phenolic cross-links to the wall are at play. As such, it is not necessarily a conundrum that the Tyr domains are retained in grass LRXs even though no extensin partners featuring cross-linking motifs are available.

As for the ferulates, it is assumed that cross-links involving aromatic residues will co-polymerize with lignin during its synthesis41. This is technically difficult to prove and remains an attractive working hypothesis for extensins while good evidence has been provided for ferulates[52]. Extensin-lignin cross-links are well documented[53,54,55] and demonstrated to be elicitor inducible[56] but the nature of the cross-links, i.e. if they are phenolic cross-links, remains to be elucidated.

Extensin content is generally low in secondary cell walls (where lignification is quantitatively most prominent), but it has been shown that extensin make a small but measureable contribution to recalcitrance to saccharification for biofuel production[57]. However, the importance of extensins in secondary wall assembly should not be underestimated. Overexpression of an extensin in rice was found to increase secondary wall thickness substantially[58].

The involvement of extensins in plant defense was realized many years ago[59,60] and the current status was most recently reviewed by Deepak et al[61]. A recent study examined the effect of heterologous expression of a fungal feruloyl esterase in Arabidopsis. An increased susceptibility to *Botrytis cinerea* infection was observed as well as a shift to cross-linked extensins[62]. Di-ferulic cross-links are not known in Arabidopsis and the authors proposed that wall-bound ferulic acid in

dicots are involved in polysaccharide-extensin cross-links. Hence, a reduction in wall associated ferulic acid led to a promotion of extensin self-crosslinking.

3 Structure and formation of cross-links and phenolic inter-unit linkages

3.1 Structure and formation of lignin inter-unit linkages

Laccase- and peroxidase-catalyzed formation of monolignol radicals, and subsequent coupling, results in the formation of lignin polymers[63]. This combinatorial coupling of monolignol radicals, which initially leads to the formation of lignin oligomers, may take place intracellularly or in the cell wall[21]. The combinatorial nature of the polymerization process of monolignol radicals contributes to the large structural diversity of lignins. The most abundant linkage between monolignols is aryl β-aryl ether (β-O-4'), followed by phenylcoumaran (β-5'/α-O-4'), resinol (β-β'/α-O-γ'/α'-O-γ), and biphenyl (5-5'), whereas dibenzodioxocin (5-5'/4-O-β''/4'-O-α''), diaryl ether (4-O-5'), and spirodienone (β-1'/α-O-α'/ β'-O-4'') linkages also occur but to a much smaller extent[64] (Figure 2). A recent study conducted on poplar natural variants suggests that monomer concentration and transport influence C-O and C-C bond content during the lignification process[65]. Thus, monomer concentrations also play a dominating role in the resulting shape of lignin[6]. Notably, conifer lignins were found to be more branched compared to lignins in grasses due to the absence of S-units and higher occurrence of dibenzodioxocin inter-unit linkages typically found in high-guaiacyl lignins[66], whereas more linear lignins can be obtained by transgenic approaches that increase S-unit content[67].

Figure 2: Common phenolic inter-unit linkages identified in lignin. **1**, aryl β-aryl ether (β-O-4'); **2**, phenylcoumaran (β-5'/α-O-4') ; **3**, biphenyl (5-5'); **4**, dibenzodioxocin (5-5'/4-O-β''/4'-O-α'');

5, diaryl ether (4-O-5'); **6**, resinol (β-β'/α-O-γ'/α'-O-γ); **7**, spirodienone (β-1'/α-O-α'/ β'-O-4''); Bonds between monolignols are presented in red color. R = H or OMe; L = Lignin backbone.

Because the polymerization process is chemically driven, phenolic radicals other than the traditional monolignols can also be incorporated into lignin if they are present during lignification[169]. For example, non-canonical lignin monomers, such as hydroxycinnamates[247] ferulate dehydrodimers[68,]52, tricin[69,70], dihydroconiferyl alcohol[71], caffeyl alcohol[72], 5-hydroxyconiferyl alcohol[73], hydroxycinnamaldehydes[74], and hydroxystilbenes[75], were identified as lignin components. Lignins from certain plants are also known to be acylated with acetate[76], *p*-coumarate[76], *p*-hydroxybenzoate[77,78], benzoate[79], vanillate[79,68], and ferulate[79] (Figure 3).

Figure 3. Examples of lignin acylation. Acyl groups (highlighted in red) can be **9**, acetate**; 10**, *p*-coumarate; **11**, *p*-hydroxybenzoate; **12**, benzoate; **13**, vanillate; **14**, ferulate.

In specific mutants, incorporation of hydroxybenzaldehydes[80], tyramine ferulate[81], and naringenin[82] into lignins have also been observed. Although never observed naturally, rosmarinic acid (a caffeate ester), epicatechin, gallate derivatives, and quercetin glycosides (e.g. hyperoside) have also been demonstrated to incorporate lignin using biomimetic systems[83,84]. These numerous examples of incorporation of non-traditional monomers emphasize the plasticity of lignification biogenesis and challenge an alternative view that proposes an ordered and non-random process of

lignin formation[85]. Observations on the relative abundances of inter-unit linkages that do not change as expected in lignin of certain mutants and transgenics featuring altered monolignol ratios led some authors to propose a controlled process for monolignol polymerization and suggest that linkage types are not simply controlled by combinatorics, i.e. the concentrations of the different monolignols[86]. Readers can refer to the study of the caffeic acid *O*-methyl transferase Arabidopsis mutant *Atomt1* for a presentation of this argument[87].

3.2 Structure and formation of cross-links involving phenolics on polysaccharides

3.2.1 Phenolics on pectic RGI

RGI is a branched heteropolymer of alternating α-1,2-linked rhamnose (Rha) and α-1,4-linked galacturonic acid (GalA) residues. The Rha residues of the RGI backbone can carry neutral side-chains of predominantly 1,4-β-D-galactose and/or 1,5-α-L-arabinose residues. These linkage types were initially discovered in endo-polygalacturonase digests of cell walls of suspension cultured sycamore cells[88,89]. RGI biosynthesis is only partly understood. Candidate arabinosyltransferases have been identified[90], as well as galactosyltransfereases that are involved in building β-1,4-galactan side-chains[91]. An assay for backbone synthesizing rhamnosyltransferases has been developed[92] and used successfully to identify a new family of rhamnosyltransferases[93].

RGI has a number of specialized roles, e.g. as mucilage[94,95], but as a general primary wall constituent it displays non-covalent self-assembly properties[96]. The core Caryophyllales side-chains of RGI carry phenolic substituents that are

potentially involved in cross-linking, Figure 4. In spinach (*Spinacia oleracea*) and beet (*Beta vulgaris*) cell walls, ferulic acid mainly esterifies the neutral sugars (arabinan and galactan) of pectic side-chains[97,98]. The exact locations of these ester linkages were determined by treating cell walls from spinach leaves and sugar beet pulp with Driselase, a commercial enzyme mixture from the fungus *Irpex lacteus* that contains several pectolytic enzymes but lacks hydroxycinnamoyl esterase activities. The side chains of sugar beet RGI can be feruloyl substituted at the O-2 in the main backbone of α-(1,5)-linked arabinan, the O-5 in the terminal arabinose98, or the O-6 in the backbone of galactan[99,]25. A pectin-extensin cross-link has been demonstrated in sugar beet[100]. The linkage was proposed to be covalent but the type of linkage and hence whether it could be phenolic remains to be determined. A covalent link between RGI and extensin has also been observed in cotton[101]. Cotton is a Rosid and is thus expected to feature very low levels of polysaccharide feruloylation2 but cotton has not been investigated in this regard and the authors do not exclude the possibility of a phenolic cross-link.

Figure 4. Simplified structure of RGI, a pectic polysaccharide with a back-bone of alternating rhamnosyl and galacturonosyl residues. Rhamnosyl residues may carry neutral side-chains and it is a speciality of the Caryophylales plant families that the side-chains are esterified with ferulic acid (shown in red). Monosaccharides are color-coded: Galacturonic acid, orange; rhamnose, dark pink; galactose, blue; arabinose, green.

3.2.2 Phenolics on xylan

Xylans comprise a backbone of β-(1→4)-linked xylose residues which can be *O*-acetylated and/or substituted at O-2 by α-Ara, α-GlcA and α-MeGlcA side-chains, giving rise to arabinoxylans, glucuronoxylans, and glucuronoarabinoxylans (GAX), respectively[102]. GAX is the major structural component of the Poaceae primary cell wall, with glucuronoxylan alone constituting up to 40% of the dry mass[103,104].

Figure 5. Maize pericarp glucuronoarabinoxylan (GAX). A Representative structure with ferulic acid (shown in red) esterified to arabinosyl residues. B and C Two additional identified feruloylated maize GAX side-chains[105], the one in C shown with a covalent linkage to lignin. Monosaccharides are colorcoded: Xylose, yellow; arabinose, green; glucuronic acid, purple; L-galactose, blue.

The GAX of the maize pericarp (Figure 5) exemplifies the fine structure of this molecule. This is the most complex xylan known and features the highest degree of feruloylation[106]. Ferulic acid is the most abundant phenolic compound (up to 90%) followed by small amounts of *p*-coumaric acid[107,108]. Side-chains comprising glucuronic acid and its 4-*O*-methyl derivative are linked to the xylan backbone[109,110]. Extensive acylation on xylose and arabinose residues is also observed[111,112]. Xylose residues can be found as 2-linked terminal xylose residues on arabinose or as 2,4-linked xylose, with evidence for an additional terminal galactose at the 4 position[113] in addition to the 3-linked L-Gal[129] shown in Figure 5. The figure provides a generalised structure of maize GAX, which is composed of a β-1,4-linked xylopyranosyl backbone decorated at the C_2-O and/or C_3-O positions with α-L-

arabinofuranose residues. The highly branched structure and few unsubstituted xylosyl residues render maize GAX recalcitrant to hydrolysis[114,115], which distinguishes maize GAX from wheat and barley GAX[116,117]. Consequently, increased enzyme mediated solubilisation using GH10 and GH11 xylanases has been observed in wheat GAX compared to maize GAX[118]. It has been suggested that no regions in maize xylan exist where several contiguous xylose residues are unsubstituted, making it inaccessible to endo-acting enzymes[119].

Ferulic acid is typically esterified to the C(O)5-hydroxy group of arabinosyl residues in (glucurono)arabinoxylan[120,26,121]. Evidence for the presence of a more alkaline resistant linkage between ferulic acid and GAX, most likely an ether linkage, has been provided by Burr and Fry[122]. Ester formation precedes formation of the more stable linkage observed, and the radical structure, which is an intermediate in oxidative coupling of ferulic acid, was proposed by the authors to form a strong *p*-hydroxybenzyl ether bond via nucleophilic attack by a sugar residue, possibly assisted by an as yet unidentified protein. All possible quinone intermediate radicals generated during the dehydrogenation of a ferulate moiety are shown in Figure 6, top. From these, radical coupling produces ferulate dehydrodimers (e.g. 8-8, 8-O-4, 8-5-linkage, etc.) following cross-linking via nucleophilic addition.

Nucleophilic addition can occur either intra- or inter-molecularily. In the case of intermolecular nucleophilic addition, an oxygen atom is either abstracted from a water molecule to form ether linkages, or from a polysaccharide to form a carbohydrate complex. In order to proceed with this reaction, the sugar nucleophilic

attack requires the presence of a specific enzyme, or possibly dirigent protein, as water is a stronger nucleophile than alcohols. As such, in a hydrated medium, water competes with the sugar and will be the prominent reactant as indicated in Figure 6.

Figure 6: Three phenolic cross-linking mechanisms, including a tentative mechanism of ether bond formation between ferulic acid and a sugar-OH group[115] here provided by an arabinosyl residue shown in red. The resonance forms shown result from oxidation of ferulate by peroxidase (this oxidation can also be catalyzed by laccase).

In Norway spruce, NMR analyses of isolated fractions enriched in various polysaccharide-lignin complexes suggested that the carbohydrate and lignin constituents are chemically bonded[123], which is further supported by the identification of ether-linked lignin nucleation points on both xylan and galactoglucomannan (GGM)[124]. A detailed analysis conducted on Japanese red pine wood using nuclear magnetic resonance spectroscopy evidenced α-ether bonds between lignin and the primary hydroxyl group of mannose residues in glucomannan[125]. A one-enzyme mechanism where laccase mediates ether linkage formation has been proposed (Figure 2a, ibid124), a reaction that likewise would suffer from competition with water unless a second, unknown enzyme is introduced[126].

A key feature of esterified ferulic acid is its ability to form cross-links by oxidative coupling, resulting in the formation of ferulate dimers, trimers, and even tetramers[127,128] (figure 7). Quantification of feruloylated GAX sidechains is problematic due to a lack of standards, but is possible through mild acidic

hydrolysis combined with C_{18}-solid phase extraction and LC-DAD/MS[129]. Another reported method to quantify free and ester-linked ferulic acid is based on liquid extraction, precipitation and reverse-phase HPLC[130]. Analysis of the presence of ferulic acid dehydrodimers and dehydrotrimers via alkaline pre-treatment and reverse-phase HPLC has also been reported[131,132].

Figure 7. Identified ferulate crosslinks in GAX. **15**, 8-O-4-DFA (or β-O-4-DFA); **16**, 8-5-DFA (or 8-5-benzofuran-DFA); **17**, 8-8 (non-cyclic)-DFA; **18,** 8-8-THF-DFA; **19**, 8-8 (cyclic)-DFA; **20**, 5-5-DFA; **21**, 4-O-5-DFA; **22**, 8-O-4/8-5(cyclic)-TriFA; **23**, 8-8(cyclic)/5-5-TriFA; **24**, 8-5(cyclic)/4-O-5/8-5(cyclic)-TetraFA. New bonds formed upon radical coupling are highlighted in red. Abbreviations are DFA (diferulic acid), THF (tetrahydrofuran), TriFA (triferulic acid) and TetraFA (tetraferulic acid).

Formation of ferulate cross-links impedes enzymatic degradation of the cell wall[133,134]. Interestingly, ferulic acid has been shown to cross-link other polysaccharides, lignin, and possibly even proteins[135]. In cell walls of maize, 5-5-coupled diferulate dimerization cross-links xylans, a process that may result in coupling to lignin52. Administration of radiolabeled arabinose to maize cell-suspension cultures showed extensive cross-linking to soluble polysaccharides, including xylans and xyloglucans[136]. Maize xylan-associated glucuronic acid substitutions mediate cross-linking between neighboring xylan chains and cellulose surfaces, and are stabilized by Ca^{2+} as shown by molecular dynamics[137]. Recent solid-state NMR and molecular genetics studies showed that the GAX fine structure, in particular the positioning of GlcA/GlcA-Me side-chains and acylation influence its interaction with cellulose. The two-fold helical screw configuration

adopted by GAX in the secondary cell wall enables interaction with hydrophilic surfaces and serves to assemble the cellulose fibrils in higher order structures[138]. This interaction has been observed in freshly hydrated cell walls indicating that water – and hydrogen bonding – plays a crucial role in this context despite the xylan conformation in water being a threefold helical screw[139]; the same conformation observed in dried cell walls. In primary cell walls, interactions between pectin and cellulose have also been reported[140,141]. As such, phenolic cross-linking of matrix polysaccharides in the wall appear to create networks that comprise cellulose microfibrils.

Mutants that affect GAX fine structure in ways that, for example, abolish points of feruloylation, are relevant to cross-linking. Xylan biosynthesis is rather well understood[142] and the sites of feruloylation have been mapped in maize[143]. However, the mechanism of xylan feruloylation remains a conundrum as shall be discussed below.

Substrate and localization of feruloylation

The substrate for feruloylation of arabinoxylan is not clearly defined but may require initial activation via feruloyl-CoA formation. In crude fractions isolated from rice tissue, ferulic acid is indeed transferred to arabinoxylan from feruloyl-CoA[144]. However, this cannot be taken as a proof of feruloyl-CoA being a substrate for the feruloylation, as the experiment did not exclude the possibility that short feruloylated polysaccharides are recognized by transferases responsible for protein feruloylation[145]. Feruloyl glucose has been proposed as an alternative substrate because increased levels of hydroxycinnamate esters in non-lignified cell wall

material was observed in a transgenic aspen line with antisense down-regulated 4-coumaroyl:CoA ligase (4CL)[146,147]. Two possible pathways for arabinoxylan feruloylation have been suggested[148]. The first involves the initial synthesis of arabinoxylan followed by feruloylation while the other proposes that the feruloylation pathway proceeds through an initial reaction of feruloyl-CoA with UDP-Ara to form UDP-Ara-Fer, which later acts as a donor to the growing arabinonoxylan chain148. Either of these possibilities point to feruloylation occurring in the secretory pathway, and this notion is backed up by experimental evidence. Pulse-chase experiments with labeled arabinose in suspension cultures of *Festuca arundinacea* documented that feruloylation of polysaccharides preceded export to the wall[149,150]. Evidence for apoplastic feruloylation has also been presented[151]: Kinetic studies of feruloylation based on ^{14}C-cinnamic acid and use of the secretory pathway inhibitor Brefeldin A (BFA) demonstrated that the BFA-treatments only had a marginal effect on the incorporation of radiolabeled ferulic acid into the wall. By contrast, the incorporation of radiolabelled arabinose was significantly impaired. The conclusion from this was that polysaccharides already deposited in the wall could be subject to feruloylation, a mechanism that could be instrumental for pathogen defense151. The substrate for feruloylation, whether intra- or extra-cellular, and the exact biochemistry of these processes are yet to be elucidated. However, identification of the acyl transferases involved in feruloylation, and direct substrate studies, would allow the mechanism to be deciphered.

