Covalent interactions between lignin and hemicelluloses in plant secondary cell walls

Oliver M. Terrett, Paul Dupree*

Department of Biochemistry, University of Cambridge, Hopkins Building, The Downing site, Tennis Court Road, Cambridge CB2 1QW, UK

Oliver M. Terrett: omt27@cam.ac.uk

Paul Dupree: pd101@cam.ac.uk

*Corresponding author

Email: pd101@cam.ac.uk

Tel: +44 1223 333340

Fax: +44 1223 333345

Abstract

The plant secondary cell wall is a complex structure composed of polysaccharides and lignin, and is a key evolutionary innovation of vascular land plants. Although cell wall composition is well understood, the cross-linking of the different polymers is only now yielding to investigation. Cross-linking between hemicelluloses and lignin occurs via two different mechanisms: incorporation into lignin by radical coupling of ferulate substitutions on xylan in commelinid monocots, and incorporation of hemicellulosic glycosyl residues by re-aromatisation of lignification intermediates. Recent genetic evidence indicates that hemicellulose:lignin cross-linking has a substantial impact on plant cell wall recalcitrance. Engineering plant biomass with modified frequencies of cross-links will have significant impacts on biomass utilisation.

Introduction

Together, lignin and hemicelluloses can comprise 40-70% of plant biomass by weight. Both polymers are deposited during secondary cell wall formation. Lignin and hemicelluloses interact with each other and cellulose in the cell wall and affect emergent properties of plant biomass such as mechanical strength, flexibility and recalcitrance to enzymatic digestion. Understanding how lignin and hemicelluloses interact with each other may enable us to tailor such properties for different products in an emerging sustainable economy in diverse areas such as construction, materials, and chemical feedstocks.

Lignin is a phenolic polymer found in the cell walls of vascular plants, mainly in the thickened secondary walls of xylem vessels and tracheids (which transport water) and sclerenchyma and xylem fibres (which provide stem mechanical support) [1,2]. Lignin is derived from monolignols synthesised via the phenylpropanoid pathway. There are three canonical monolignols: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. However, an array of additional monomers are incorporated into lignin in many species, highlighting the flexibility in the polymerisation reactions that generate lignin [1]. For example monocots incorporate tricin, whereas some species have been shown to incorporate hydroxystilbenes [3,4]. Once synthesised in the cytosol, the monolignols and other monomers are exported to the cell wall [1]. In the wall, monolignol radicals are generated by laccase and peroxidase enzymes. Monolignol radicals couple combinatorially, forming oligolignols, which can also form radicals to then polymerise further by endwise radical coupling with further monomer radicals [5]. In this way, large polymers with a variety of linkages are formed. The polymerisation and structural diversity of lignins are reviewed elsewhere in this issue.

In the lignified cells of seed plants, the major hemicelluloses are xylan and galactoglucomannan (figure 1) [6]. Hemicelluloses are polysaccharides that usually have an equatorial β-1,4-linked glycosyl residue backbone [6]. This means they have similar backbone linkages as the glucan chains that make up crystalline cellulose. The backbone structure may enable binding to cellulose, in a similar fashion to the binding of glucan chains in the cellulose microfibril [7,8]. Hemicelluloses are synthesised in the Golgi apparatus and secreted into the cell wall during secondary wall deposition [9]. Xylan and galactoglucomannan are synthesised at a similar time as cellulose, whereas at least some of the lignification occurs after polysaccharide synthesis [10].

Xylan is a significant component of all vascular plant secondary cell walls [6]. It is the main hemicellulose in angiosperm (hardwood) secondary cell walls, contributing 20-40% of biomass weight. Xylan can also constitute up to 15% of gymnosperm (softwood) cell walls. The backbone consists of β-1,4 linked xylosyl residues. The hydroxyl groups on carbon-2 or -3 of the xylosyl residues are often substituted [6], but these substitutions are diverse, and vary phylogenetically [6,11]. The different substitutions affect the self-association properties of the xylan polymer, and also its interaction with cellulose and lignin [8,12]. The only xylan substitution ubiquitous in vascular plant cell walls is 2-linked glucuronic acid (in most species this is 4-O-methylated), suggesting particular importance. Eudicot xylans are acetylated, whereas monocot xylans are acetylated and highly arabinosylated. The conifers, which make up the majority of gymnosperm species, are devoid of xylan acetylation and have only arabinosyl and glucuronosyl substituents on their xylan [11]. Feruloylated and *p*-coumaroylated arabinosyl substitutions of the xylan backbone are thought to be confined to the commelinid monocots (including grasses such as the crops rice, maize, wheat and sugarcane) [13]. There is some indirect evidence of ferulate in conifer primary cell walls, and this may also be true of secondary cell walls, but the identity of the feruloylated wall component remains to be demonstrated [14].

Softwood cell wall hemicelluloses are dominated by galactoglucomannan, which forms 15-25% of their cell walls by weight, see figure 1 [6]. Galactoglucomannan is a lower proportion (up to 5% by weight) of angiosperm cell walls. The galactoglucomannan backbone is

composed of around 80% mannosyl residues, and the rest are glucosyl residues [15]. Carbons 2 and 3 of the mannosyl residues can be acetylated, and carbon 6 can be galactosylated. The extent to which galactoglucomannans vary in structure phylogenetically remains to be determined.

Hemicelluloses and lignin covalently cross-link in the plant cell wall. Cross-linking arises from two characteristics of lignin polymerisation in the wall. Firstly, due to weak substrate specificity, laccases and peroxidases have oxidative effects on monolignol-related compounds, such as the ferulate substitutions of commelinid monocot xylan [16,17]. In addition, oxidised monolignols may oxidise other phenolic compounds, producing radicals non-enzymatically [18]. Generated radicals of feruloylated xylan enable coupling of xylan with monolignols and growing lignin polymers (Figure 2A). Secondly, the coupling of monolignols via their β carbon (the majority of linkages in native lignins [19]) produces a quinone methide intermediate. To form the mature lignin polymer, re-aromatisation of the quinone methide by nucleophilic addition at the α position must occur [20]. This addition is most likely to occur with water acting as the nucleophile, but could occur with any polysaccharide hydroxyl or carboxylic acid (Figure 2B) [20,21].