3.2.3 Genes involved

Many studies have focused on elucidating the structures of the ferulate conjugating components of the cell wall, and how these cross-links are formed. Interest in biofuel has led to progress within the last few years in elucidating the cell biology of feruloylation. So far, a few of the arabinonoxylan feruloyl transferase (AFT) genes are known. Based on the assumption that genes encoding AFTs involved in arabinoxylan biosynthesis in grasses would be more highly expressed than in their dicot orthologs, transcriptome profiling suggests that the AFTs could belong to the BAHD family of transferases[152]. BAHD enzymes are also implicated in lignification, section 4.1.2, and they are named after the first four members that were biochemically characterized in this family (i.e. benzylalcohol acetyltransferase, BEAT; anthocyanin hydroxycinnamoyl transferase, AHCT; anthranilate hydroxycinnamoyl/benzoyl transferase, HCBT; deacetylvindoline acetyltransferase, DAT[153]). They are also designated as members of the PF02458 family of domain-containing transferases, which are known to include hydroxycinnamoyl transferases[154] and acetyl transferases[155].

Experimental evidence for transferases from the PF02458 family being involved in feruloylation of arabionoxylan was obtained from rice[156], *Brachypodium distachyon*[157], and *Setaria viridis*[158]. It was reported that, in rice, the ferulic acid and *p*-coumaric acid content correlated with the transcription level of specific BAHD encoding genes. Twelve transferases clustered together in a neighbor-joining tree and were assigned to 5 classes (I-V). Direct evidence for their involvement in arabinoxylan acylation was obtained using RNAi transgenic plants and comparing

ferulic and *p*-coumaric acid levels to the control plants. Down regulation of genes encoding the group III and IV transferases specifically reduced the content of ferulic acid, whereas down-regulation of genes encoding group I and II transferases had a modest effect on *p*-coumaric acid content in the stem. Genes encoding group V transferases were only expressed at low levels in the roots and vegetative tissue and were not included in further experiments. The authors proposed that the processes affecting the incorporation of ferulic and *p*-coumaric acid may not be connected and instead are controlled separately by different transferase members of the BAHD family[156]. Table 1 lists grass BAHDs implied to be involved in arabinoxylan acylation.

Table 1: BAHDs genetically involved in arabinoxylan acylation

Gene name	Accession number / BAHD clade	Approach to modify gene expression	Observed phenotype	Reference
OsAT10	*Os06g39390* / IV	Overexpression by activation tagging in rice	60% reduction and 300% increase in cell wall esterified ferulates and *p*-coumarate, respectively, in young leaves	159
BdAT1	*Bradi2g43520* / I	1) RNA silencing and 2) *p35S* overexpression in *Brachypodium distachyon*	1) 35% reduction in cell wall esterified ferulates and diferulates in both leaves and stems 2) 58% and 47% increase in cell wall esterified ferulates and diferulates in leaves and stems, respectively	157
Os05g08640 *Os06g39470* *Os01g09010* *OsAT10*	*Os05g08640* / III *Os06g39470* / III *Os01g09010* / IV *Os06g39390* / IV	Simultaneous RNAi silencing in rice	19% reduction in cell wall esterified ferulates in leaves	156
SvBAHD01	Sevir.5G130000 / IV	RNAi silencing in *Setaria viridis*	~65% and ~35% reduction in arabinoxylan feruloylation in stems and leaves, respectively. 30-150% increase in arabinoxylan *p*-coumaroylation	158
BdBAHD01	*Bradi2g05480* / IV	RNAi silencing in *Brachypodium distachyon*	10-20% reduction in arabinoxylan feruloylation in stems	158

More recently, using a transgenic approach, a BAHD from clade I in *Brachypodium* (BdAT1) was shown to participate in arabinoxylan feruloylation157. Overexpression and downregulation of *BdAT1* resulted in an increase and a decrease of cell-wall ferulate levels, respectively. Similarly, two BAHDs from clade IV in *Setaria viridis* (SvBAHD01) and *Brachypodium* (BdBAHD01) have been implicated in arabinoxylan feruloylation since their silencing also resulted in reduced ferulate levels158. Interestingly, in the case of *Setaria viridis*, the decrease of ferulate was

accompanied by an increase of *p*-coumarate esters on arabinose and an improvement of biomass saccharification.

The group of BAHD acyltransferases contains more proteins than initially thought. At present, twenty members potentially involved in xylan acetylation have been identified in various bioinformatics studies159, whereas Mitchell's clade152 comprised only 12. An acyltransferase *Os*AT10 mutant in rice was characterised and it was found that increased expression of *OsAT10* (encoding a BAHD from group IV) resulted in an increased level of *p*-coumaric acid conjugation and a reduced level of conjugated ferulic acid159. This indicates that the enzyme encoded by *OsAT10* is responsible for the incorporation of *p*-coumaric acid into a precursor of arabinoxylan. Changes observed in hydroxycinnamic acids levels are related to the TFA-soluble matrix polysaccharides but not to lignin. *p*-Coumaric acid predominantly acylates lignin and feruloylated arabinoxylan. However, no evidence that *Os*AT10 has *p*-coumaric acid monolignol acyltransferase activity was found. Recently, overexpression of *OsAT10* in switchgrass also modified the ratio of cell-wall-bound *p*-coumaric acid to ferulic acid, which resulted in an improvement of biomass saccharification efficiency[160].

The stage in arabinoxylan formation at which the coupling of ferulic acid to arabinose occurs has been investigated in rice[161]. XAX1, which belongs to glycosyltransferase family GT61, is a xylosyltransferase required for xylosyl substitution of the arabinosyl residue that is optionally feruloylated (Figure 5). It was found that insertional mutants of the *xax1* gene presented a decreased feruloyl and

coumaroyl ester content. This implies that a side-chain exclusively built by Ara residues is a poorer substrate for feruloylation than an Ara-Xyl side-chain. If BAHD enzymes acylate an intermediate substrate to be transported into the secretory pathway, then UDP-arabinofuranose (UDP-Ara*f*) would be a suitable candidate. However, if UDP-Ara*f* were a substrate for feruloylation of xylan, the decrease in ferulate esters would not be observed in the *xax1* mutant. This led to the conclusion that another, yet unidentified, compound is involved as a feruloylation intermediate. The feruloylated protein mentioned above might be considered as the "unknown intermediate". Presently, feruloylated proteins have not been identified and it is not obvious how transport into the secretory pathway would be mediated. One candidate set of proteins is the GT75s/Reversible Glycosylated Polypeptides, which act as UDP-Ara*p*/Ara*f* mutases[162] and are located on the cytoplasmic face of Golgi[163]. In this position they are, therefore, exposed to the cytoplasmic BAHDs. UDP-Ara*f*-Fer would again be the compound required to pass into the Golgi lumen, leaving the *xax1* mutant phenotype unaccounted for.

Characterized BAHDs use aromatic and lipophilic acceptors[164,165,166] and this also applies to Mitchell's clade of BAHDs in grasses (discussed below). As such, the possibility that the intermediate is a small lipophilic hydroxyl-cinnamate ester that can pass passively or be easily transported into the secretory pathway should be entertained and would suggest that the target enzyme for the final acylation of GAX is a trans-esterase. However, it has been suggested that the intermediate is an

arabinosyl-ferulate ester, likewise calling for a trans-esterase being responsible for the final acylation21.

3.2.4 Other hydroxycinnamates in the cell wall

p-Coumaric acid and sinapic acid are phenylpropanoids structurally related to ferulic acid. They are also detected in the cell wall but are much less abundant. Although *p*-coumaric acid can undergo cyclodimerization, and theoretically take part in the cross-linking of the polysaccharide chains, its oxidative coupling products were not detected *in planta*[167]. Sinapic acid and its dehydrodimerization products (8-O-4 and 8-8 linkage type) have been isolated from cereal grain dietary fibers (wheat, spelt, rye, and wild rice), and supposedly play the same role as ferulates in the plant cell wall. Sinapate-ferulate structures were also observed. In combination with ferulic acid, sinapic acid can – in addition to 8-O-4 and 8-8 bonds – form 8-5 and 5-5 bonds. Heterogeneous phenolic coupling provides an opportunity for formation of more complex structures[168,169].

3.3 Structure and formation of cross-links in extensins

Extensins are characterized by their distict glycosylation pattern, featuring short arabinoside chains appended to hydroxy-proline (Hyp) residues, see Figure 8A.

Figure 8. A. Simplified contiguous Pro glycosylation motif featuring the four arabinosides, Hyp-Ara_1 to Hyp-Ara_4, found in most plants and single Gal side-chain on Ser. Glycosyltransferases responsible for building the side-chains are indicated in blue. The ordering of the side-chains shown is arbitrary as the arrangement of the arabinosides is

unknown. **B**. Staggered partial alignment of Arabidopsis Ext3 favours cross-links featuring pulcherosine[45]. Hydrophobic residues are shaded; the darkest are those involved directly in the cross-links. O is the one-letter code for hydroxyproline. **C.** Isodityrosine, cross-linking of two extensins with pulcherosine[182] as indicated in panel B, and di-isodityrosine[181]. Monosaccharides are color-coded: arabinose, green; galactose, blue.

The positioning of the Tyr residues, as well as the glycan structure, is integral to cross-linking in extensins. The importance of extensin *O*-glycosylation, and in particular the fourth Ara*f* residue, for cross-linking has been substantiated via *in vitro* studies[170], which demonstrated that the initial rate of cross-linking was primarily determined by the number of cross-linking motives in the protein backbone and by Hyp-Ara_4 content (arabinoside chain-length distribution is considered specites specific[171] with cotton fibres being the only cell type for which developmental control of chain-lengths has been documented[172]). Recent *in vivo* studies corroborate this observation of the importance of arabinosides: Extensins are important to root-microbe interactions and are present also in secreted mucilage. Lesions in RRA or xeg113 affect cross-linking and lead to modified mucilage compositon[173]. Grasses are thought not to cross-link their extensins due to the absence of the YXY motif. As such, the protein backbone sequences vary significantly across the plant Kingdom, with notable differences between grasses and other flowering plants155, whereas extensin *O*-glycosylation is as old as the green plant lineage[174], hence the use of gene and protein names that relate to Arabidopsis in the tables below.

A prerequisite for glycosylation is the hydroxylation of Pro residues to yield Hyp. The motifs required for this have recently been reviewed[175]. In Arabidopsis, knock-out and RNAi-studies of the responsible proline-4-hydroxylases (P4Hs) demonstrated that cell walls of root hairs are compromised if P4H2, P4H5 or P4H13 are downregulated[176,177,178]. Interestingly, Hyp formation, while necessary, is not sufficient for correct root hair formation. Arabinosylation is also required as knocking-out RRA and XEG113 arabinosyltransferases also lead to compromised root hairs177.

In *Chlamydomonas*, arabinosylation of cell wall proteins leads to an extended conformation of the peptide backbone[179]. This may be of relevance to cross-linking via the YXY crosslinking motif. Meanwhile, intrachain cross-linking of this motif to yield isodityrosine has been demonstrated[180]. The isodityrosine structure cannot be formed between neighboring Tyr residues but is sterically feasible based on the YXY sequence motif. The formation of interchain cross-links, both the tetrameric Tyr cross-link, di-isodityrosine[181], the pulcherosine cross-link[182], and arabinosylation of sequences interspacing the YXY motives were suggested to provide a conformational contraint that favors interpolypeptide cross-links since the glycosylation restricts polypeptide folding. In Arabidopsis, direct visualization of self-assembled extensin-3 (*At*EXT3) has been accomplished[183]. Knockout mutants in this gene are embryo-lethal as this extensin is directly involved in the assembly of new cross-walls as cells divide. The *At*EXT3 sequence features 11 repeats with YXY cross-linking motifs and sites for arabinosylation, which indicates that the

staggered alignment of cross-linking motifs (Figure 8b, and 8c) provides the scaffold for the establishment of extensin cross-links. It is tempting to speculate that the isodityrosine moiety would form in the Golgi and the di-isodityrosine cross-link would form in the apoplast. However, so far this has not been investigated.

In Arabidopsis, a study of phenotypic revertants of the AtEXT3 knockout mutant enabled classification of 20 extensins according to isodityrosine content[184]. Extensins, P4Hs, and extensin decoration glycosyltransferases are listed in Table 2.

Table 2: Extensin genes and genes involved in post-translational modifications that influence tyrosine cross-linking of extensins

Gene name	Arabidopsis AGI locus	Approach to modify gene expression	Observed phenotype / Function / Expression/ Other	Reference
AtEXT3	At1g21310	T-DNA line and in vitro assay	*rsh* (ROOT-SHOOT-HYPOCOTYL DEFECTIVE, RSH) Essential for cell plate formation, hence the knock out mutant *AtEXT3* is lethal	185 , 183
AtEXT6/ (S7)	AT2G24980	T-DNA line (SALK_010820)	Impaired roothair phenotype	177
AtEXT7 (S11)	AT4G08400	T-DNA line (Sail_882_D02)	Impaired roothair phenotype	177
EXT8	At2g43150			
EXT9	At3g28550			
EXT10 / S14)	AT5G06640	T-DNA line (SALK_099527)	Impaired roothair phenotype	177
EXT11 (S16)	AT5G49080	T-DNA line (SALK_035869)	Impaired roothair phenotype	177
EXT12 (S18)	AT4G13390	T-DNA line (Sail_1249_F11)	Impaired roothair phenotype	177
EXT13	At5g06630			
EXT14	At5g06640			
EXT15	At5g35190			
EXT1/4	At1g76930			
EXT19 (S)	At5g19810			
(22S)	At4g08370			

(21S)	At1g26250			
(20S)	At1g26240			
P4H2	At3g06300	T-DNA line P4H2.1 (SALK_042128), P4H2.2 (SALK_118623)	EXT type prolyl-C4-hydroxylase . Impaired roothair phenotype and reduced HYP coexpressed with six different EXTsBlee et al (2001)	176 177 178
P4H5	At2g17720	T-DNA line (SALK_152869)	Impaired roothair phenotype and reduced HYP	177
AtP4H13	AT2G23096	TDNA line (SAIL 425_H02)	Impaired roothair phenotype and reduced HYP	177 178
AtSGT1	At3g01720	*sgt1-1* (SALK_059879) *sgt1-2* (SALK_054682)	serine O-α-galactosyltransferase, GT96, ER and Golgi localized. Longer roots, larger rosettes	186
AtHPAT1,2,3	At5g25265 At2g25260 At5g13500	*hpat1* (GABI_298B03), *hpat2* (SAIL_178_H04), *hpat3* (SALK_047668)	hydroxyproline *O*-β-arabinosyltransferase Golgi localized. GT95 Impaired pollen tube growth, defects in cell wall thickening, enhanced hypocotyl elongation, shorter root hairs.	187 188
AtRRA1, 2, 3	At1g75120 At1g75110 At1g19360	T-DNA line ((SAIL_590_G09 Garlic_76_G04), *rra2* (Garlic_244_A03 SAIL_70_D08) *rra3* (GABI_233B05))	β-1,2-arabinosyltransferase, Golgi localized. GT77 Reduced root hair growth and reduced levels of arabinose in the mutant. RRA2 has meristem specific expression	189 177 39
AtXEG113	At2g35610	*T-DNA line (xeg113–1* (SALK_066991), *xeg113–3* (SALK_058092))	β-1,2-arabinosyltransferase, Golgi localized, GT77. Reduced root hair growth and reduced levels of arabinose in the mutant	190 177
AtExAD	At3g57630	T-DNA lines (exad1-1 (SAIL_843_G12), exad-2 (SALK_206288C) exad1-3 (SALK_204414C))	α-1,3-arabinosyltransferase GT47, Golgi localized. Reduced root hair growth and reduced levels of arabinose in the mutant	191

3.4 Lignin nucleation and co-polymerization

Ferulic acid is a key compound in the cell wall, tethering lignin and polysaccharides in grasses via ether-linkages between lignin monomers and ferulic acid (Figure 5C). As previously described, ferulic acid may also act as a nucleation site for lignification167,192. Recent studies on fractionation of wheat straw lignin revealed a distinct fraction with lower lignin content and higher ratios of arabinoxylan chains esterified by ferulic acid forming dimers, structures which were considered to represent lignin nucleation sites193. The isolation of ether-linked ferulic acid-coniferyl alcohol from saponified wheat and oat straw extracts provides evidence for the lignin-ferulate cross-link. The first identified structures of ferulate-monolignol coupling products were two isomers of β-O-4 dimers194. Subsequently 8-5 and 8-β dimers were also identified195, 121, see Figure 2. The proposed order of cross-linking reactions in the cell walls is as follows: first the ferulate is esterified to arabinoxylan and then the feruloyl arabinoxylan is linked to lignin via ether bonds. Eventually the amount of ether-bound ferulate equals that of the esterified form196. We will return to this subject in section 4.1.2 (BAHD transferases) and in section 6.4 (Engineering lignin cross-links).

p-Coumaric acid and other hydroxycinnamic acids are structurally related to ferulic acid with variations in their aromatic ring hydroxylation pattern and methoxylation, which results in different *in vivo* properties. *p*-Coumaric acid, which was isolated following mild saponification of the cell wall, mainly acylates lignin (and to a minor extent arabionoxylans) in immature tissues197,198. Some studies have shown that syringyl units are enzymatically pre-acylated with *p*-coumaric acid before their

incorporation into lignin. The observation that a higher *p*-coumaric acid content is detected in stems than in leaves, and that lignification is higher in stems than in leaves, corroborates the notion that *p*-coumaric acid is involved in the lignification process156. *p*-Coumarate essentially acts as a 'radical catalyst' and has been proposed to be involved in the radical transfer mechanism required for lignin formation[199,]192. However, even though *p*-coumaric acid is readily oxidized to produce radicals, it does not undergo oxidative coupling. In addition, no dimerization product has been detected in plants. Oxidized *p*-coumaric acid transfers the radical to sinapyl alcohol producing more stable radicals and facilitating the polymerization of lignin167.