In this review we discuss the recent and historic evidence for the two modes of hemicellulose:lignin cross-linking. We also discuss the biological significance of cross-linking hemicelluloses with lignin and the possibility for altering the properties of cell walls by genetically altering the structure of hemicelluloses or lignin.

In commelinid monocots, feruloylated arabinosyl substitutions of xylan enable cross-linking between xylan and lignin by radical coupling.

One of the characteristic features of commelinid monocot secondary cell walls is the presence of 5-*O*-feruloylated and 5-*O*-*p*-coumaroylated α-1,3 arabinosyl substitutions on the xylan backbone [6]. Ferulate is produced by oxidation of coniferaldehyde from the general phenylpropanoid pathway [22]. It has been well established that two or more ferulate substitutions of xylan can undergo radical coupling in a manner analogous to lignification, forming xylan:xylan cross-links [23]. Due to this propensity for radical coupling with other ferulate substitutions and the structural similarity of ferulate to monolignols it was hypothesised that xylan ferulate substitutions can participate in lignin polymerisation reactions, and that such cross-links may be important for cell wall properties [24].

Studies of *in vitro* lignification reactions of feruloylated arabinose and monolignols demonstrated that ferulate could couple with coniferyl alcohol at the 4, 5 and 8 positions of the ferulate [24]. Further studies showed that feeding maize suspension cell systems with monolignols and hydrogen peroxide reduced the amount of alkali-labile ferulates on xylan, indicating the ferulate substitutions were incorporated into the lignin polymers [25]. These studies provided evidence that ferulate substitutions are compatible with radical coupling reactions, suggesting ferulate-mediated xylan:lignin cross-linking may occur *in vivo*. In 1995, Nuclear Magnetic Resonance (NMR) analysis was used to definitively demonstrate that ferulate was 8-β coupled to coniferyl and sinapyl alcohol *in vivo* [26]. Other linkages were found in gas-chromatography/mass-spectrometry (GC/MS) analysis, identifying coniferyl

alcohol β-O-4 linked to ferulate, as in figure 2A [27]. This *in vivo* evidence shows that ferulate substitutions participate in the radical coupling reactions of lignification. It has also been demonstrated that diferulates incorporate into lignin *in vitro*, suggesting even more complex structures involving multiple xylan molecules linked to each other can cross-link to lignin [28]. Quantification of ether-linked ferulates in grass cell walls, which includes xylan:xylan cross-links and xylan:lignin cross-links, suggests that 20-80% of ferulate is ether-linked [29,30].

There is significant evidence suggesting that ferulate-mediated xylan:lignin cross-linking is important for recalcitrance to enzymatic degradation. Studies of *in vitro* lignification systems have shown that ferulate-mediated xylan:lignin cross-linking impedes the degree of enzymatic digestion[31]. *In vivo* evidence comes from a maize mutant (*sfe*) with reduced feruloylation. The *sfe* mutant has higher *in vitro* rumen digestibility [30,32]. Consistent with this, in animal feeding trials the *sfe* mutant led to reduced feeding but increased milk production, suggesting it is more digestible *in vivo* [33]. Collectively, these studies suggest that the level of ferulate linked xylan to lignin has a biological function in the enzymatic recalcitrance of cell walls.

Modulating ferulate-mediated xylan:lignin cross-linking could be an important strategy for tailoring commelinid monocot biomass for commercial uses, like feed or fuel production. As ferulate substitutions participate in the radical coupling reactions of lignification, and the radical generation can occur non-enzymatically, modulating lignin structure to prevent ferulate-mediated xylan:lignin cross-linking may not work. However, it should be possible to target xylan biosynthetic enzymes to reduce the level of α-1,3 arabinosylation or the feruloylation of arabinoxylan. Family 61 glycosyl transferase enzymes (GT61) add arabinosyl substitutions to the xylan backbone [34]. RNAi knockdown of the GT61 *XAT1* reduced the amount of ferulate in soluble arabinoxylan of wheat endosperm [35]. In rice, a GT61 knockout mutant, *xax1*, was found to have 60% reduced feruloylation and 50% reduced *p*-coumaroylation of xylan [36]. These alterations in *xax1* cell wall composition led to a significant decrease in recalcitrance. Unfortunately, the *xax1* mutant is dwarfed, so general reduction of XAX1 activity is not a suitable engineering strategy. It is unclear why the plant is dwarfed, but it may be due to a loss of wall strength in specific cells such as xylem vessels. A more complex genetic strategy such as vessel complementation has been effective in rescuing the dwarf phenotypes of some Arabidopsis xylan and lignin mutants while maintaining reduced recalcitrance, and may be a solution for grass cell wall modification [37,38].

Based on high relative expression in grasses of GT61s and other xylan synthesis genes, it was proposed that BAHD acyltransferases are good candidates for feruloylation of the arabinosyl substitutions on xylan [39]. It remains unclear how these cytosolic enzymes are involved in xylan synthesis, but feruloylation of the UDP-arabinose sugar nucleotide, which is then transported into the Golgi for xylan synthesis, is possible [40]. Consistent with this, using an RNA interference (RNAi) approach four *BAHD* genes were downregulated in rice, resulting in a 20% decrease in feruloylation of xylan [41]. In addition, the feruloylation level in *Brachypodium distachyon* correlates with *BAHD* gene expression and overexpression of one

BAHD gene increased xylan feruloylation [42,43]. Recently, a single *BAHD* acyltransferase gene was used as an RNAi target in *Setaria viridis* [44], resulting in a decrease of unetherified ferulate by 70-90% and of ferulate dimers by over 50%. Lignin-etherified ferulates were not measured. Importantly, the authors found an increase in sugar release by saccharification of 40-60%. The *Setaria* plants showed normal biomass production, suggesting BAHD enzymes are a promising target for reducing recalcitrance in commelinid monocots.

Reduction of feruloylation of xylan leads to improved digestibility of grasses, and so is a promising approach for engineering reduced hemicellulose:lignin links. As some modifications lead to growth impairment or to minor changes in cell walls, a moderate reduction of the feruloylation and genetic redundancy of the BAHD enzymes needs to be considered in grass engineering approaches.