Polysaccharides esterified with *p*-coumaric acid have been isolated but are present in minor amounts compared to ferulic acid-acylated polysaccharides[197,]198.

Finally, tricin was recently established as a true lignin monomer in some commelinid monocot species[69,]70 (Figure 9). After oxidation, both tricin and monolignols (or monolignol acetate or *p*-coumarate conjugates) may associate via radical coupling reactions to form tricin-(4'-*O*-β)-linked dimers[69,200]. As tricin is incorporated into a polymer in the form of 4'-*O*-β-coupled products and their higher oligomers, each tricin unit should localize at the initiating end of its lignin chain. Consequently, tricin acts as a nucleation site for lignin chain extension in monocots, a role that is also played by ferulate on arabinoxylans[201].

Figure 9. Tricin derivatives have been identified in lignin in grasses and other commelinid monocots. Three families of tricin derivaties, a, b and c, are whown with T = tricin; G = guaiacyl unit; S = syringyl unit; HP = *p*-hydroxyphenyl unit.

4 Cross-linking enzymes

4.1 Enzymes involved in lignin polymerization

Both peroxidases and laccases are responsible for catalyzing oxidative coupling reactions. Secreted peroxidases (class III) have been extensively gene duplicated and neofunctionalized. Peroxidases play roles in both scission of cell wall polymers and in the formation of cross-links in the wall[202,203]. In addition, peroxidases appear to be the prime enzyme catalysts for ferulic acid cross-linking[204] and the activity appears to be specific122. Laccases, at least the microbial enzymes, catalyze both cleavage and polymerization of lignin *in vitro*[205].

4.1.1 Laccases and peroxidases

Laccase and class III peroxidase enzymes belong to large multigene families. The overlapping expression patterns of the different members, as well as their apparent functional redundancy, make it challenging to assess the role of individual oxidoreductases in lignification. Arabidopsis contains 17 laccase genes[206] and 73 peroxidase genes[207].

The number of genes encoding putative class III peroxidases appears to be even higher in grasses, with respectively 143, 138, and more than 300 candidates in *Brachypodium*[208], rice[209], and the bioenergy crop switchgrass[210]. Recent reverse genetic studies have implicated several members in lignification (Table 3).

Table 3: Peroxidases and laccases genetically implicated in lignification.

Gene name[a]	Accession number / alternative name (group)[b]	Approach to modify gene expression	Observed phenotype	Reference
AtPrx2	*At1G05250 / AtP11 (L)*	T-DNA insertion	11% reduction of lignin content. 25% reduction in the *atprx2*/*atprx25* double mutant	211
AtPrx4	*At1g14540 / AtP46 (B)*	T-DNA insertion	37% reduction of lignin content under long-day conditions. Decrease of S-units	230
AtPrx17	*At2g22420 / AtP25 (D)*	T-DNA insertion 35S Overexpression	Reduction of lignin content. Increased lignin content	212
AtPrx25	*At2G41480*	T-DNA insertion	12% reduction of lignin content. 25% reduction in the *atprx2*/*atprx25* double mutant	211
AtPrx52	*At5g05340 / AtP49 (B)*	T-DNA insertion	12%-22% reduction of lignin content. Decrease of S-units	213
AtPrx64	*At5g42180 / AtP17* (H)	Endodermis-specific artificial microRNA knockdown	Delay in the formation of functional Casparian strips	214
AtPrx71	*At5g64120 / AtP15 (I)*	T-DNA insertion	Increased S/G	215
AtPrx72	*At5g66390*	T-DNA insertion	60% reduction of lignin content, reduced S/G	231
NtPrx60	AF149251	Antisense downregulation in tobacco	40-50% reduction of lignin content	216
PtrPO21		downregulation in Populus trichocarpa	20% reduction of lignin content	217
PrxA3a	*D38050*	Antisense downregulation in hybrid aspen	Up to 20% reduction of lignin content	218
tpx1	*L13654*	35S Overexpression in tomato	40% - 225% increase of lignin content	219
BdLAC5	*Bradi1g66720*	TILLING mutant	10% reduction of lignin content.	220
PtLAC2	*Potri.008G064000*	RNA silencing	No change in lignin content. Increased S/G	221
AtLAC4	*At2g38080*	T-DNA insertion	13.5% reduction of lignin content. 20-40% reduction in the *lac4*/*lac17* double mutant	222
AtLAC11	*At5g03260*	T-DNA insertion	Growth arrest and no lignin staining in the roots in the *atlac4*/*atlac17*/*atlac11* triple mutant.	223

AtLAC15	*At5g48100*	T-DNA insertion	30% reduction of lignin content in seeds	224
AtLAC17	*At5g60020*	T-DNA insertion	14% reduction of lignin content. Reduced G-units in interfascicular fibers. 20-40% reduction in the *lac4*/*lac17* double mutant	222
GhLac1	*KX822020.1*	35S Overexpression and RNA silencing in cotton	Increased and decreased lignin content, respectively	225
PtrLAC1 *PtrLAC2* *PtrLAC8* *PtrLAC14* *PtrLAC15* *PtrLAC18* *PtrLAC23* *PtrLAC24* *PtrLAC26* *PtrLAC30* *PtrLAC40* *PtrLAC41* *PtrLAC43* *PtrLAC44* *PtrLAC45* *PtrLAC46* *PtrLAC49*	*POPTR_0001s14010* *POPTR_0001s18500* *POPTR_0004s16370* *POPTR_0006s09830* *POPTR_0006s09840* *POPTR_0008s07370* *POPTR_0009s15840* *POPTR_0009s15860* *POPTR_0010s19090* *POPTR_0011s12100* *POPTR_0016s11950* *POPTR_0016s11960* *POPTR_0019s11820* *POPTR_0019s11830* *POPTR_0019s11850* *POPTR_0019s11860* *POPTR_0958s00200*	Downregulation in *Populus trichocarpa* by overexpression of the negative regulator Ptr-MIR397a	12%-22% reduction of lignin content	226
SofLAC	*SCVPRZ3027A08.g*	Overexpression in Arabidopsis *lac17* mutant	Restores lignin content to wild-type levels in the *lac17* mutant	227
ZmLAC3		Overexpression in maize	11%-20% increase of lignin content in mature stems	234

[a]Peroxidase gene names are according to Peroxibase[228]. [b]Names based on the Arabidopsis nomenclature and phylogeny from Duroux and Welinder[229].

Within the peroxidases, disruption of *At*Prx2211, *At*Prx4[230], *At*Prx17[212], *At*Prx25[211], *At*Prx52[213], or *At*Prx72[231] reduces lignin content in Arabidopsis, while disruption of *At*Prx71 increases the lignin S/G ratio[215]. Among these peroxidases, *At*Prx2, *At*Prx17, and *At*Prx25, are localized to the cell wall[211,212]. Similarly, down-regulation of the anionic class III peroxidases PrxA3a and *Ptr*PO21 in aspen[218] and *Populus trichocarpa*[217] respectively, and of the cationic peroxidase *Nt*Prx60 in tobacco[216],

results in reduced lignin content, indicating a role in lignification for these enzymes. The tomato basic peroxidase tpx1 is also involved in lignification as its overexpression results in an increase in the lignin content of leaves[219].

It is interesting to consider whether the peroxidases involved in lignin formation are recognisable by their sequences, i.e. whether they are closely related or belong to distinct phylogenetic clades.

Figure 10. Maximum likelihood phylogeny of rice and Arabidopsis peroxidases – overview and key to Figures 11 and 12. Clade names, where clade structure is maintained after the merger across the two species are indicated according to Duroux and Welinder[229] for Arabidopsis (marked in green) and according to Passardi et al.[209] for rice (marked in blue). Dashed lines indicate subclades for which enlarged and annotated views are provided in Figures 11 and 12.

Figure 10 provides an overview of the phylogeny of rice and Arabidopsis peroxidases, while Figure 11 shows clades harboring the peroxidases listed in Table 3.

Figure 11. A-D: Expanded view of clades from Figure 10 harboring Arabidopsis peroxidases implied in lignin formation (Table 3) indicated with arrows. Color-coding as in Figure 10.

Peroxidases involved in lignin synthesis appear to be encoded by small groups of very similar genes embedded across the mixed Arabidopsis-rice clades.

For laccases, truncation of *Bd*LAC5 reduced lignin content by 10% in *Brachypodium.* The modest reduction observed may be explained by the

compensatory activity of *Bd*LAC6, another cell wall localized laccase expressed in *Brachypodium* stems220. Disruption of AtLAC15, a gene coding for a laccase preferentially expressed in Arabidopsis seeds, resulted in a 30% decrease of lignin content224. Meanwhile, silencing the laccase encoded by *PtLAC2*, which is preferentially expressed in xylem in poplar, resulted in a change of the lignin S/G ratio but no change in lignin content, suggesting a role in the oxidation of cell wall-related phenolics for this laccase221. Disruption of *AtLAC4* or *At*LAC17 in Arabidopsis reduces lignification in stems222, and localization of these laccases in secondary cell wall domains directs lignification in culture systems for xylem cell differentiation[232]. Simultaneous disruption of *At*LAC4 and *At*LAC17 reduces lignin content further compared to the single mutants222. The involvement of the sugarcane laccase, *Sof*LAC, in lignification is supported by its expression profile in lignifying tissues and its capacity to complement the Arabidopsis *lac17* mutant227. The functional redundancy of laccases for lignification is also supported by the simultaneous disruption of *At*LAC4, *At*LAC17, and *At*LAC11 in Arabidopsis, which almost completely abolished root lignification and resulted in a growth arrest phenotype223. Interestingly, lignification of the Casparian strip, an apoplastic diffusion barrier in plants, is unaffected in this triple mutant. This corroborates the involvement of peroxidases in this precise lignification process223,214. In this process, an NADPH oxidase involved in the supply of H_2O_2 for peroxidase-mediated lignification has been identified and shown to be brought into proximity of localized peroxidases through the action of Casparian strip domain proteins214.

Deposition and maintenance of the Casparian strip in the endodermis are highly regulated processes[233]. Moreover, artificial microRNA knockdown of a peroxidase gene (*At*Prx64) specifically in the endodermis delayed the formation of this lignified diffusion barrier214. Recently, overexpression and downregulation of the laccase *GhLac1* gene in cotton resulted in an increase and a decrease of lignin content, respectively225. Increased lignin contents were also observed in maize lines that overexpress the laccase gene *ZmLAC3* [234] Finally, the simultaneous downregulation of 17 laccase genes in poplar (out of 49 sequences present in the genome) via the overexpression of a microRNA resulted in reductions of both laccase activity and lignin content226.

Table 3 does not comprise any enzymes annotated to be involved in lignan cross-linking for the simple reason that these have attracted much less interest than the dirigent proteins (4.1.3) and none are known. The seminal paper on the dirigent proteins[235] implied a laccase, and an extract from *Piper regnellii* leaves capable of producing (+)-conocarpan, a neolignan, was observed to be devoid of detectable peroxidase activity[236].

4.1.2 BAHD Transferases

Acyl transferases from the BAHD enzyme family153 have also been implicated in lignin acylation (Table 4). In this case there is no subcellular location conundrum, as was the case for xylan feruloylation. Withers et al.[237] have identified a rice *p*-coumaroyl CoA:hydroxycinnamyl alcohol transferase (*Os*PMT) that catalyzes the coupling of *p*-coumarate with monolignols via *p*-coumaroyl-CoA (see Figure 3).

*Os*PMT has a high affinity for sinapyl alcohol and *p*-coumaryl alcohol (as acyl acceptors) *in vitro*, yielding sinapyl-*p*-coumarate and *p*-coumaryl-*p*-coumarate. *Os*PMT homologs have also been identified in *Brachypodium*164 and maize[238]. Mutation of *Bd*PMT almost completely depleted the fraction of *p*-coumarate acylating lignin, whereas overexpression both increased the amount of *p*-coumarate groups on lignin by three-fold and decreased total lignin content. Interestingly, the amount of *p*-coumarate acylating arabinosyl units on arabinoxylans was unchanged in these transgenics, highlighting the specificity of *Bd*PMT for monolignols164. Similarly, *Zm*PMT was shown to use *p*-coumaroyl-CoA as a donor and monolignols as acceptors, with a strong preference for sinapyl alcohol. Downregulation of *Zm*PMT in maize led to a reduction of *p*-coumarate attached to lignin, and decreased levels of S-units in lignin, although the lignin content remained unchanged238.

The enzyme responsible for the transfer of benzoate groups onto monolignols has not been identified at present. Such a transferase likely belongs to the BAHD enzyme family, in which several members are known to use both hydroxycinnamoyl-CoA and benzoyl-CoA as donors[239,240]. Interestingly, a *Populus trichocarpa* BAHD transferase from clade V (*Pt*ACT47) that transfers benzoyl-CoA onto coniferyl alcohol has been discovered[241]. Whether *Pt*ACT47 uses 4-hydroxybenzoyl-CoA and sinapyl alcohol as substrates for lignin acylation remains to be determined. Alternatively, certain enzymes from the Serine CarboxyPeptidase

Like (SCPL) acyltransferase family, whih is capable of using hydroxycinnamoyl and benzoyl glucose esters as donors, are also plausible candidates[242].

The enzyme responsible for the transfer of acetate groups onto monolignols for lignin acetylation also remains to be identified. An acetyl-CoA:coniferyl alcohol transferase (*Ph*CFAT) from the BAHD family, specifically expressed in *Petunia* flowers, was shown to participate in the synthesis of the volatile compound eugenol[243]. Overexpression of *Ph*CFAT was conducted in poplar, aspen, Arabidopsis and tobacco in order to increase the eugenol content in these plants, but the impact on the levels of acetylation of lignin was not analyzed[244, 245]. The putative acetyl-CoA:monolignol transferase of the BAHD enzyme family involved in lignin acetylation in plants such as sisal and kenaf remains to be identified.

A couple of transferases that catalyze the synthesis of monolignol ferulate ester conjugates have been identified in Chinese angelica and rice[246,247]. These two enzymes, *As*FMT and *Os*FMT1 (AT5), belong to the BAHD family. *As*FMT preferentially uses feruloyl-CoA as a donor and monolignols as acceptors. Overexpression of *Os*FMT1 in rice and *As*FMT in poplar resulted in an increase of coniferyl ferulate in lignin of transgenic plants[246,247].

Table 4: BAHDs genetically involved in lignin acylation

Gene name	Accession number / BAHD clade	Approach to modify gene expression	Observed phenotype	Reference
OsPMT	*Os01g18744* / V	*p35S* and *pAtCesa7* overexpression in poplar and Arabidopsis, respectively	Incorporation in lignin of monolignol *p*-coumarate conjugates	248
BdPMT	*Bradi2g36910* / V	1) Missense mutant and RNA silencing 2) *pZmUbi1* overexpression in *Brachypodium* 3) *pAtC4H* overexpression in Arabidopsis	1) Reduction of lignin bound *p*-coumarate 2) Increase of lignin bound *p*-coumarate and S/G ratio 3) Increase of lignin bound *p*-coumarate	164 249
ZmPMT	BT042717.1 / V	RNA silencing	Reduction of cell-wall bound *p*-coumarate and lignin S units	238
AsFMT	AHL24755.1 / III	*p35S* and *pPtCesa*8 overexpression in poplar	Incorporation in lignin of monolignol ferulate conjugates. Slight increase of lignin S units	246
OsFMT1 / AT5	*Os05g19910* / V	Activation tagging and *pZmUbi1* overexpression	Incorporation in lignin of monolignol ferulate conjugates	247

4.1.3 Dirigent Proteins

Dirigent (DIR) proteins mediate regio- and stereoselective coupling of two phenoxy radicals generated by laccases or peroxidases during lignan biosynthesis. As examples, a DIR protein discovered in *Forsythia suspensa*[250] direct the coupling of coniferyl alcohol radicals to give the 8,8'-linked lignan dimer (+)-pinoresinol, whereas *At*DIR6 from Arabidopsis favors the formation of the 8,8'-linked dimer (-)-pinoresinol[251]. Such selective coupling of radicals is not observed in the absence of DIR proteins: The activity of laccases or peroxidases alone towards coniferyl alcohol molecules result in a racemic mixture of approximately equal amounts of (+/-) 8,8'-, (+/-) 8,5'-, and 8-*O*-4'-linked products251. DIR proteins have been

suggested to be involved in lignin biosynthesis by directing the polymerization of coniferyl alcohol[252,253], but so far, strong evidence for the participation of DIR proteins in the biosynthesis of lignin is missing. The exact function of lignans (8-8-linked monomers) and neolignans (8-5- or 5-5-linked monomers) in plants is not fully resolved, and their incorporation into lignin has never been evidenced. The occurrence of glycosylated lignans in the vacuole suggests their intracellular relocation, rather than crosslinking to cell wall components following synthesis in the apoplast22. Nevertheless, cotton plants overexpressing a DIR-like gene showed a higher accumulation of lignin in the epidermis and vascular bundles of leaves[254], whereas disruption of Pinoresinol Reductase 1 (PrR1) in Arabidopsis leads to decreased lignin content in stems[255], and a protective role against lignification-induced oxidative damage has been proposed for such reductases[256]. Finally, although not considered to be involved in the lignification of xylem vessels and fibers, one DIR domain-containing protein (*At*DIR10) has been implicated in lignin synthesis in the Casparian strip in the root of Arabidopsis, but its function remains speculative[257].

4.2 Peroxidases in extensin cross-linking

Both the formation of intra- and interchain cross-links in extensins are generally thought to be catalyzed by peroxidases. Fry149 demonstrated that isodityrosine (the intra-chain link) could be formed by peroxidase isoforms. Cross-linking to yield di-isodityrosine interchain cross-links is also catalyzed by peroxidases as demonstrated in elegant experiments[258] in which the substrates – glycopeptide

modules featuring isodityrosine moieties – were produced using synthetic modules expressed *in planta* followed by cross-linking *in vitro* using an extensin peroxidase purified from tomato suspension cultures. The *in vivo* situation was addressed using NMR showing that di-isodityrosine cross-links were at the limit of detection[259]. Four extensin peroxidases have been biochemically characterized and cloned. The sequence of one of these, GvEP1 from grapevine[260,261], has been lost. Biochemical evidence for the activity of the grapevine enzyme is strong and the *Vitis vinifera* genome comprises peroxidases that are very closely related to the three other extensin peroxidases. These are included in table 5, and in Figure 12.

Figure 12. A and **B:** clades of Figure 10 comprising biochemically characterized extensin peroxidases (Table 5) indicated with arrows. Colour-coding as in Figure 10.

The CG5 peroxidase in tomato, Solyc02g094180[262], has been shown to cross-link EXTs under physiological conditions. Lupin LEP1 peroxidase[263] and its close relative from French bean, FBP1[264], have been shown to cross-link extensins *in vitro,* but at a pH higher than normally found in the apoplast, and the two peroxidases were hypothesized to be activated in response to external stress stimuli.

The existence of peroxidases dedicated to extensins is not *a priori* necessary, but it is striking that the three characterized extensin peroxidases (and their putative grapevine orthologs) occupy subclades with no rice sequences and with no peroxidases implied in lignin formation (see Figure 12), suggesting specialization

and labour division among peroxidases. No Arabidopsis peroxidase(s) has been experimentally shown to cross-link EXT YXY motifs. It is possible that enzyme mass may correlate negatively with glycan decoration and that a higher isoelectric point may provide increased mobility within the cell wall allowing the peroxidase to more easily access the dense secondary cell wall231.

Table 5 includes the most similar Arabidopsis sequences to the three characterized extensin peroxidases. Expression of Arabidopsis peroxidases related to the tomato enzyme correlates to some degree with extensin expression, but extensin expression and expression of the enzymes responsible for post-translational modifications of extensins are generally not correlated191. Furthermore, given the role of extensins and cell wall cross-linking in pathogen defense[265], expression patterns of peroxidases often reflect this biological function, as exemplified by Prx37 in Table 5.

Table 5: Peroxidases demonstrated to be involved in extensin cross-linking or implied to be by phylogeny

Gene name	Accession number / alternative name (group)[a]	Approach to characterize enzyme or modify gene expression	Observed phenotype / Function / Expression/ Other	Reference
CG5 *(tomato extensin peroxidase pI 4.6)*	Solyc02g094180	Heterologous expression in *E.coli*	Covalently crosslinked tomato P1 extensin and P3-type extensin analogs *in vitro*	262
AtPrx35	AT3G49960.1 / AtP21 (M)			
AtPrx50	AT4G37520.1 / AtP9 (M)			
AtPrx16	AT2G18980.1 / AtP22 (M)			
AtPrx55	AT5g14130.1 / AtP20 (M)			
LEP1 *Lupinus albus* (Lupin)	AF403735	Purified from vegetative organs	Apoplastic extensin cross-linking activity in vegetative organs	263
FBP 1 – (*French Bean Peroxidase 1*)	AAD37427 (AF149277_1)	Expressed in *Pichia pastoris*	FBP1 covalently crosslinked tomato P1 extensin and P3-type extensin analogs *in vitro*. FBP1 and other members of the family, were all induced in cell cultures by elicitors	264
AtPrx22	AT2G38380 / AtPEa (A)	Supressed expression in roots by Zn^{2+}		266
AtPrx23	AT2G38390 / AtP34 (A)			
AtPrx32	AT3G32980 / AtP16 (A)			
AtPrx37	AT4G08770 / AtP38 (A)	*AtPrx37* promotor*::GUS* expression;35S::*AtPrx37* over expression; Ectopic over expression and knock out of transcription factor DEWAX	Constitutive over expression confers a dwarf phenotype, delayed development and an increase in the amount of esterified phenolic material	261 267

			associated with their walls.	
AtPrx38	AT4G08780 / (A)	Ectopic over expression of transcription factor DEWAX	Over expression of *PRX38* leads to increased expression of the transcription factor *DEWAX*	267
AtPrx58	AT5G19880 / AtP42 (A)			
AtPrx54	AT5G06730 / AtP29 (A)			

4.3 Peroxidases involved in cross-linking of feruloylated GAX

As with extensin peroxidases, it is not a given that specialized peroxidases are required to form ferulate cross-links. In fact, to the best of our knowledge, no peroxidase has yet been associated specifically with GAX cross-linking. However, pronounced differences in GAX cross-linking activity have been found between enzymes secreted by maize suspension cultures and horseradish peroxidases, indicative of a specialization of peroxidases[122,268]. In rice, *Os*Prx110-114 have been implicated in ferulate cross-linking, however solely based on their expression profiles[268]. While *in vitro* activity is still missing it is interesting that these peroxidases belong to clade IV3, one of the clades with no Arabidopsis accessions. It should be noted however that Figure 9 comprises several clades or sub-clades that are found only in rice.

5 Cross-linking enzymes in biotechnology

5.1 Fiber cell composites

Cross-linking enzymes (e.g. oxidase-type enzymes) as an emerging tool have gained increasing attention in relation to fabrication of green cellulose-based materials, such as composites and hydrogels. A composite is a multi-phase material that exhibits a significant proportion of the properties of the constituent phases in such way that an improved combination of mechanical characteristics, such as stiffness, toughness, and ambient and high-temperature strength, is created [269]. Many composite materials are composed of just two phases: a continuous phase (the matrix), which surrounds the reinforcing material (dispersed

phase). In principle, the mechanical properties of composite materials are dependant on both the fibers and the interfacial properties between the matrix and the reinforcement phase. In the cellulose-based composites discussed here, the dispersed phase of reinforcement refers to different types of cellulose fibers (e.g. whole fiber cells, microcelluloses and nanocelluloses), while the matrix can be any polymeric material.

Oxidase enzymes (i.e. laccases and peroxidases) are the most commonly used enzymes in cellulose-based material fabrication in order to improve their mechanical and physical properties and reduce the consumption of synthetic polymers. In general, oxidase enzymes have three different roles in cellulose-based material fabrication (Table 6): (1) Polymerization and cross-linking of lignin or other phenols in lignocellulosic materials to enclose cellulose fibers in the lignified or cross-linked network[270]; (2) Enzyme-catalyzed grafting of hydrophobic chemical agents[271,272] (e.g. alkyl gallate and lauryl gallate) onto the surface of cellulosic fibers to enhance the compatibility between fibers and hydrophobic polymers; (3) Enzyme-catalyzed grafting of germicidal agents or antimicrobial compounds (e.g. natural phenols, chitosan and catechin) onto the surface of cellulosic fibers to develop or to improve antimicrobial properties of fibers286[,273].

Table 6 Application of oxidase enzymes in lignocellulosic materials.

Lignocellulosic material	**Enzyme**	**Matrix polymer**	**Chemicals used for fiber surface modification**	**Manufacturing process**	**Role of laccase treatment**	**Reference**
Hemp bast fiber	Laccase (*Trametes versicolor*)	Epoxy	/	Vaccum infusion moulding	Polymerization and crosslinking of lignin	274
Plantain fiber	Laccase (*Aspergillus oryzae*)	None (self-bonding board)	/	Hot pressing	Polymerization and crosslinking of lignin	275
Hemp hurds and flax shives	Laccase (*Aspergillus sp.*)	None (self-bonding board)	/	Hot pressing	Polymerization and crosslinking of lignin	276
Jute fiber	Laccase (*Trametes versicolor*)	Polypropylene	/	Hot pressing	Polymerization and crosslinking of lignin	277
Kraft pulp	Laccase (*Trametes pubescens*)	Ultra-filtered lignin	/	Cold pressing followed by hot pressing	Polymerization and crosslinking of lignin	278
Rubber wood fiber	Laccase	Hydrolyzed lignin or urea formaldehyde	/	Cold pressing followed by hot pressing	Polymerization and crosslinking of lignin	279
Jute fiber	Laccase (*Aspergillus sp.*)	Polypropylene	Alkyl gallate	Hand lay-up followed by hot pressing	Polymerization and crosslinking of lignin	280
Wood fiber	Peroxidase SP 502	None (self-bonding board)	/	Hot pressing	Polymerization and crosslinking of lignin	281
Microfibrillated cellulose	Laccase (*Trametes versicolor*)	Glycerol	Galactoglucomannan-lignin network	Gelation followed by solution casting	Polymerization and crosslinking of lignin	282
Cellulose nanocrystals	Laccase (*Pycnoporus cinnabarinus*)	/	Feruloylated arabinoxylan oligomers	Gelation followed by solution casting	Polymerization and crosslinking of phenols	283 284
Kraft pulp	Laccase (*Trametes villosa*)	/	Lauryl gallate	/	Hydrophobization of cellulose fiber	285
Linen fiber	Laccase (*Ascomycete myceliophthora thermohpila*)	/	Chitosan and catechin	/	Grafting germicidal agents onto fiber surface	286
Flax pulp	Laccase (*Pycnoporus cinnabarinus*)	/	Syringaldehyde acetosyringone *p*-coumaric acid	/	Grafting natural phenols onto fiber surface	287

Oxidase enzymes have long been used to produce medium-density (MDF) and high-density fiberboards (HDF) from wood fiber or pulp either with fewer or completely without binders (e.g. urea-formaldehyde). Oxidase enzymes catalyze cross-linking of lignin moieties and covalent binding of the phenolic compounds to wood fiber. As such, binder-less or binder-free fiberboards can be made via hot-pressing after enzyme treatment275,288,281,289,281,290. It has been found that binder-less and binder-free fiberboards have comparable mechanical properties with traditional fiberboards.In general, the concept of using oxidase enzymes to produce binder-less fiberboards not only reduces consumption of synthesis binders but also aims to modify cellulose fiber surfaces to increase the hydrophobicity of the fibers and, therefore, improve the compatibility between the fibers and matrix polymers (e.g. lignin or synthetic binder).

Grafting of hydrophobic chemical agents onto jute fabric by laccase has been accomplished using gallate carrying linear alkyl chains of different lengths280. The hydrophobicity of the jute fibers was assessed by measuring the water contact angle, which increased from 106.61º in the control fibers to 117.54º, 121.70º, and 133.01º respectively for gallates carrying alkyl chains of length 3, 8, and 12 carbons. Consequently, water absorption of fibers decreased from about 25 weight percent in the control fibers to about 20 in the treated fibers, and improved interfacial bonding between fibers and polypropylene (PP) was achieved in jute/PP composites280.

Fillat et al.287 attempted to graft different phenolic compounds, including syringaldehyde, acetosyringone and *p*-coumaric acid, onto the surface of unbleached flax fibers using laccases to obtain fibers with improved antimicrobial properties. All treated fibers showed antimicrobial activity in inhibiting the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*, and acetosyringone and *p*-coumaric acid grafted fibers showed a high antibacterial activity against *K. pneumonia* compared to syringaldehyde-grafted fibers.

5.2 Cellulose nanofibril composites

The demand for green materials with functionality, uniformity, and high mechanical strength-to-weight performance is increasing. These qualities cannot be achieved in materials based on whole fiber cells. Instead, cellulose nanofibrils with a width below 100 nm can be derived from the plant cell wall and used to form nanostructured materials[291,292]. Compared to whole fiber cells, cellulose nanofibrils (high purity of cellulose) contain no structural elements from plants other than the cellulose microfibril. The intra- and inter-chain hydrogen bonds make such fibrils remarkably stiff and stable. Crystalline cellulose fibrils have been estimated to have an axial elastic modulus greater than Kevlar, as well as low density[293].

Due to their inherent strength and self-interacting properties, cellulose nanofibrils have shown a strong reinforcement effect in composite materials made with various polymer-matrices[294,295]. However, the performance of these materials is still far from the theoretical potential of the fibrils. A major bottleneck is the hydrophilicity of cellulose which causes poor fibril-matrix adhesion and agglomeration during formation of composites. These problems may partly be overcome by chemical

introduction of functional groups to the surface of cellulose. However, altering the chemistry of cellulose ultimately has negative effects on its structural stability and interactions within a final composite[295].

The plant cell wall supposedly achieves cellulose-matrix adhesion using an alternative approach by bridging via other biopolymers. Therefore, a biomimetic approach has been explored where functional groups were enzymatically and chemically introduced into xyloglucans, potentially allowing cellulose to be indirectly attached to a hydrophobic polymer[296,297]. More recently, bioinspired materials have been developed based on evidence that complex networks between hemicelluloses and lignin can be synthesized in vitro using laccase[282,298]. It has further been shown that cellulose nanofibrils can be entrapped in the formed hemicellulose-lignin network enabling the synthesis of composites and films[298,282]. These authors further reported that they made barrier films and composites from cellulose, the galactoglucomannan-lignin network, and glycerol. Nanofibrils, produced from carboxymethylated cellulose, were first mixed with galactoglucomannan-lignin, and then cross-linked between lignin-moieties with laccases to enclose cellulose in the formed networks. Finally, the composites were produced by reinforcing the formed cellulose-hemicellulose-lignin networks in a plasticizer. Even though the material strength and stiffness were not on par with plant cell walls, the materials exhibited low oxygen permeability.

In a similar approach, bioinspired assemblies of secondary cell walls were made from cellulose nanofibrils and ferulic acid-substituted arabinoxylans283. Laccase

was used to cross-link the ferulic acids and thus embed the cellulose nanofibrils in a matrix. This study did not comprise the mechanical properties of the materials. Instead, the mobility of fluorescent probes and cellulose binding domains within the assemblies were examined to reveal features influencing enzymatic activity on lignocellulosic biomass [283,284].