Nucleophilic moieties present in hemicelluloses can participate in lignification reactions, cross-linking hemicelluloses and lignin

In eudicots, arabinosyl substitutions are not found on secondary wall xylan, and in gymnosperms secondary wall arabinosyl substitutions may not be feruloylated, so linkage through ferulate esters is not a major hemicellulose:lignin linkage in these clades. However, nucleophilic groups of xylan and galactoglucomannan have been proposed to re-aromatise the quinone methide intermediate of lignols that are coupling at the β position. Depending on whether the nucleophile is a hydroxyl or carboxylic acid this reaction would form ether or ester linkages to lignin.

Recently, an ether hemicellulose:lignin cross-link was verified *in vivo*; by sequential extractions and enzyme digestions, a fragment of galactoglucomannan cross-linked to lignin was extracted from Japanese red pine [45]. Using multidimensional NMR experiments, it was possible to identify with unambiguous multi-bond correlations that the carbon 6 of a backbone mannosyl residue was ether-linked to the α carbon of a β-O-4-linked unit of lignin, as in figure 2B. It will be important to investigate whether other hydroxyls of galactoglucomannan or xylan can also form α ethers. Currently, there is no conclusive data to suggest that these α ethers have a biological function in cell walls, as has been demonstrated for ferulate-mediated xylan:lignin cross-links in grass biomass recalcitrance.

There is indirect evidence that glucuronic acid substitutions on xylan can also act as a nucleophile in the described reaction, and that glucuronic acid-mediated xylan:lignin cross-linking may occur. The presence of esterified glucuronic acid substitutions of xylan has been described in wood [46]. NMR has also been used to assign glucuronic acid-mediated xylan:lignin α or γ ester bond signals based on similarity with model compounds, but the experiments were performed on complex mixtures, making the spectra difficult to interpret [47,48]. Other indirect evidence for glucuronic acid-mediated xylan:lignin cross-linking comes from identification of glucuronyl esterase activities in carbohydrate esterase family 15 (CE15), which act on model compounds [49]. When tested on proposed xylan:lignin complexes extracted from wood, the enzymes increase the amount of carboxylic acid groups and hydroxyl groups, suggesting the hydrolysis of esters [48]. Moreover, the

glucuronyl esterases decrease the molecular weight distribution of proposed xylan:lignin complexes in size exclusion chromatography, and synergistically boost GH10 xylanase activity on xylan:lignin complexes [50].

Additional indirect evidence for glucuronic acid-mediated xylan:lignin cross-links is the finding that glucuronic acid is important for biomass recalcitrance in Arabidopsis [51]. Enzymatic saccharification of the *gux1 gux2* mutant, which lacks glucuronic acid on secondary cell wall xylan, shows that up to twice as much glucose is released and five times more xylose in limited-saccharification studies. This difference could be due to the generation of glucuronic acid-mediated xylan:lignin cross-links in wild type but not *gux1 gux2* mutants, but might also be a consequence of other changes to the wall such as the mode of xylan-binding to cellulose or changes in the charge of xylan. However, other Arabidopsis xylan substitution mutants, including a reduced acetylation mutant with xylan that no longer binds to cellulose, have little effect on cell wall recalcitrance, suggesting glucuronic acid is uniquely important [8,51].

Glucuronic acid removal seems to be an effective route to reduced recalcitrance. However hydroxyl groups are inherent to hemicelluloses, so engineering hemicellulose synthesis is a poor route to preventing hemicellulose:lignin ether cross-linking. On the other hand, substantially altering lignin composition may prevent such cross-linking. During monolignol synthesis, prior to methylation by caffeic acid *O*-methyl transferase (COMT), the phenyl ring is first hydroxylated by *p*-coumaroyl quinate/shikimate 3'-hydroxylase (C3'H) and (in the case of converting coniferyl alcohol to sinapyl alcohol) ferulate 5-hydroxylase (F5H). When COMT activity is reduced, plants produce an unconventional monolignol with an additional ring hydroxyl, 5-hydroxyconiferyl alcohol [52]. Lignin containing caffeyl alcohol, another unconventional monolignol with an additional ring hydroxyl, is synthesised in the seedcoats of some species [53]. During radical coupling at the β-position of such catechol units, the hydroxyl of the phenyl ring in the polymerising lignin acts as an internal nucleophile that attacks the quinone methide intermediate, producing benzodioxane units, see figure 3 [52–54]. Polysaccharides are not able to cross-link to the lignin by nucleophilic attack of the quinone methide as they cannot compete with such intramolecular trapping. COMT downregulation significantly reduces recalcitrance to enzymatic digestion and increases the proportion of benzodioxane units in the lignin of multiple species [54–57]. A reduction in hemicellulose:lignin cross-linking remains to be demonstrated, but altering COMT expression is a viable route to producing plants with reduced recalcitrance. *In vitro*, phenylpropanoid conjugates, e.g., rosmarinic acid, have been used as monolignol alternatives that also produce benzodioxane units in the resultant lignin [58]. Engineering rosmarinic acid synthesis into plants would require introducing additional genes such as rosmarinic acid synthase [59].

There are alternative routes for reducing hemicellulose:lignin cross-links. For instance, mutants in cinnamyl alcohol dehydrogenase (CAD) enzymes cannot reduce the aldeyhyde of monolignol precursors to a hydroxyl, so incorporate hydroxycinnamaldehydes into their lignin. When coupling at the 8 position (equivalent to the β position of a monolignol, see figure 3D), the hydroxycinnamaldehydes eliminate a proton rather than undergoing

nucleophilic attack of the quinone methide [52]. *In vivo* this results in the presence of an unsaturated bond between carbons 7 and 8 in the 8-O-4 and 8-8 linkages [52]. Multiple studies have shown that CAD mutation or downregulation reduces recalcitrance or increases extractability of hemicelluloses or lignin, but any reduction in hemicellulose:lignin crosslinking remains to be investigated [60–65]. The recalcitrance differences observed in CAD and COMT mutants may be partially explained by reductions in lignin content.

Conclusions

Two mechanisms for cross-linking of hemicelluloses and lignin have been described: radical coupling resulting in ferulate-mediated xylan:lignin cross-linking, and re-aromatisation of the quinone methide intermediate by polysaccharide nucleophiles, leading to formation of ether or ester bonds between hemicelluloses and lignin. There is an emerging body of evidence that suggests both modes of cross-linking have a role in the recalcitrance of plant cell walls. However, open questions remain, specifically in regard to re-aromatisation of lignification intermediates by hemicelluloses. It will be important to characterise the structural diversity of these cross-links and address the extent and functionality of this type of cross-linking in the plant cell wall. Future research will have to face the challenge of modulating hemicellulose:lignin cross-linking in order to engineer improved biomass for a variety of applications. Biofuel generation may require a reduction of cross-linking, whereas applications such as construction materials or pathogen-resistant crops could profit from increased cross-linking.

Acknowledgements

O.M.T was a recipient of an iCASE studentship from the BBSRC and Novozymes (Reference BB/M015432/1). P.D. was supported by the Leverhulme Trust Centre for Natural Material Innovation and the OpenPlant Synthetic Biology Research Centre BB/L014130/1. We would like to thank Nadine Anders for critical reading of the manuscript.

References

1. Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph SA, Christensen JH, Boerjan W: **Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl- propanoids**. *Phytochem Rev* 2004, **3**:29–60.

2. Evert F. R: *Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development*. John Wiley & Sons, Inc.; 2006.

3. Lan W, Lu F, Regner M, Zhu Y, Rencoret J, Ralph SA, Zakai UI, Morreel K, Boerjan W, Ralph J: **Tricin, a flavonoid monomer in monocot lignification**. *Plant Physiol* 2015, **167**:1284–1295.

4. Río JC del, Rencoret J, Gutiérrez A, Kim H, Ralph J: **Hydroxystilbenes are monomers in palm fruit endocarp lignins**. *Plant Physiol* 2017, **174**:2072–2082.

5. Morreel K, Ralph J, Kim H, Lu F, Goeminne G, Ralph SA, Messens E, Boerjan W: **Profiling of oligolignols reveals monolignol coupling conditions in lignifying poplar xylem.** *Plant Physiol* 2004, **136**:3537-3549.

6. Scheller HV, Ulvskov P: **Hemicelluloses**. *Ann Rev Plant Biol* 2010, **61**:263–289.

7. Simmons TJ, Mortimer JC, Bernardinelli OD, Pöppler A-C, Brown SP, deAzevedo ER, Dupree R, Dupree P: **Folding of xylan onto cellulose fibrils in plant cell walls revealed by solid-state NMR**. *Nat Comms* 2016, **7**:ncomms13902.

8. Grantham NJ, Wurman-Rodrich J, Terrett OM, Lyczakowski JJ, Stott K, Iuga D, Simmons TJ, Durand-Tardif M, Brown SP, Dupree R, Busse-Wicher M, Dupree P.: **An even pattern of xylan substitution is critical for interaction with cellulose in plant cell walls**. *Nat Plants* 2017, **3**:859.

9. Taylor JG, Owen TP, Koonce LT, Haigler CH: **Dispersed lignin in tracheary elements treated with cellulose synthesis inhibitors provides evidence that molecules of the secondary cell wall mediate wall patterning**. *Plant J* 1992, **2**:959–970.

10. Smith RA, Schuetz M, Roach M, Mansfield SD, Ellis B, Samuels L: **Neighboring parenchyma cells contribute to Arabidopsis xylem lignification, while lignification of interfascicular fibers is cell autonomous**. *Plant Cell* 2013, **25**:3988–3999.

11. Busse-Wicher M, Li A, Silveira RL, Pereira CS, Tryfona T, Gomes TCF, Skaf MS, Dupree P: **Evolution of xylan substitution patterns in gymnosperms and angiosperms: implications for xylan interaction with cellulose**. *Plant Physiol* 2016, **171**:2418-2431.

12. Busse-Wicher M, Gomes TCF, Tryfona T, Nikolovski N, Stott K, Grantham NJ, Bolam DN, Skaf MS, Dupree P: **The pattern of xylan acetylation suggests xylan may interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of *Arabidopsis thaliana***. *Plant J* 2014, **79**:492–506.

13. de O. Buanafina MM: **Feruloylation in grasses: current and future perspectives**. *Mol Plant* 2009, **2**:861–872.

14. Carnachan SM, Harris PJ: **Ferulic acid is bound to the primary cell walls of all gymnosperm families**. *Biochem Syst Ecol* 2000, **28**:865–879.

15. Hannuksela T, Hervé du Penhoat C: **NMR structural determination of dissolved O-acetylated galactoglucomannan isolated from spruce thermomechanical pulp**. *Carbohyd Res* 2004, **339**:301–312.

16. Carunchio F, Crescenzi C, Girelli AM, Messina A, Tarola AM: **Oxidation of ferulic acid by laccase: identification of the products and inhibitory effects of some dipeptides**. *Talanta* 2001, **55**:189–200.

17. Ward G, Hadar Y, Bilkis I, Konstantinovsky L, Dosoretz CG: **Initial steps of ferulic acid polymerization by lignin peroxidase**. *J Biol Chem* 2001, **276**:18734–18741.

18. Ralph J: **Hydroxycinnamates in lignification**. *Phytochem Rev* 2010, **9**:65–83.

19. Shi J, Pattathil S, Parthasarathi R, Anderson NA, Kim JI, Venketachalam S, Hahn MG, Chapple C, Simmons BA, Singh S: **Impact of engineered lignin composition on biomass**

recalcitrance and ionic liquid pretreatment efficiency. *Green Chem* 2016, **18**:4884-4895.

20. Mottiar Y, Vanholme R, Boerjan W, Ralph J, Mansfield SD: **Designer lignins: harnessing the plasticity of lignification**. *Curr Opin Biotech* 2016, **37**:190–200.

21. Vanholme R, Morreel K, Darrah C, Oyarce P, Grabber JH, Ralph J, Boerjan W: **Metabolic engineering of novel lignin in biomass crops**. *New Phytol* 2012, **196**:978–1000.

22. Nair RB, Bastress KL, Ruegger MO, Denault JW, Chapple C: **The *Arabidopsis thaliana REDUCED EPIDERMAL FLUORESCENCE1* gene encodes an aldehyde dehydrogenase involved in ferulic acid and sinapic acid biosynthesis**. *Plant Cell* 2004, **16**:544–554.

23. Ralph J, Quideau S, Grabber JH, Hatfield RD: **Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls**. *J Chem Soc Perk T 1* 1994, **0**:3485-3498.

24. Ralph J, Helm RF, Quideau S, Hatfield RD: **Lignin–feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers**. *J Chem Soc Perk T 1* 1992, **0**:2962-2969.