5.3 Hydrogels

Hydrogels are insoluble polymer networks that can retain large amounts of water upon formation. Hydrogel technology is applied to a wide range of applications, such as drug delivery systems, tissue engineering, food additives etc.[299]. Additional chemical cross-linking of the polymers in the hydrogels is sometimes necessary to make a robust hydrogel, for example when used as support-material in damaged tissue. Among in-organic polymers, proteins, and polysaccharides, cellulose is considered an excellent polymer for preparing hydrogels due to its hydrophilicity, abundance, non-toxicity, and biocompatibility[300]. In addition, the surface chemical properties of cellulose provide possibilities for functionalization. For example, covalent incorporation of tyramine onto carboxymethyl cellulose was achieved via formation of di-tyrosine cross-linked hydrogels. Cross-link formation was catalysed by horseradish peroxidase and hydrogen peroxide. As di-tyrosine cross-linked hydrogels can be formed under mild conditions with minimal side reactions, there is potential for making these materials *in situ*, e.g. for incorporation of cells or tissue re-generation[301].

Pectin has also been used in synthesis of hydrogels. A number of studies have been conducted to improve the viscosity and gelling performance of sugarbeet

pectin. The major deficiency of sugar beet pectin is a very low average molecular mass of 10 000 Dalton, compared to, for example, lime pectin which has an average mass of 150 000 Dalton. Sugarbeet pectin is rich in RGI encompassing ester bound ferulic acid residues on the side-chains (Figure 4). Sugarbeet pectin has been extracted by an enzymatic process using commercial enzymes (Driselase or SP584) affording low molecular mass fractions. Arabinan side chains are very sensitive to acid extraction but mild enzyme-based extraction procedures may preserve the structure of the arabinan chains and give rise to appreciable number of arabinan chains with high ferulic acid content. Several studies have used this arabinan fraction for oxidative cross-linking[302,303,]. The feruloylated arabinan contains 2-14 arabinose residues, and 1 to 2 feruloylated residues on each chain after separation on a feruloylated affinity column. When this mixture of feruloylated arabinans was subjected to oxidative reaction (horseradish peroxidase and H_2O_2), the content of monomeric feruloyl residues declined while di-ferulic residues increased. The product also expressed high-gelling and high-viscosity performance, which has commercial interest.

6 Cleaving and engineering cross-links

Lignin represents a very large resource of aromatic feedstock for a wide variety of applications. Lignin depolymerization with the aim of exploiting its building blocks involves mainly non-enzymatic catalysis and has recently been reviewed[304]. For other applications the cleavage of lignin inter-unit linkages is relevant as a means of overcoming biomass recalcitrance. It is of interest, for example, in the manufacture of nanocellulose for advanced biomaterials (preceeding section) and in biofuel

production. Harsh chemical treatments, usually with hypochlorite, are common in nanocellulose production. Environmentally friendly enzyme-mediated alternatives are desirable[305] and relevant in an attempt to overcome the recalcitrance of biomass also for biofuel production. Ester bonds between polysaccharides and phenolic moieties are by far the most labile. However, they make up a minor proportion of the bonds found in cross-linked material. Ether bonds are much more common in phenolic cross-links but cleaving these requires stronger agents. As such, it is attractive to introduce more labile bonds into biopolymers in order to improve biomass for biofuel production.

6.1 Feruloylesterases as auxiliary enzymes

In transgenic tall fescue (*Festuca arundinacea*) or wheat overexpressing the *Aspergillus niger* ferulic acid esterase gene (faeA), a reduction of esterified ferulates was observed[306,307]. In a subsequent experiment, larvae of the leaf grazing fall armyworm (S*podoptera frugiperda*) were fed leaves of the transgenic tall fescue. Larvae growth, mortality and food utilization were found to negatively correlate with the degree of feruloylation in the transgenic leaves[308]. The aim of these studies was to modify the cell wall in order to improve its degradability and increase its solubility and extractability. Ferulates were cleaved off from the polysaccharide chain either *in muro* or during their transport through the Golgi. Potential drawbacks to utilizing such approaches are the phenotypic consequences on the plant yield306.

6.2 Reduction of lignin-xylan linkages

Several studies have documented the occurrence of γ-ester linkages between hydroxyl groups from lignin building blocks and glucuronic acids from glucuronoarabinoxylans in pine, birches, and beechwood[309,310]. Glucuronoyl esterases (EC 3.1.1.-) are accessory enzymes that act on the esters of 4-O-methyl-D-glucuronic acid (MeGlcA), which are grouped into the Carbohydrate Esterase (CE) family 15 according to the CAZy classification112,[311]. Expression of genes encoding CE15 enzymes was attempted in Arabidopsis and hybrid aspen to reduce the presence of lignin-xylan crosslinks and hereby improve biomass saccharification. Counterintuitively, transgenic aspen that expressed a gene encoding a cell-wall targeted version of a CE15 from the white-rot basidiomycete *Phanerochaete carnosa* (*Pc*GCE) showed higher lignin content (+ 40%) and a reduced amount of cellulose and MeGlcA groups. Nevertheless, saccharification of the biomass after acid pretreatment resulted in an increase in cellulose conversion in the transgenic lines, indicating that intermolecular cross-linking targeted by *Pc*GCE inhibits the enzymatic digestibility of lignocellulose[312]. These results partially corroborate the results from an original study on the heterologous expression of *Pc*GCE in Arabidopsis cell walls[313]. In this study, transgenic Arabidopsis plants displayed reduced glucose and xylose content, as well as reduced cell wall thickness in the interfascicular fibres. Interestingly, xylose recovery was improved despite the fact that the content of insoluble lignin was unchanged.

6.3 Lignin modifying enzymes

Cleavage of phenolic cross-links in nature is mainly observed in the context of wood-decaying processes mediated by fungi and bacteria[377]. Next to a whole battery of polysaccharide-degrading enzymes (e.g. cellulases, hemicellulases, and pectinases), some fungi and bacteria express genes encoding oxidative enzyme systems, which predominantly serve to solubilise lignin in the plant cell wall to increase accessibility to, and enable hydrolysis of, cellulose and hemicelluloses. Most wood-decaying fungi are not able to grow on lignin[314,315]. However, there are examples of both fungi and bacteria that rely on lignin as their sole carbon source (Table 7).

Table 7. Lignolytic microorganisms

Taxa	Phylum	References
Aspergillus sp.	Fungi	316 317
Achremonium sp Fusarium oxysporum Trichoderma sp Verticillium sp Verticillium sp Trichocladium canadense	Fungi	316
Phoma herbarum	Fungi	318
Bacillus sp.	Bacteria	319 320
Aneurinibacillus aneurinilyticus	Bacteria	321
Burkholderia sp.	Bacteria	322 319
Serratia sp., Pseudomonas chlororaphis, Stenotrophomonas maltophilia, Mesorhizobium sp.	Bacteria	323
Acinetobacter sp.	Bacteria	324
Tolumonas lignolytica	Bacteria	325
Oceanimonas doudoroffii	Bacteria	326
Aquitalea sp., Cupriavidus sp., Gordonia sp., Paenibacillus sp.	Bacteria	319
Enterobacter lignolyticus	Bacteria	327

Pandoraea sp.	Bacteria	328
Comamonas sp.	Bacteria	329

Lignin-degrading peroxidases are mainly found in class II of non-animal heme-peroxidases, which are comprised of secreted fungal peroxidases[330]. Among those peroxidases four subgroups are defined: (A) lignin peroxidases (LiP), (B) manganese peroxidases (MnP), (C) versatile peroxidases (VP), and (D) dye-decolorizing peroxidases (DyP). These are heme-dependent enzymes with a high redox-potential of 0.8-1.5 V and a relatively broad substrate specificity; active on phenolic substrates, nonphenolic lignin model compounds and a range of organic compounds in the presence of $H_2O_2$314.

Figure 13. The mechanism of radical mediated lignin depolymerisation. The reaction can be started either by a peroxidase or by a laccase-mediator-system (LMS). Black structures in the bracket represent radical resonance structures. Blue structures are linked to polymeric lignin while red structures are released degradation products.

Laccases are another class of lignolytic enzymes and are present in all life kingdoms. They are grouped into true fungal *sensu stricto* laccases, fungal pigment multicopper oxidases, fungal ferroxidases, ascorbate oxidases, and plant and insect laccases. Laccases are copper-dependent enzymes with a broad substrate range and a slightly lower redox potential of 0.3-0.8 V compared to the peroxidases205. Laccases usually catalyze the formation of phenoxy radicals

through removal of a single electron from a phenolic hydroxyl group along with reduction of O_2 to H_2O. Radical mediated lignin cleavage is outlined in Figure 13. Upon radical generation in the lignin several reactions may occur: ring cleavage, demethoxylation, benzylic oxidation, β-O-4 cleavage or α-β scission[205]. Recent thermodynamic considerations appear to support the hypothesis that laccases alone are insufficient to catalyze lignin cleavage[331]. In contrast, recent EPR studies on laccase activity on lignin revealed that these are indeed able to generate radicals in lignin, and that the redox potential is not predictive of the reaction efficiency: The laccase from *Myceliophthora thermophila* (redox potential 0.4 V) showed a somewhat higher radical generation rate in lignin than the laccase from *Trametes versicolor* (redox potential 0.8 V)[332]. Nevertheless, the use of a so-called laccase mediator system is considered to catalyze bond cleavage in lignin substrates205. The laccase-mediator interactions may explain why some lignin-degrading microorganisms do not possess peroxidase-encoding genes in their genomes. For the brown rot basidiomycete *Postia placenta*, it was shown that laccases expressed by the fungus utilized 2,5-dimethoxyhydroquinone as a mediator for reduction of Fe^{3+} to Fe^{2+} with concomitant H_2O_2 production. Hydroxyl radicals (•OH) generated via the Fenton reaction were then, due to their small size, able to diffuse into the plant cell wall to start the radical reaction necessary to degrade lignin and cellulose[333] (Figure 13). In the natural eco-systems involved in carbon turnover of the complex plant cell wall matrix, fungi and bacteria form a complex synergistic and competitive community, in which they not only compete for

nutrients but also for space. The extent of the complexity of the interactions in this process is far from understood. Data based on DNA-sequencing shows that fungi dominate the decomposition in the upper soil horizons, while bacteria are more important in deeper ones[334]. This synergistic action was recently demonstrated using a laccase as an isolated enzyme and a soil bacterium[335]. Bacterial growth on lignin was significantly improved when a laccase was added to the cultivation medium. The addition of reactants for the Fenton reaction led to further improvement of growth. A comprehensive overview of the enzymes, these organisms and their evolution has been provided by Janusz and colleagues[336].

6.4 Engineering inter-unit linkages in lignin

Modification of inter-unit linkages within lignin is a promising approach to alter lignin structure and reduce its recalcitrance during biomass pretreatments. These modifications may be carried out in ways that do not compromise agronomic performance. Engineering plants for the expression of endogenous laccases represents one such strategy to enhance biomass quality[337]. The downregulation of laccase enzymes involved in lignin formation resulted in an improvement of biomass saccharification efficiency in *Arabidopsis*222, *Brachypodium*220, and poplar221. Modification of lignin by incorporation of non-canonical monomers has also showed promising results[338, 339,]126. In particular, incorporation of hydroxycinnamate esters, such as coniferyl ferulate, into the lignin backbone of poplar by overexpressing a feruloyl-CoA:monolignol transferase (FMT) enhanced both biomass saccharification efficiency and chemical pulping246[,340,341].

These positive results are attributed to a higher degree of readily cleavable ester linkages present in the lignin backbone due to the incorporation of the hydroxycinnamate ester. Similar results have been achieved by incorporation of rosmarinic acid (another hydroxycinnamate ester) within lignin as previously illustrated *in vitro*[84]. Incorporation of catechol (e.g., caffeyl and 5-hydroxyconiferyl alcohols) represents another approach to reduce biomass recalcitrance. Indeed, catechol groups reduce the formation of benzyl ether and ester cross-links between hemicelluloses and lignin during the β-O-4 coupling of monomers due to internal trapping of the quinone radical intermediate and formation of benzodioxane structures[342,343]. Moreover, linear lignin polymers consisting of benzodioxane units may have properties that make them amenable for use in value-added products, such as lignin-based carbon fibers[344].

Interfering with the elongation process of lignin chains has been successfully demonstrated to enhance biomass utilization. To this end, *in planta* synthesis of monomers that lack either a β-carbon or a free para-hydroxyl group have been achieved[345,346]. For example, expression in Arabidopsis of a *Pseudomonas fluorescens* hydroxycinnamoyl CoA hydratase-lyase generated plants that accumulated hydroxybenzaldehydes, which are known to form end groups in lignins[80,]345. The resulting transgenic plants contained lignins with shorter chains and showed increased biomass digestibility. A similar result might be obtained through expression of the gene encoding vanillin synthase, which catalyzes the conversion of ferulic acid into vanillin[347,348,349]. An isoeugenol *O*-methyltransferase

from *Clarkia breweri* was engineered to catalyze the methylation of para-hydroxyl groups on monolignols, preventing them from β-O-4 coupling with the free monomer. Introduction of the engineered enzyme into poplar resulted in an altered lignin content and structure, and improved biomass saccharification efficiency346. A similar approach would be to increase the content of tricin in lignins since it can only incorporate lignin via 4-O-β-coupling with a monolignol, and therefore, can only appear at the initiating end of the lignin chain (Figure 2). The recent discovery of tricin biosynthetic enzymes provides the prerequisite for such studies[350,351,352,353]. Biomass recalcitrance to degradation may be reduced by altering the lignin biosynthetic pathway to change the monomeric composition and linkage types of lignin[354]. This is the case for the aldehyde-rich lignin obtained via reduction of cinnamyl alcohol dehydrogenase activity[355,356]. Such lignins have a more hydrophobic surface that reduces its association with hemicelluloses[357]. Overexpression of ferulate 5-hydroxylase (F5H) leads to the formation of linear S-lignin, in which monomers are attached with β-O-4 linkages, whereas downregulation of coumarate 3-hydroxylase (C3H) leads to the formation of H-rich lignin. In both cases, biomass saccharification efficiency after ionic liquid pretreatment of these transgenic plants is enhanced compared to the control plants[358]. Downregulation of caffeoyl-CoA *O*-methyltransferase (CCoAOMT) was shown to decrease the content of G monomers and to improve the efficiency of biomass enzymatic hydrolysis in alfalfa[359].

Lastly, identifying the transferase responsible for attaching acetate groups on lignin could represent a promising target for biomass engineering since a positive correlation between acetate content in biomass and saccharification efficiency was recently observed following certain biomass pretreatments in poplar[360].

Another approach to modify lignin structure is *in planta* degradation of β-aryl ether bonds through expression of bacterial lignin-degrading enzymes. In Arabidopsis, expression of the gene encoding the Cα-dehydrogenase (LigD) from *Sphingobium* sp strain SYK-6 altered the lignin structure, which was reflected by an increased level of G-type α-keto-β-O-4 linkages[361]. In a more extensive approach, co-expression of the genes encoding cell wall targeted LigD, LigF and LigG (Figure 14) resulted in the disruption of a detectable portion of the β-aryl ether bonds in lignin. The number of oxidized G and S units was increased but these units were no longer bound in α-keto-β-O-4 linkages. This resulted in improved biomass saccharification efficiency[362].

Figure 14. Pathway for degradation of β-O-4-linked units by Lig enzymes from *Sphingobium paucimobilis* exemplified with the model compound β-hydroxypropiovanillone[363]. GS, glutathione; GSSG, glutathione disulfide (oxidized GS).

The enzymes LigF and LigG are glutathione transferase enzymes (GSTs) (see Figure 14). GSTs of the plant specific lambda class (GSTLs) display *in vitro* activities orthogonal to that of LigG, namely the reductive cleavage of glutathione conjugates[364,365], as demonstrated recently *in planta*[366]. While no endogenous GST-

mediated cleavage of lignin has yet been identified *in planta*, phenolic profiling in the studies of Mnich et al.362 indicated that a LigDFG-like pathway operates in wildtype Arabidopsis plants. Glutathione as well as GSTs (and other glutathione dependent enzymes) are present in the apoplast[367,368,369,370,371,372]. The possibility that an endogenous GST-mediated pathway for cleaving lignin crosslinks exists is intriguing but it remains to be investigated whether the hypothetical LigDFG-like pathway is apoplastic or, more likely, acts on intracellular substances such as lignans. Elucidation of this proposed pathway can open up new possibilities for engineering lignin structure, although the low apoplastic levels of glutathione may continue to be a bottleneck.

Recently, increased efforts have been made in exploring Lig enzymes from *Sphingobium sp*. SYK-6[373,374,375,376] and recently discovered, related enzymes from other bacteria[377,378]. These research initiatives aim to optimize the Lig enzyme systems for use in “Green chemistry”, i.e. industrial scale *in vitro*-cleavage of lignin as a source of phenolics for the chemical industry as an alternative to the current production from fossil fuels. Use of Lig enzyme systems may also pave the way for the environmentally benign enzyme-based depolymerization of lignin in order to circumvent the current bottleneck in biofuel production. Several studies show that Lig enzymes tolerate extensive variation of side-chain structures for their substrates. This includes the ability to cleave both lignin- and non-lignin-type model substrates, and the ability to release many different monomers from softwood and hardwood lignins[373,377,378]. The released monomers included the high-value

compound vanillin and ferulic acid373. The latter opens up the interesting possibility that Lig enzymes may also be able to cleave the lignin-ferulate or the ether 8-O-4 ferulate-ferulate crosslinks that are prevalent in grasses[379,380]. Identification of the hypothetical plant pathway for cleavage of α-keto-β-O-4 linkages may also prove valuable to the green chemistry approach. For instance, such enzymes may prove to be highly stable in a strongly oxidizing environment if their natural habitat is found to be in the apoplast.