25. Grabber JH, Hatfield RD, Ralph J, Zoń J, Amrhein N: **Ferulate cross-linking in cell walls isolated from maize cell suspensions**. *Phytochemistry* 1995, **40**:1077–1082.

26. Ralph J, Grabber JH, Hatfield RD: **Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins**. *Carbohyd Res* 1995, **275**:167–178.

27. Jacquet G, Pollet B, Lapierre C, Mhamdi F, Rolando C: **New ether-linked ferulic acid-coniferyl alcohol dimers identified in grass straws.** *J Agr Food Chem* 1995, **43**:2746–2751.

28. Quideau S, Ralph J: **Lignin–ferulate cross-links in grasses. Part 4.1–3 Incorporation of 5–5-coupled dehydrodiferulate into synthetic lignin**. *J Chem Soc Perk T 1* 1997, **0**:2351-2358.

29. Lam TB-T, Iiyama K, Stone BA: **Hot alkali-labile linkages in the walls of the forage grass *Phalaris aquatica* and *Lolium perenne* and their relation to in vitro wall digestibility**. *Phytochem* 2003, **64**:603–607.

30. Hatfield RD, Jung H, Marita JM, Kim H: **Cell wall characteristics of a maize mutant selected for decreased ferulates**. *American J Plant Sci* 2018, **09**:446-466.

31. Grabber JH, Ralph J, Hatfield RD: **Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize**. *J Agr Food Chem* 1998, **46**:2609–2614.

32. Jung HG, Phillips RL: **Putative seedling ferulate ester (*sfe*) maize mutant: morphology, biomass yield, and stover cell wall composition and rumen degradability**. *Crop Sci* 2010, **50**:403–418.

33. Jung HG, Mertens DR, Phillips RL: **Effect of reduced ferulate-mediated lignin/arabinoxylan cross-linking in corn silage on feed intake, digestibility, and milk production1**. *J Dairy Sci* 2011, **94**:5124–5137.

34. Anders N, Wilkinson MD, Lovegrove A, Freeman J, Tryfona T, Pellny TK, Weimar T, Mortimer JC, Stott K, Baker JM, Defoin-Platel M, Shewry PR, Dupree P, Mitchell RAC: **Glycosyl transferases in family 61 mediate arabinofuranosyl transfer onto xylan in grasses**. *PNAS* 2012, **109**:989–993.

35. Freeman J, Ward JL, Kosik O, Lovegrove A, Wilkinson MD, Shewry PR, Mitchell RAC: **Feruloylation and structure of arabinoxylan in wheat endosperm cell walls from RNAi lines with suppression of genes responsible for backbone synthesis and decoration**. *Plant Biotech J* 2017, **15**:1429–1438.

36. Chiniquy D, Sharma V, Schultink A, Baidoo EE, Rautengarten C, Cheng K, Carroll A, Ulvskov P, Harholt J, Keasling JD, Pauly M, Scheller HV, Ronald PC: **XAX1 from glycosyltransferase family 61 mediates xylosyltransfer to rice xylan**. *PNAS* 2012, **109**:17117–17122.

37. Meester BD, Vries L de, Özparpucu M, Gierlinger N, Corneillie S, Pallidis A, Goeminne G, Morreel K, Bruyne MD, Rycke RD, Vanholme R, Boerjan W: **Vessel-specific reintroduction of CINNAMOYL-COA REDUCTASE1 (CCR1) in dwarfed *ccr1* mutants restores vessel and xylary fiber integrity and increases biomass**. *Plant Physiol* 2018, **176**:611–633.

38. Petersen PD, Lau J, Ebert B, Yang F, Verhertbruggen Y, Kim JS, Varanasi P, Suttangkakul A, Auer M, Loqué D, Scheller HV: **Engineering of plants with improved properties as biofuels feedstocks by vessel-specific complementation of xylan biosynthesis mutants**. *Biotech Biofuels* 2012, **5**:84.

39. Mitchell RAC, Dupree P, Shewry PR: **A novel bioinformatics approach identifies candidate genes for the synthesis and feruloylation of arabinoxylan**. *Plant Physiol* 2007, **144**:43–53.

40. Rennie EA, Scheller HV: **Xylan biosynthesis**. *Curr Opin Biotech* 2014, **26**:100–107.

41. Piston F, Uauy C, Fu L, Langston J, Labavitch J, Dubcovsky J: **Down-regulation of four putative arabinoxylan feruloyl transferase genes from family PF02458 reduces ester-linked ferulate content in rice cell walls**. *Planta* 2010, **231**:677–691.

42. Molinari HB, Pellny TK, Freeman J, Shewry PR, Mitchell RAC: **Grass cell wall feruloylation: distribution of bound ferulate and candidate gene expression in *Brachypodium distachyon***. *Front Plant Sci* 2013, **4**:50.

43. Buanafina MM de O, Fescemyer HW, Sharma M, Shearer EA: **Functional testing of a PF02458 homologue of putative rice arabinoxylan feruloyl transferase genes in *Brachypodium distachyon***. *Planta* 2016, **243**:659–674.

44. de Souza WR, Martins PK, Freeman J, Pellny TK, Michaelson LV, Sampaio BL, Vinecky F, Ribeiro AP, da Cunha BADB, Kobayashi AK, de Oliveira PA, Campanha RB, Pacheco TF, Martarello DCI, Machiosi R, Ferrarese-Filho O, dos Santos WD, Tramontina R, Squina FM, Centeno DC, Gaspar M, Braga, MR, Tine MAS, Ralph J, Mitchell RAC, Molinari HBC: **Suppression of a single BAHD gene in *Setaria viridis* causes large, stable decreases in cell wall feruloylation and increases biomass digestibility**. *New Phytol* 2018, **218**:81–93.

45. Nishimura H, Kamiya A, Nagata T, Katahira M, Watanabe T: **Direct evidence for α ether linkage between lignin and carbohydrates in wood cell walls**. *Sci Rep UK* 2018, **8**:6539.

46. Watanabe T, Koshijima T: **Evidence for an ester linkage between lignin and glucuronic acid in lignin–carbohydrate complexes by DDQ-oxidation**. *Agr Biol Chem Tokyo* 1988, **52**:2953–2955.

47. Balakshin MY, Capanema EA, Chang H: **MWL fraction with a high concentration of lignin-carbohydrate linkages: Isolation and 2D NMR spectroscopic analysis**. *Holzforschung* 2007, **61**:1–7.

48. Arnling Bååth J, Giummarella N, Klaubauf S, Lawoko M, Olsson L: **A glucuronoyl esterase from *Acremonium alcalophilum* cleaves native lignin-carbohydrate ester bonds**. *FEBS Lett* 2016, **590**:2611-2618.

49. Špániková S, Biely P: **Glucuronoyl esterase – Novel carbohydrate esterase produced by *Schizophyllum commune***. *FEBS Letters* 2006, **580**:4597–4601.

50. Mosbech C, Holck J, Meyer AS, Agger JW: **The natural catalytic function of CuGE glucuronoyl esterase in hydrolysis of genuine lignin–carbohydrate complexes from birch**. *Biotech Biofuels* 2018, **11**:71.

51. Lyczakowski JJ, Wicher KB, Terrett OM, Faria-Blanc N, Yu X, Brown D, Krogh KBRM, Dupree P, Busse-Wicher M: **Removal of glucuronic acid from xylan is a strategy to improve the conversion of plant biomass to sugars for bioenergy**. *Biotech Biofuels* 2017, **10**:224.

52. Ralph J, Lapierre C, Marita JM, Kim H, Lu F, Hatfield RD, Ralph S, Chapple C, Franke R, Hemm MR, Van Doorsselaere J, Sederoff RR, O'Malley DM, Scott JT, MacKay JJ, Yahiaoui N, Boudet A, Pean M, Pilate G, Jouanin L, Boerjan W: **Elucidation of new structures in lignins of CAD-and COMT-deficient plants by NMR**. *Phytochemistry* 2001, **57**:993–1003.

53. Chen F, Tobimatsu Y, Havkin-Frenkel D, Dixon RA, Ralph J: **A polymer of caffeyl alcohol in plant seeds**. *PNAS* 2012, **109**:1772–1777.

54. Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M, Chen F, Foston M, Ragauskas A, Bouton J, Dixon RA, Wang ZY: **Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass**. *PNAS* 2011, **108**:3803–3808.

55. Weng J-K, Mo H, Chapple C: **Over-expression of F5H in COMT-deficient Arabidopsis leads to enrichment of an unusual lignin and disruption of pollen wall formation**. *Plant J* 2010, **64**:898–911.

56. Baxter HL, Mazarei M, Fu C, Cheng Q, Turner GB, Sykes RW, Windham MT, Davis MF, Dixon RA, Wang Z-Y, Neal Stewart C Jr: **Time course field analysis of COMT-downregulated switchgrass: lignification, recalcitrance, and rust susceptibility**. *Bioenerg Res* 2016, **9**:1087–1100.

57. Li M, Pu Y, Yoo CG, Gjersing E, Decker SR, Doeppke C, Shollenberger T, Tschaplinski TJ, Engle NL, Sykes RW, Davis MF, Baxter HL, Mazarei M, Fu C, Dixon RA, Wang ZY, Neal Stewart C Jr, Ragauskas AJ: **Study of traits and recalcitrance reduction of field-grown COMT down-regulated switchgrass**. *Biotech Biofuels* 2017, **10**:12.

58. Tobimatsu Y, Elumalai S, Grabber JH, Davidson CL, Pan X, Ralph J: **Hydroxycinnamate conjugates as potential monolignol replacements: In vitro lignification and cell wall studies with rosmarinic acid**. *ChemSusChem* 2012, **5**:676–686.

59. Petersen M, Häusler E, Meinhard J, Karwatzki B, Gertlowski C: **The biosynthesis of rosmarinic acid in suspension cultures of *Coleus blumei***. *Plant Cell Tiss Organ Cult* 1994, **38**:171–179.

60. Zhao Q, Tobimatsu Y, Zhou R, Pattathil S, Gallego-Giraldo L, Fu C, Jackson LA, Hahn MG, Kim H, Chen F, Ralph J, Dixon RA: **Loss of function of cinnamyl alcohol dehydrogenase 1 leads to unconventional lignin and a temperature-sensitive growth defect in *Medicago truncatula***. *PNAS* 2013, **110**:13660–13665.

61. Bouvier d'Yvoire M, Bouchabke-Coussa O, Voorend W, Antelme S, Cézard L, Legée F, Lebris P, Legay S, Whitehead C, McQueen-Mason SJ, Gomez LD, Jouanin L, Lapierre C, Sibout R: **Disrupting the *cinnamyl alcohol dehydrogenase 1* gene (*BdCAD1*) leads to altered lignification and improved saccharification in *Brachypodium distachyon***. *Plant J* 2013, **73**:496–508.

62. Baucher M, Chabbert B, Pilate G, Doorsselaere JV, Tollier MT, Petit-Conil M, Cornu D, Monties B, Montagu MV, Inze D, Jouanin L, Boerjan W: **Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar**. *Plant Physiol* 1996, **112**:1479–1490.

63. Fornalé S, Capellades M, Encina A, Wang K, Irar S, Lapierre C, Ruel K, Joseleau J-P, Berenguer J, Puigdomènech P, Rigau J, Caparrós-Ruiz D: **Altered lignin biosynthesis improves cellulosic bioethanol production in transgenic maize plants down-regulated for cinnamyl alcohol dehydrogenase**. *Mol Plant* 2012, **5**:817–830.

64. Anderson NA, Tobimatsu Y, Ciesielski PN, Ximenes E, Ralph J, Donohoe BS, Ladisch M, Chapple C: **Manipulation of guaiacyl and syringyl monomer biosynthesis in an**

Arabidopsis cinnamyl alcohol dehydrogenase mutant results in atypical lignin biosynthesis and modified cell wall structure. *Plant Cell* 2015, **27**:2195–2209.

65. Van Acker R, Déjardin A, Desmet S, Hoengenaert L, Vanholme R, Morreel K, Laurans F, Kim H, Santoro N, Foster C, Goeminne G, Légée F, Lapierre C, Pilate G, Ralph J, Boerjan W: **Different routes for conifer- and sinapaldehyde and higher saccharification upon deficiency in the dehydrogenase CAD1**. *Plant Physiol* 2017, **175**:1018–1039.

Figure 1 Hemicellulose structural diversity across major phylogenetic groups: The known structures of the secondary cell wall hemicelluloses, xylan and galactoglucomannan, are shown, along with the proportion of dry biomass by weight that hemicellulose forms in each major phylogenetic group. A dash indicates that the hemicellulose structure or weight content is not known.