7 Concluding remarks

In this review, we have provided an overview of the phenolic cross-links that occur in plant cell walls. These structures, as well as the genes and enzymes responsible for their metabolism, have evolved to serve mainly structural purposes in the plants. We may see the structures as a source of inspiration for biomaterials or as impediments to the use of plant material for animal feed or biofuel. Our understanding of phenolic cross-links, their biological function, and how they are formed and cleaved is substantial, yet leaves us with a number of obvious shortcomings that future research should address:

- The precise biochemistry and subcellular localization of polysaccharide feruloylation remains to be elucidated.
- Interplay between plant peroxidases involved in extensin and lignin inter-unit linkages and the non-redundant roles of laccases and peroxidases in lignin polymerization have been demonstrated, but it remains to be discovered which peroxidases and/or laccases that catalyse GAX and lignan cross-linking. A

comprehensive phylogenetic analysis of the peroxidase gene family resulting in consistent naming of clades across species would be conducive.

- Formation of intra-molecular vs. inter-molecular polysaccharide cross-links is difficult to predict and requires further studies in both Poacea and Caryophyllales.
- A realistic mechanism for the introduction of ether bond formation between sugars and phenyl propanoids may require the identification of a yet unrecognized type of enzyme.
- Elucidating the fine structure of cross-links between biopolymer families, extensin-RGI, extensin-lignin, lignin-mannan etc. is instrumental to the study of higher-order structures.
- Considering the importance of plant-microbe interactions in the environment and the impact of roots in shaping the rhizosphere, in-field evaluations of crops designed with modified lignin structure and/or phenolic cross-links should be prioritized.

A more thorough understanding of these issues would allow the plant breeder or the plant biotechnologist to more effectively breed for cell wall traits, which in turn will be conducive to developing cell wall deconstruction technologies.

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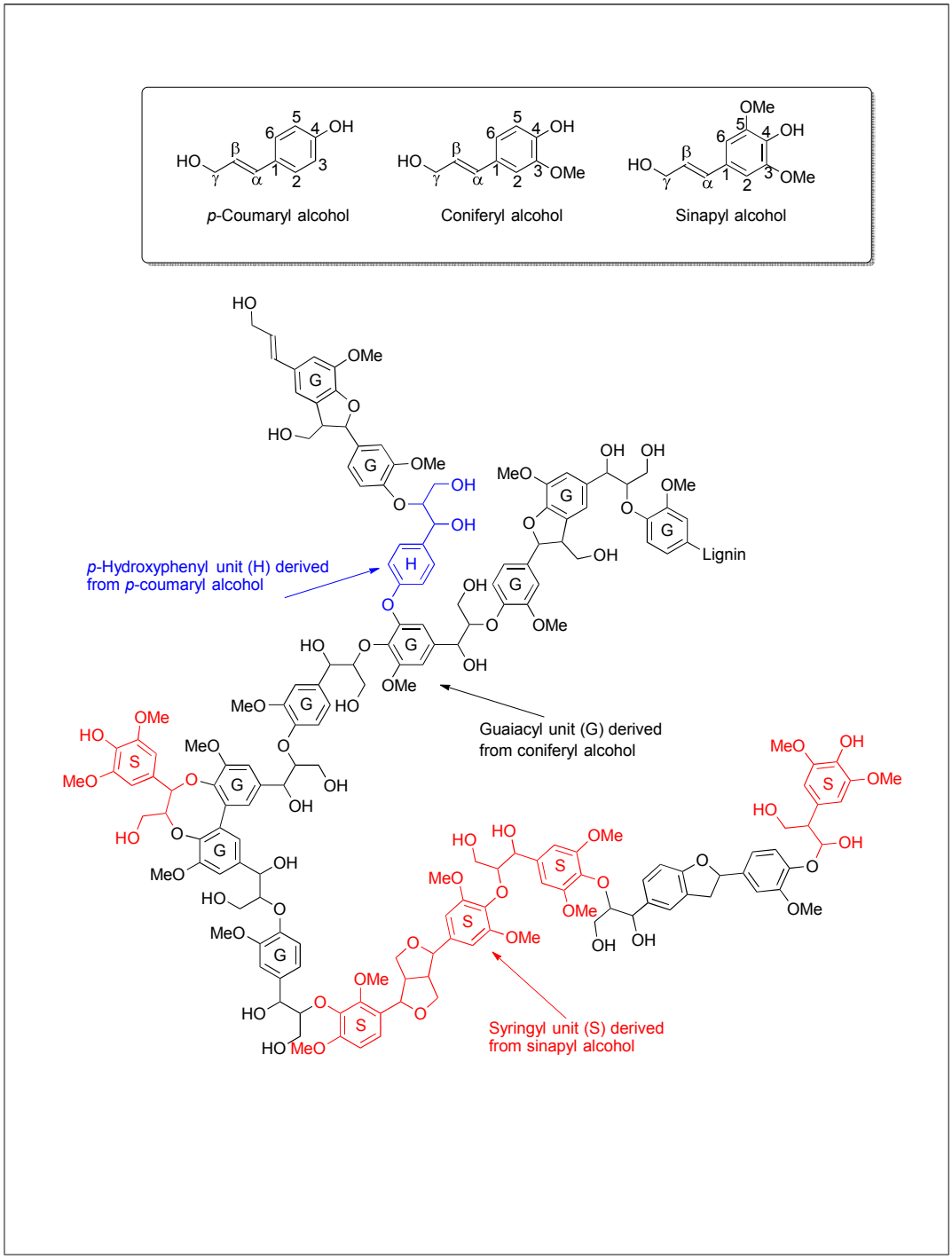

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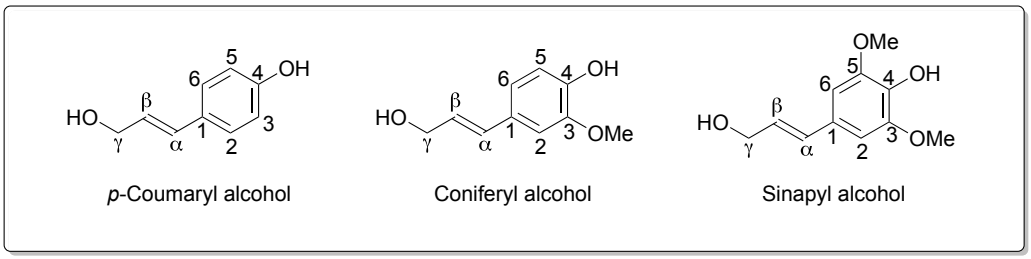

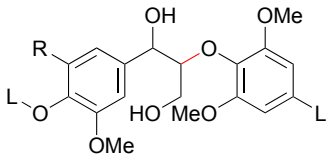

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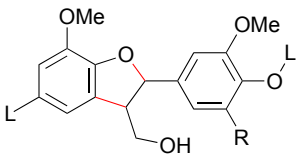
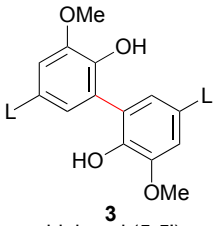
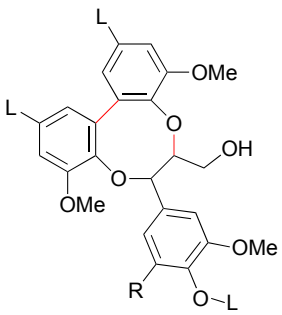
aryl α-aryl ether (α-O-4')



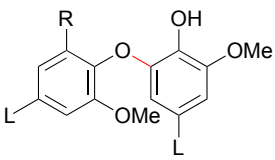
2
phenylcoumaran (β-5'/α-O-4')



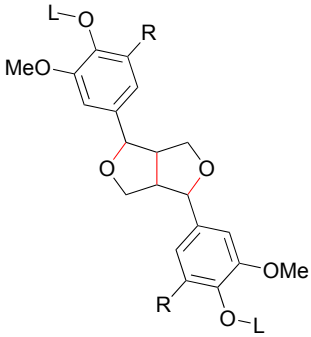
3
biphenyl (5-5')



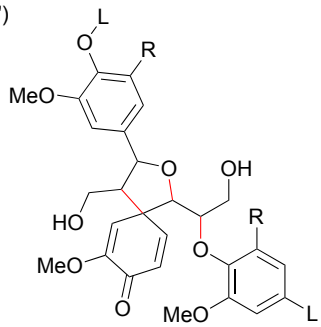
4
dibenzodioxocin (5-5'/4-O-β''/4'-O-α'')



5
diaryl ether (4-O-5')



6
resinol (β-β'/α-O-γ'/α'-O-γ)



7
spirodienone (β-1'/α-O-α'/ β'-O-4'')

R = H or OMe
L = Lignin backbone

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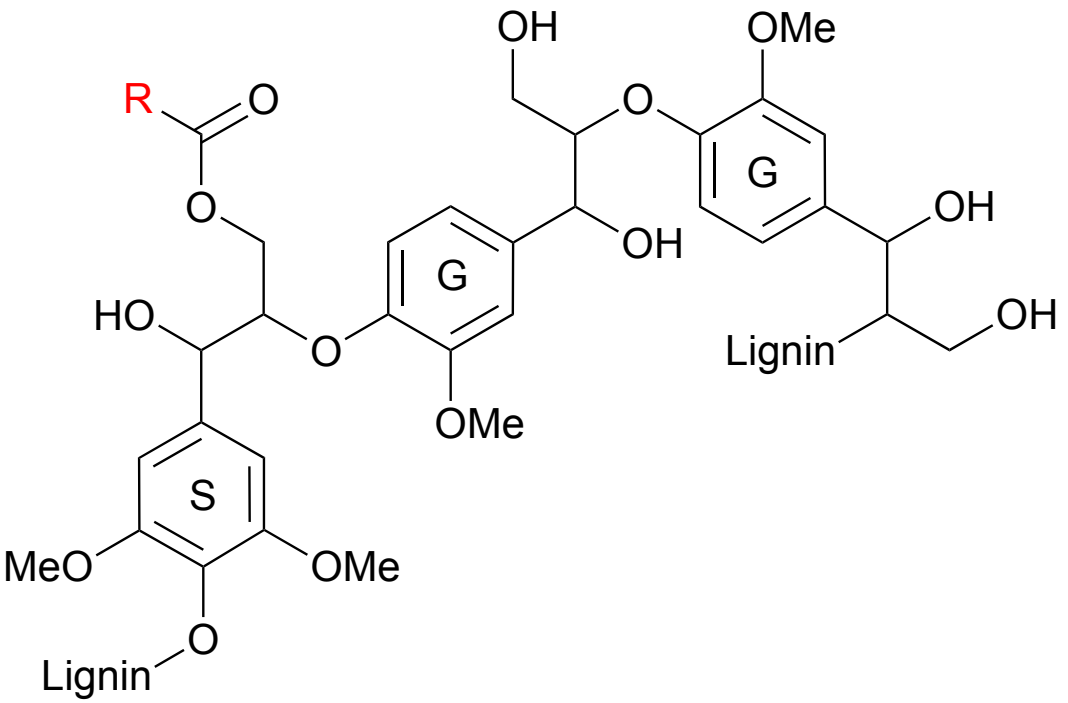
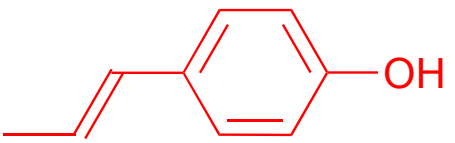


R =

9 $-CH_3$

10



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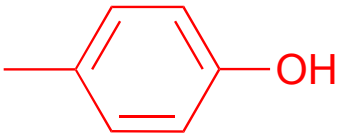

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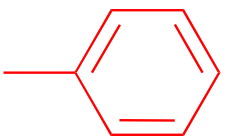

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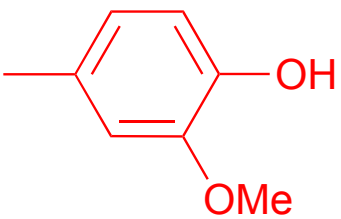

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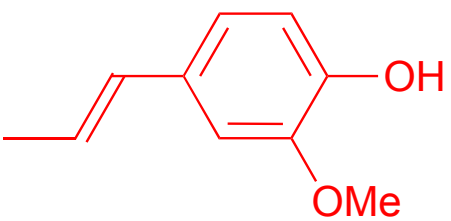

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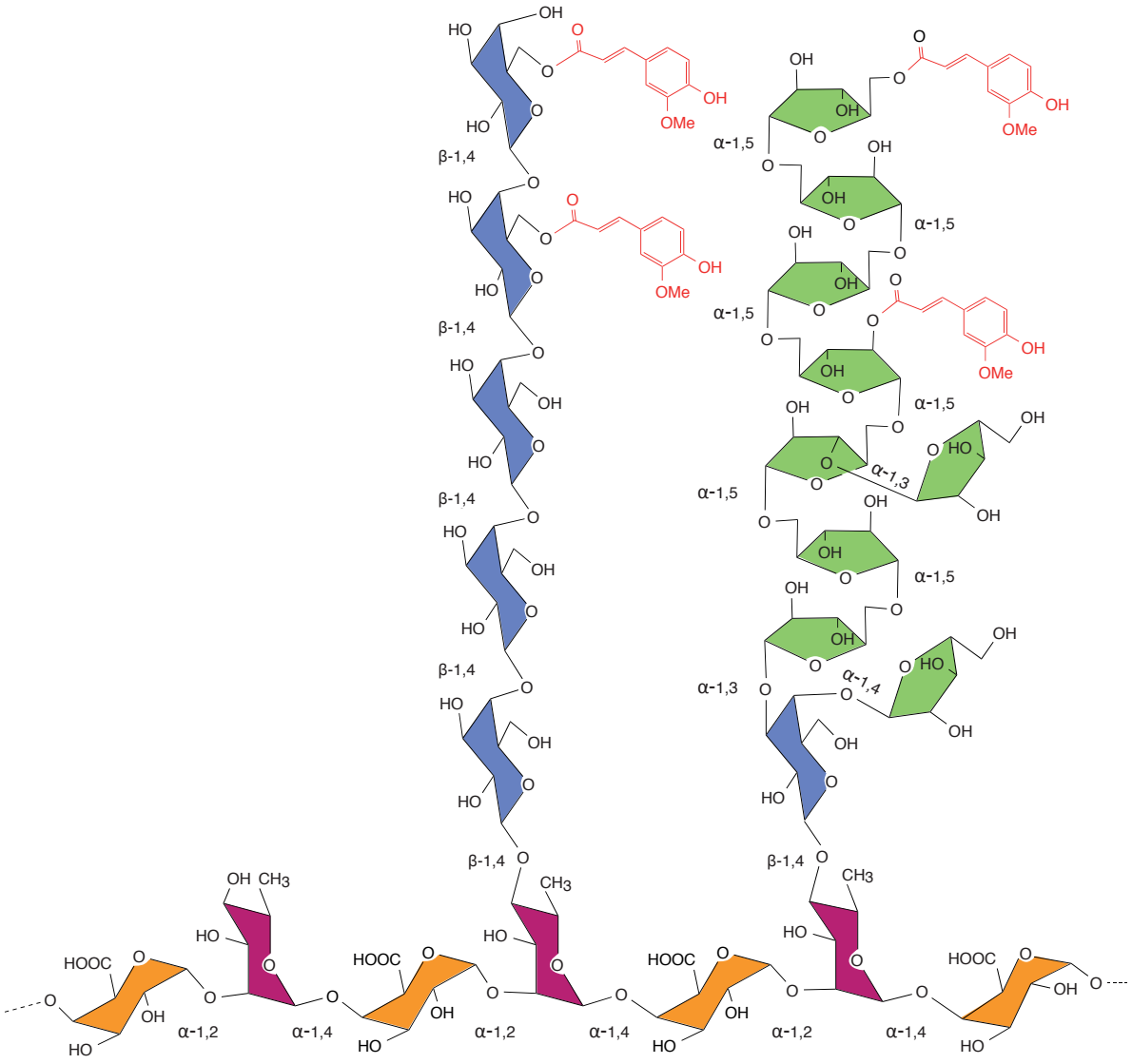