Figure 2 Two mechanisms for hemicellulose:lignin cross-linking. A) Radical coupling of a monolignol to ferulated xylan. The backbone xylosyl residue (grey) is susbtituted by 3-linked arabinosyl residue (pink), which is modified by a ferulate (brown). A β-O-4 linkage between the ferulate and a monolignol is shown, but other linkages can occur. B) Re-aromatisation of the quinone methide intermediate by hemicellulose nucleophiles. The quinone methide intermediate of two monolignols forming a β-O-4 linkage is shown. The intermediate is re-aromatised by the carbon 6 hydroxyl of a mannosyl residue of galactoglucomannan. A mannosyl residue (blue) ether-linked to a dilignol is shown, but other glycosyl residues could form the bond. Curly arrows show the movement of electrons. Further lignin polymerisation can occur at carbons marked by orange dotted lines.

Figure 3 Down-regulation of COMT or CAD activity are potential lignin engineering strategies to prevent hemicellulose:lignin cross-linking via re-aromatisation of the quinone methide intermediate A) Down regulation of COMT produces a monolignol with an additional aromatic ring hydroxyl, 5-hydroxyconiferyl alcohol. During re-aromatisation of the quinone methide intermediate, the additional ring hydroxyl acts as an internal nucleophile, forming benzodioxane units. B) Downregulation of CAD activity produces hydroxycinnamaldehydes. During re-aromatisation of the quinone methide intermediate of hydroxycinnamaldehydes, the proton at position 8 is eliminated, resulting in unsaturated bonds between position 7 and 8. Curly arrows show the movement of electrons.

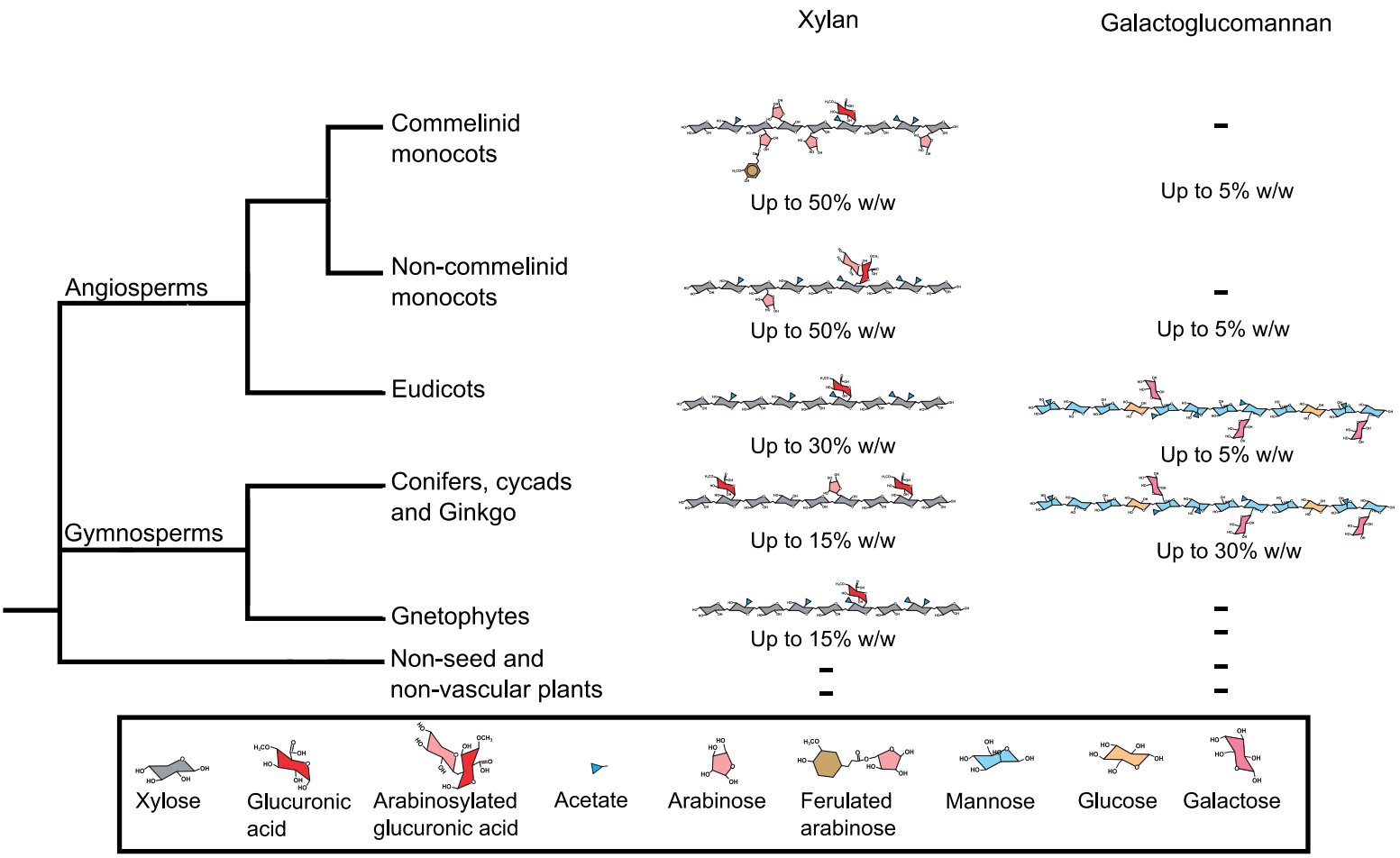
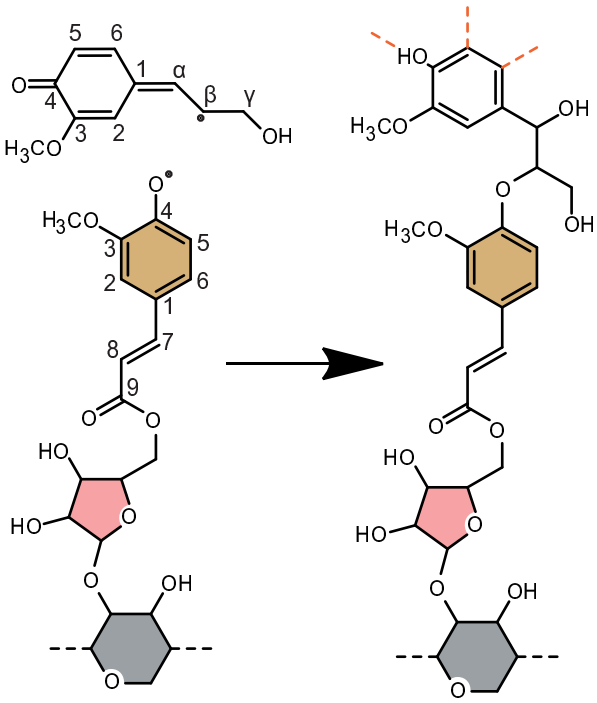