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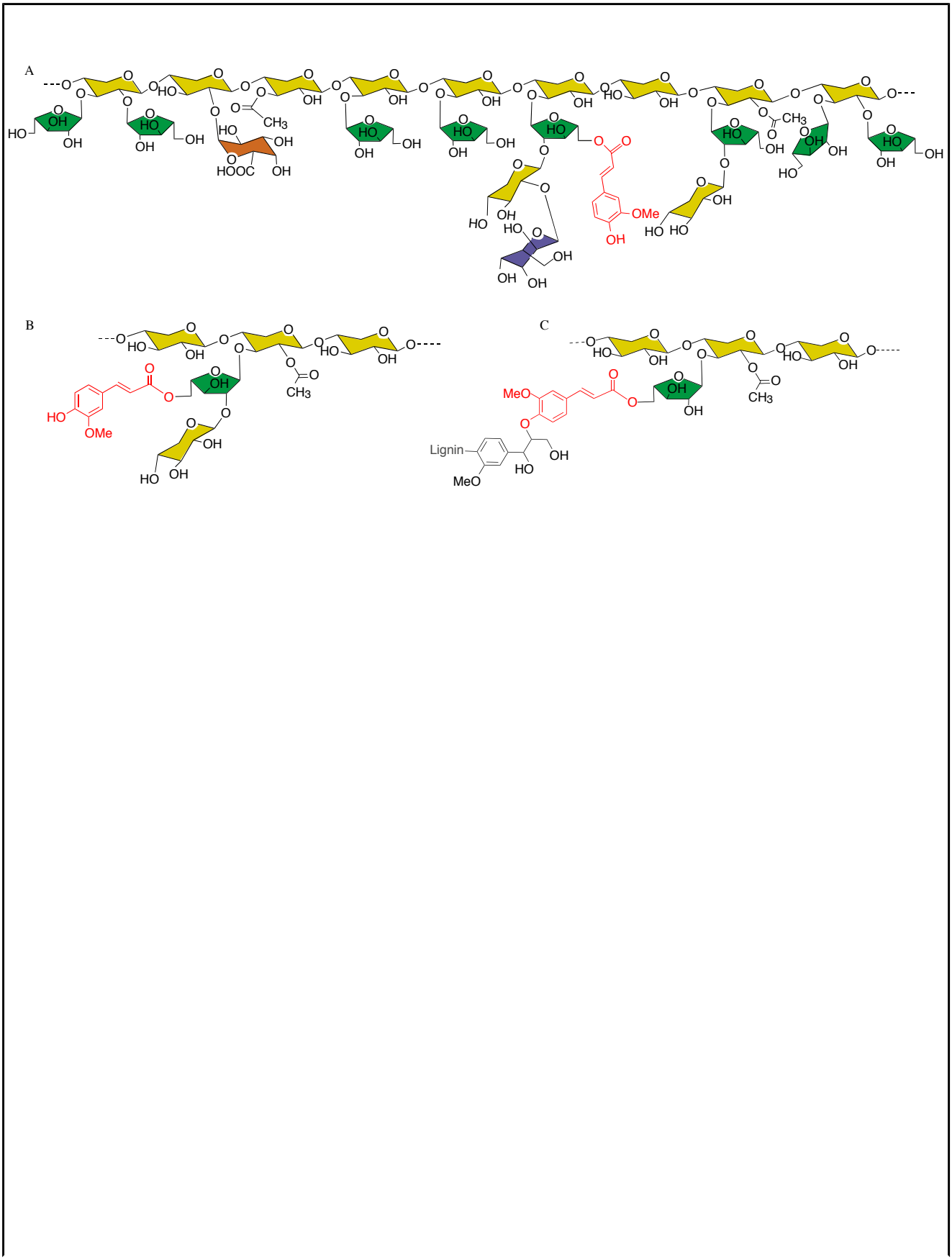

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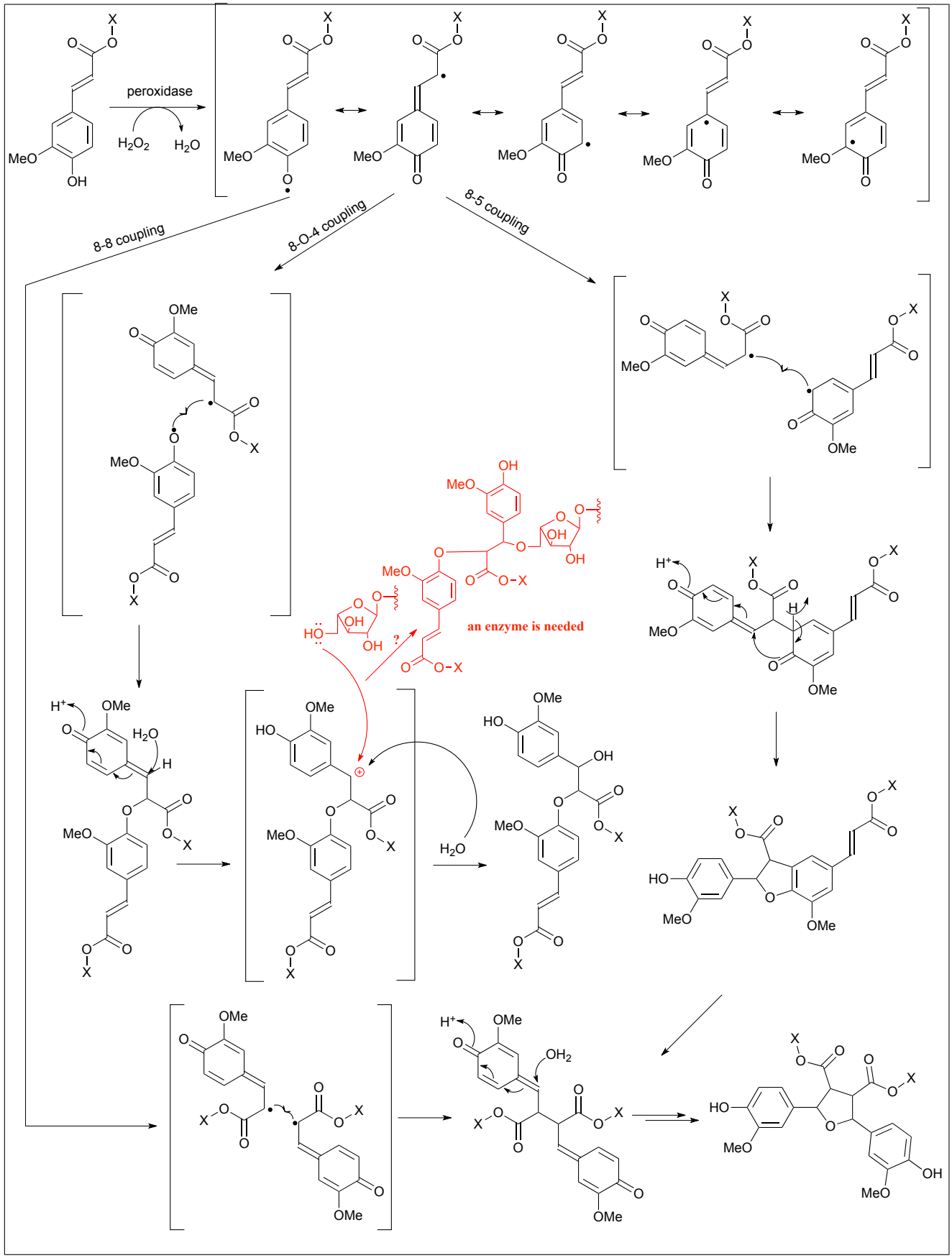

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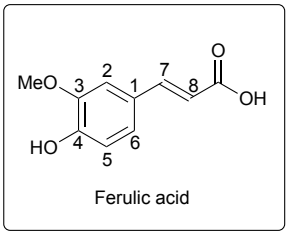
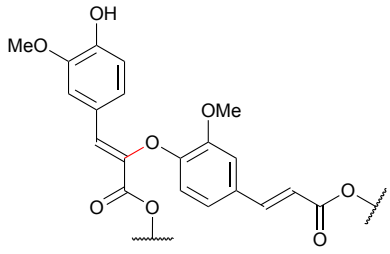


Ferulic acid



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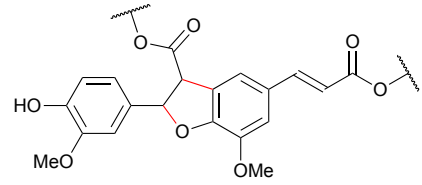

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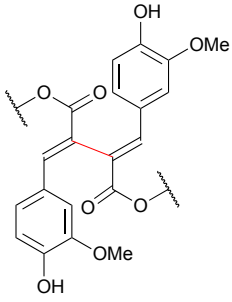

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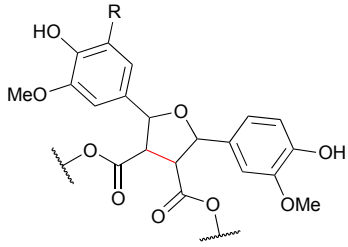

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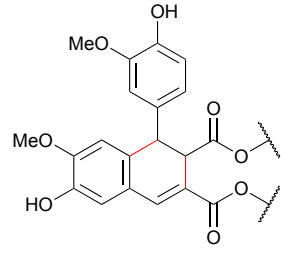

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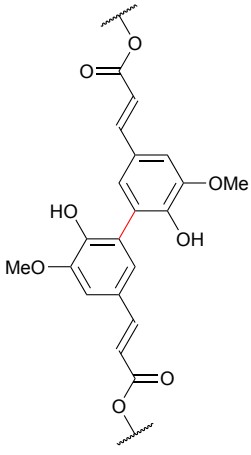

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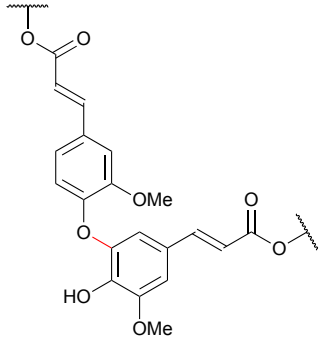

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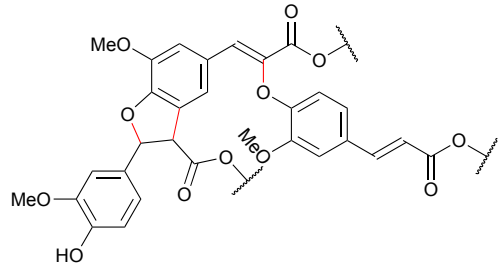

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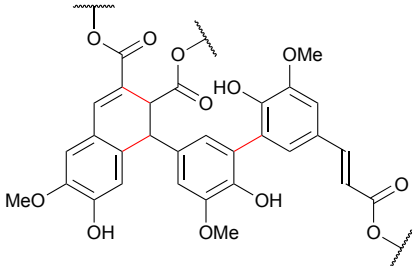

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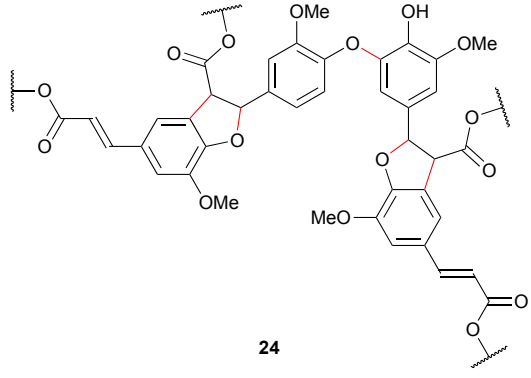
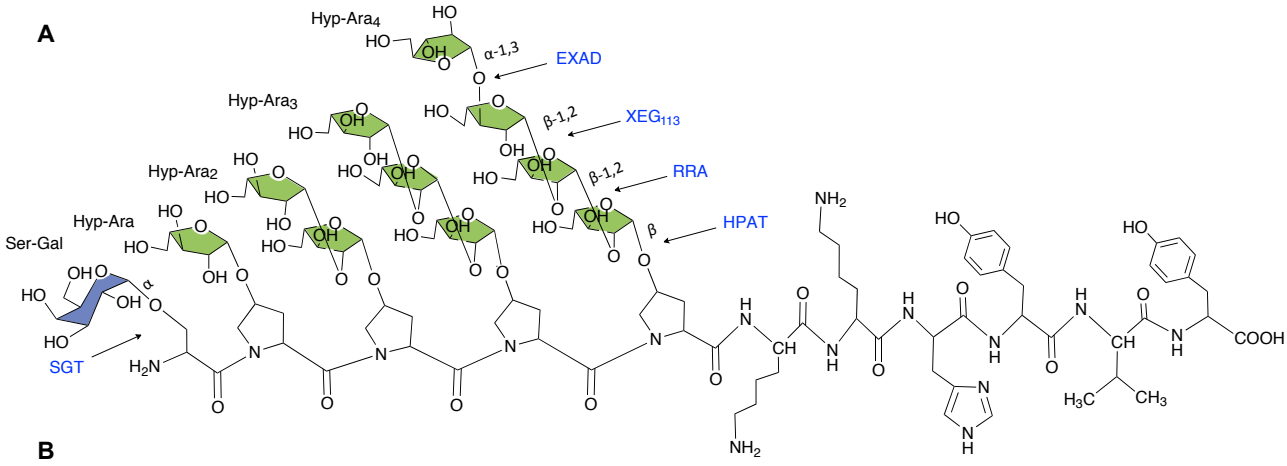


24

All text and images must be placed within the frame.



B

S0000KKHYVYKS0000VKHYS000VYHS0000KKHYVYKS0000VKHYS000VYH
S0000KKHYVYKS0000VKHYS000VYHS0000KKHYVYKS0000VKHYS000VYHS0000KKHYVYKS0000VKHYS000VYH
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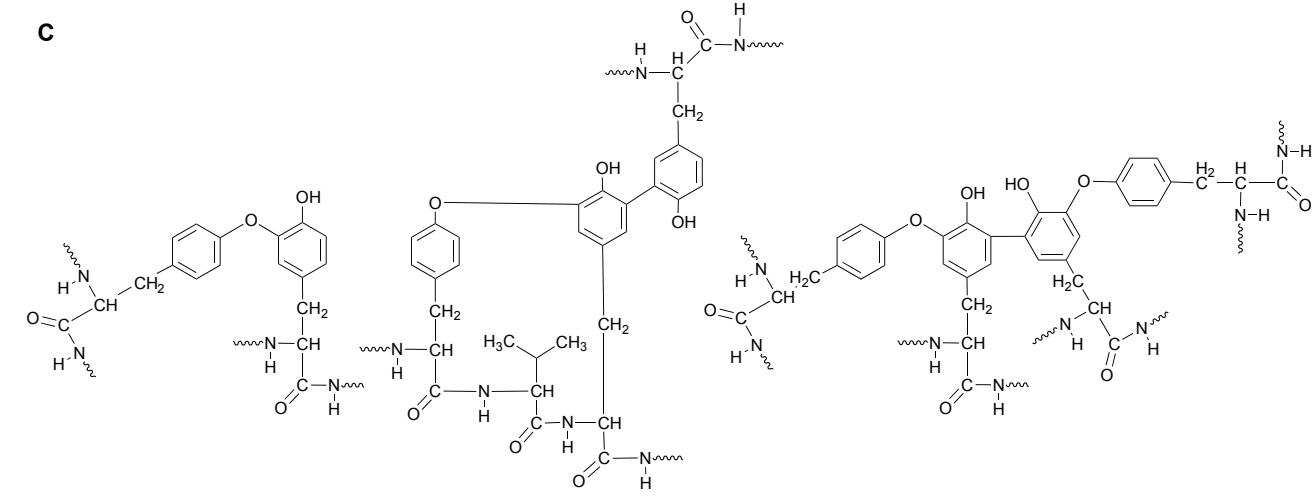
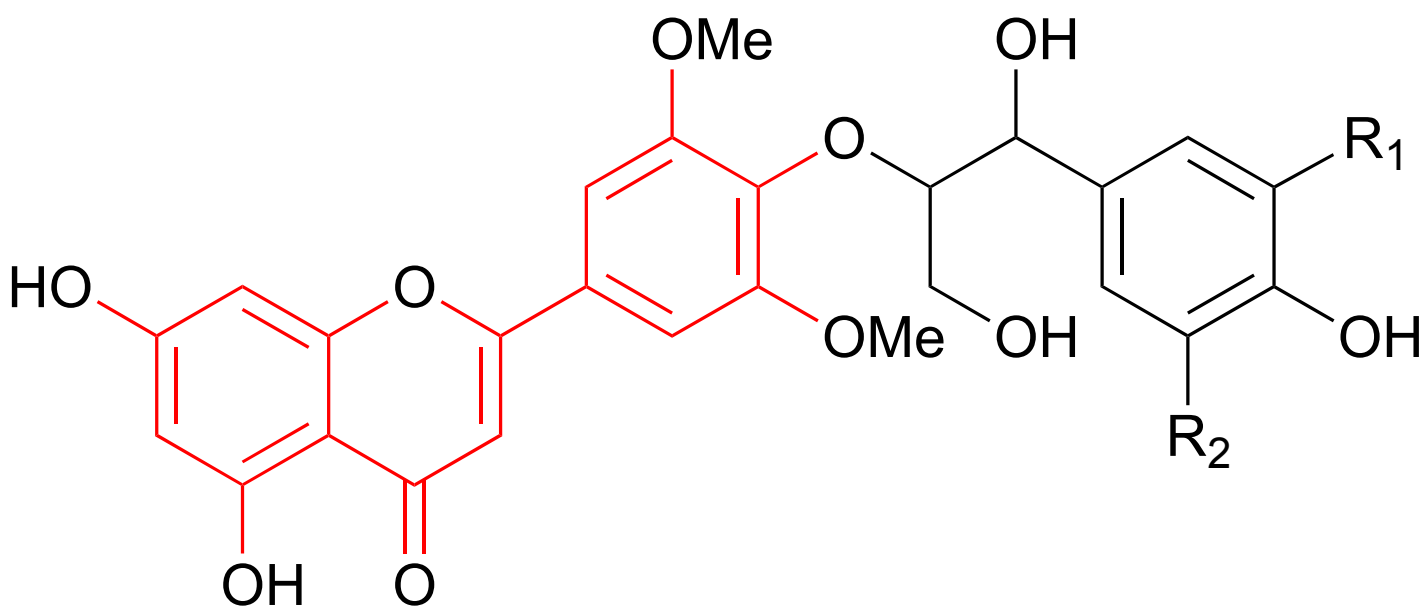


Isodityrosine

Pulcherosine YVY:Y cross-link

Di-isodityrosine

All text and images must be placed within the frame.



a: R_1 = H, R_2 = OMe [T-(4'-O-β)-G]
b: R_1 = R_2 = OMe [T-(4'-O-β)-S]
c: R_1 = H, R_2 = H [T-(4'-O-β)-HP]

RSC Author Templates - ChemDraw (CDX) - Double Column Artwork

All text and images must be placed within the frame.

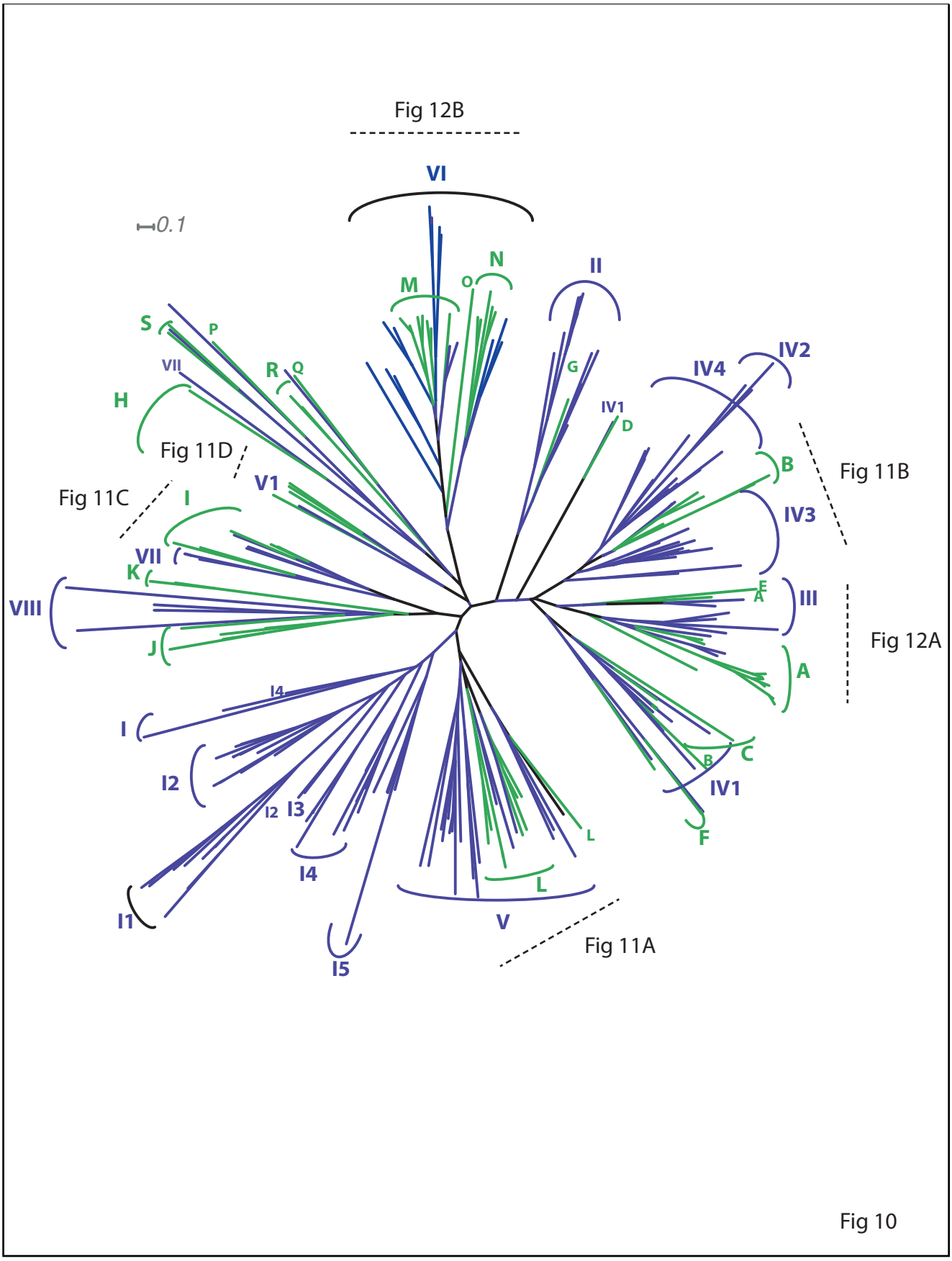


Fig 10

RSC Author Templates - ChemDraw (CDX) - Single Column Artwork

All text and images must be placed within the frame.

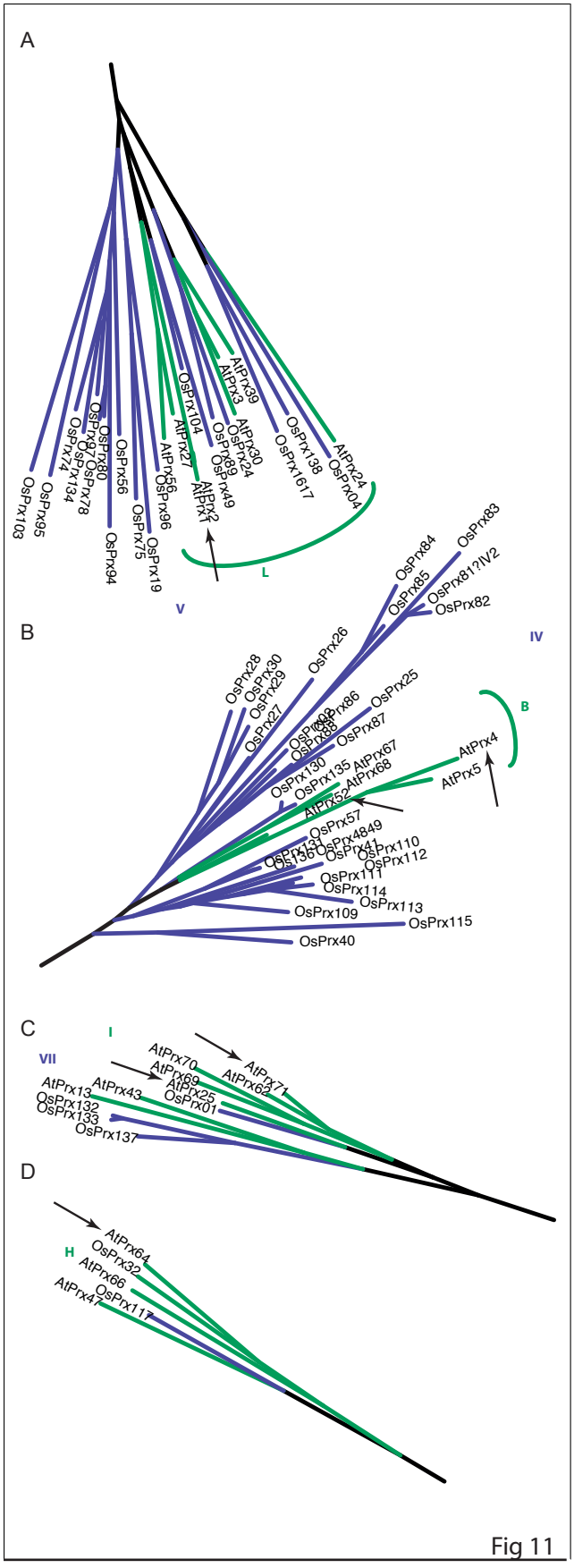


Fig 11

RSC Author Templates - ChemDraw (CDX) - Single Column Artwork

All text and images must be placed within the frame.

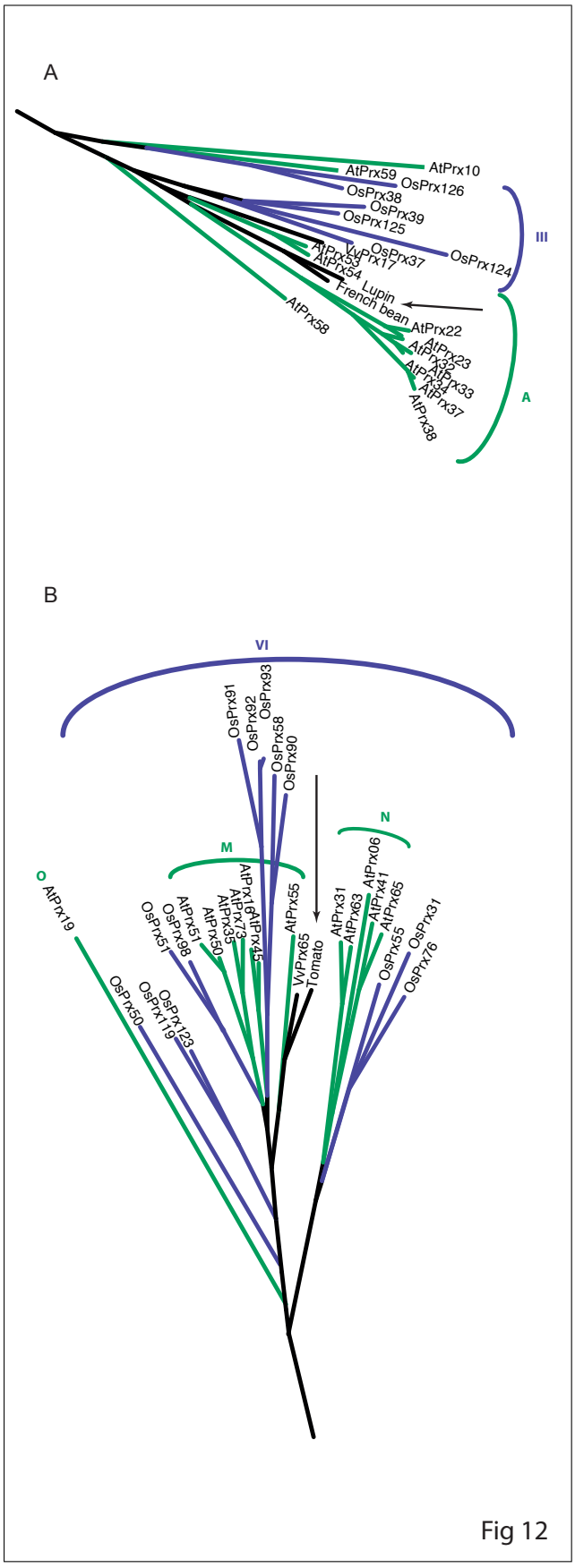
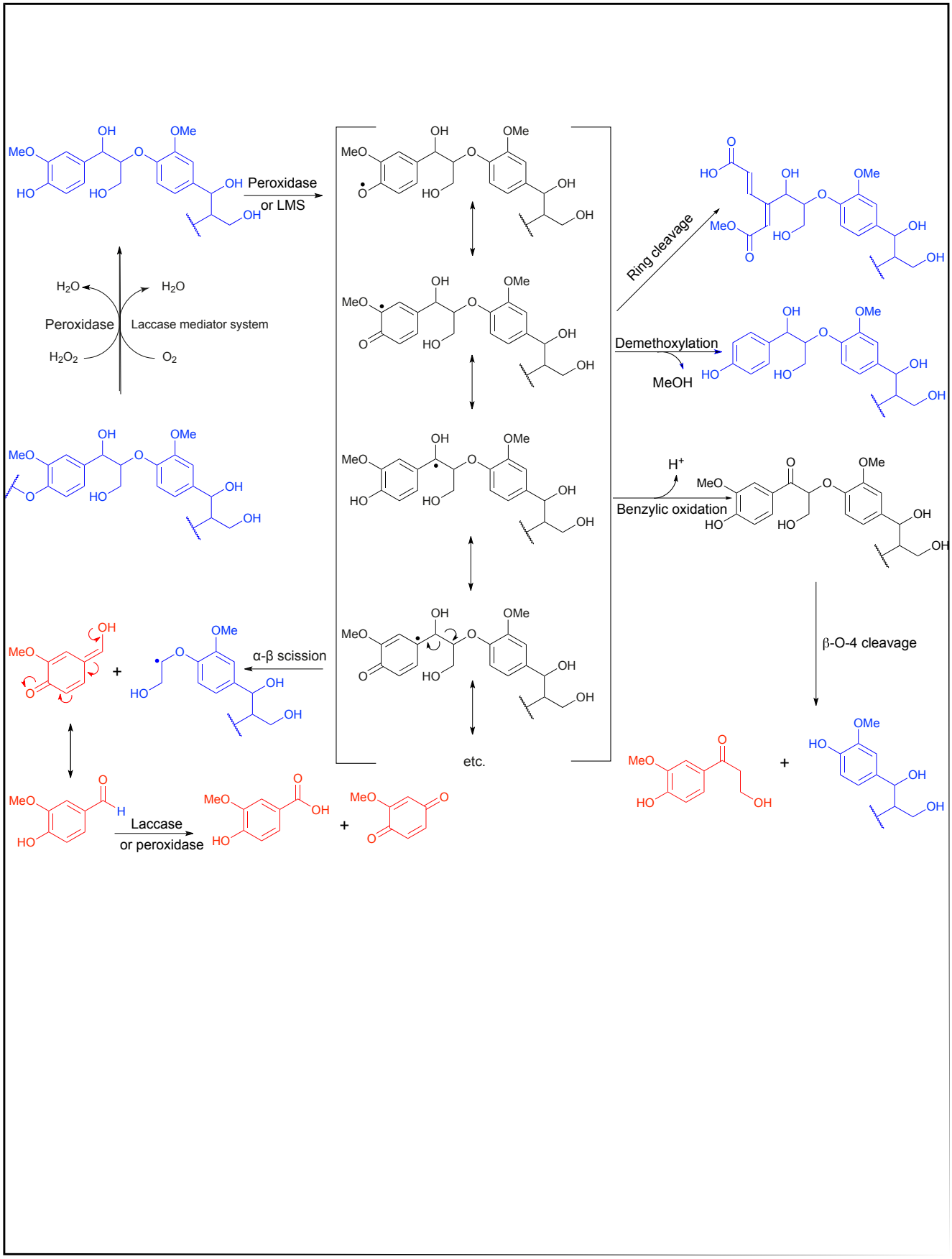


Fig 12

All text and images must be placed within the frame.



RSC Author Templates - ChemDraw (CDX) - Single Column Artwork

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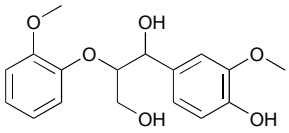
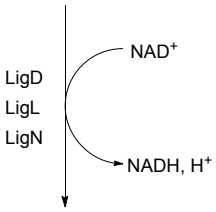
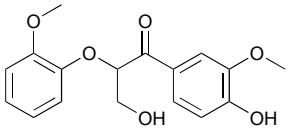
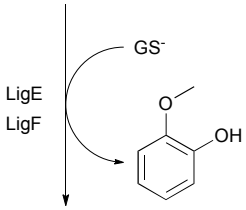


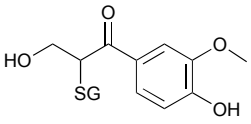
guaiacylglycerol-β-guaiacyl ether



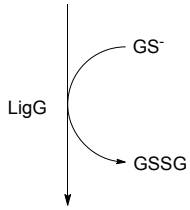


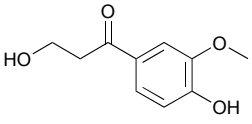
α-(2-methoxyphenoxy)-β-hydroxypropio-vanillone





α-glutathionyl-β-hydroxypropiovanillone





β-hydroxypropiovanillone