Xylan
Galactoglucomannan
Commelinid monocots
Up to 50% w/w
Up to 5% w/w
Non-commelinid monocots
Up to 50% w/w
Up to 5% w/w
Angiosperms
Eudicots
Up to 30% w/w
Up to 5% w/w
Conifers, cycads and Ginkgo
Up to 15% w/w
Up to 30% w/w
Gymnosperms
Gnetophytes
Up to 15% w/w
Non-seed and non-vascular plants
Xylose
Glucuronic acid
Arabinosylated glucuronic acid
Acetate
Arabinose
Ferulated arabinose
Mannose
Glucose
Galactose

Figure 1

A



B

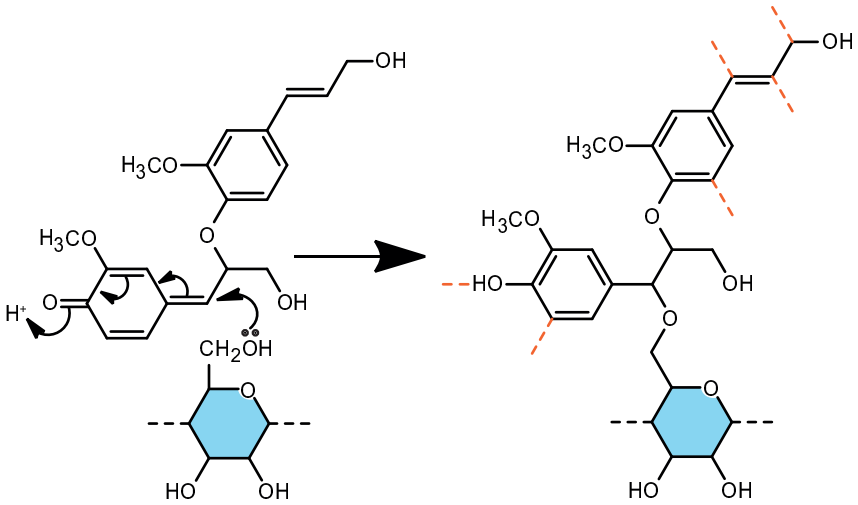
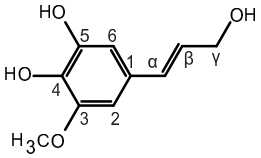
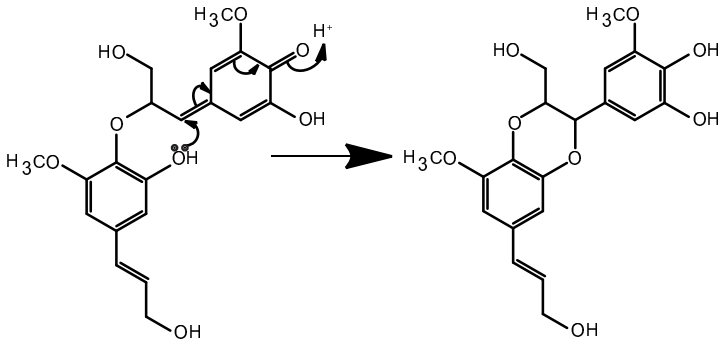


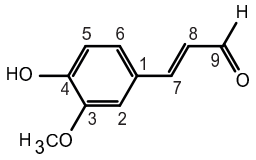
Figure 2

A


HO
OH
HO
H3CO
α
β
γ


H3CO
H+
HO
O
OH
OH
H3CO
OH
H3CO
HO
OH
OH
H3CO
O
OH

B


H
HO
H3CO
O

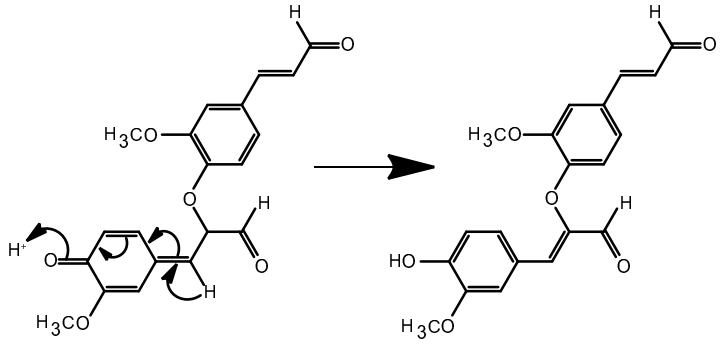
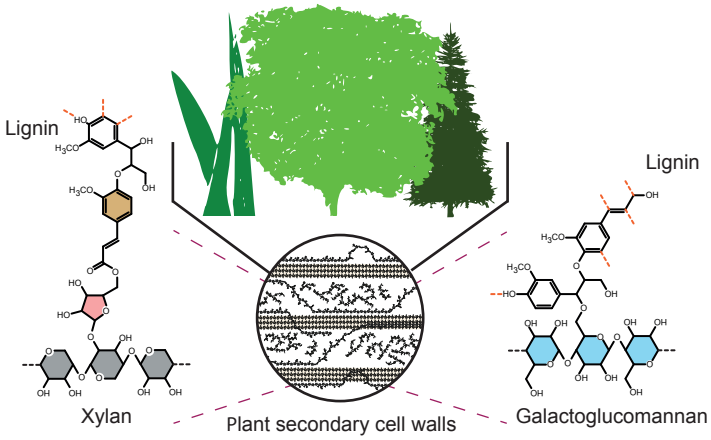

H
O
H3CO
O
H
H+
O
H
O
H3CO
H
H
O
H3CO
O
H
HO
O
H3CO

Figure 3


Lignin
Xylan
Plant secondary cell walls
Lignin
Galactoglucomannan