Review article

Altered lignin biosynthesis using biotechnology to improve lignocellulosic biofuel feedstocks

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Summary

Lignocellulosic feedstocks can be converted to biofuels, which can conceivably replace a large fraction of fossil fuels currently used for transformation. However, lignin, a prominent constituent of secondary cell walls, is an impediment to the conversion of cell walls to fuel: the recalcitrance problem. Biomass pretreatment for removing lignin is the most expensive step in the production of lignocellulosic biofuels. Even though we have learned a great deal about the biosynthesis of lignin, we do not fully understand its role in plant biology, which is needed for the rational design of engineered cell walls for lignocellulosic feedstocks. This review will recapitulate our knowledge of lignin biosynthesis and discuss how lignin has been modified and the consequences for the host plant.

Lignocellulosic biomass as a source for biofuel

Lignocellulosic biomass is a potential large-scale biofuel feedstock wherein entire aboveground biomass would be converted to fuels. The major candidates of lignocellulosic biomass are dedicated biofuel crops such as switchgrass (*Panicum virgatum* L.), *Miscanthus*, and poplar (*Populus* spp.), as well as 'waste' products from forest biomass, crop residuals, and household refuse. In the USA alone, biofuels could sustainably replace nearly 30% of current petroleum used in transportation fuels (Perlack and Stokes, 2011). Dedicated perennial biomass crops could have many environmental and resource advantages over nonrenewable fossil fuels as well as annual crops. An advantage of perennials over annuals is that they regrow each year after harvest, reducing the time and energy spent for replanting each season. Another benefit is that they translocate minerals and carbohydrates into belowground storage at the dormancy stage, making the crop more suitable for the bioconversion process as well as reducing fertilizer needs in the subsequent growing season (Propheter and Staggenborg, 2010). Advantageous environmental qualities associated with perennial grass crops include reduced soil erosion, improved water quality, and the ability to sequester significant amounts of carbon belowground (McLaughlin *et al.*, 2002). A 5-year field study led to projections that lignocellulosic biofuels derived from switchgrass, a leading perennial biofuel candidate, would decrease greenhouse gas emissions and would act as a carbon sink compared with fossil fuels equivalents (Schmer *et al.*, 2008).

Carbohydrate-rich plant cell walls are the major energy sink in plant biomass (Hisano *et al.*, 2009). After growth and harvest of biomass, the major biofuel expense is pretreatment to loosen/breakdown cell walls, followed by saccharification and their conversion into biofuels (Rubin, 2008). Although lignocellulosic biofuels potentially provide benefits like reducing greenhouse gases, the high costs of pretreatment, saccharification, and fermentation can render them economically nonviable when taken in context of other fuel opportunities (Hisano *et al.*, 2009). Therefore, a major challenge is to decrease the effort and cost to convert these feedstocks into fuel. Fortunately, advances in our knowledge of the lignin biosynthetic pathway and plant biotechnology can be used to reduce the resistance of lignocellulosic biomass to breakdown into simple sugars and increase biofuel production; we now have effective tools to address the recalcitrance problem.

Why lignin needs to be manipulated in lignocellulosic feedstocks

Cellulose is the most prevalent polymer on earth and a primary source for the production of biofuels (Lynd *et al.*, 2008). Cellulose is difficult to convert into fermentable sugars because of its crystallinity and occlusion by lignin; decreasing lignin in cell walls can enhance saccharification (Saballos *et al.*, 2012; Vanholme *et al.*, 2008, 2010b). It is thought that lignin occludes cellulose by physically shielding it from microbial or enzymatic decomposition (Berlin *et al.*, 2006; Vanholme *et al.*, 2010a). Lignin can also adsorb hydrolytic enzymes to prevent them from breaking down cellulose into monosaccharides (Converse *et al.*, 1990; Lee *et al.*, 1994). In addition, lignin degradation products can inhibit the performance of yeast for fermentation of monosaccharides

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(Keating *et al.*, 2006). Such breakdown products or derivatives of lignin can also negatively affect the growth of microorganisms that can be used in consolidated bioprocessing (Agbor *et al.*, 2011). Thus, lignin has received significant attention in biofuels research with the goal of improving conversion efficiency. One of the most promising solutions to the lignin problem is to decrease its biosynthesis in the plant itself, as this approach might be more efficient and cost effective than removing it at the biorefinery. Pretreatment methods, such as acid hydrolysis, alkaline hydrolysis, and enzymatic hydrolysis, are currently being used to breakdown the lignocellulosic matrix and remove lignin (Kumar *et al.*, 2009; Mosier *et al.*, 2005). Expensive pretreatment might be decreased or eliminated by the use of low-lignin feedstocks (Chen and Dixon, 2007; Hisano *et al.*, 2009).

Evolution and biosynthesis of lignin

It is widely accepted that the adaptation of vascular plants to terrestrial habitats was made possible by lignin (Figure 1), which provides structural support and affiliated traits required for an erect growth habit (Jones *et al.*, 2001). Lignin, once believed to be limited to vascular plants, has recently been discovered in the bryophyte *Marchantia polymorpha* (Espineira *et al.*, 2011). Gene network studies have revealed the earliest appearance of the monolignol biosynthetic gene families in the moss *Physcomitrella patens,* but lignin and lignin monomers have not been found (Xu *et al.*, 2009). Studies by Martone *et al.* (2009), however, have identified the existence of secondary walls and lignin in the marine red alga *Calliarthron cheilosporioides* through derivatization followed by reductive cleavage (DFRC), a β-O-4 linkage specific lignin analysis technique. The relevant pathways might have evolved more than one billion years ago, prior to the divergence of red and green algae, or have developed convergently in *C. cheilosporioides* and land plants (Martone *et al.*, 2009). The presence of syringyl monomers (S-lignin) in the lignin polymer, which was thought to be a unique characteristic of angiosperms, has been found in some lycophytes (*Selaginella*), ferns (*Dennstaedtia bipinnata*), and gymnosperms (*Podocarpus macrophyllus* and *Tetraclinis articulate*). Thus, it might have evolved independently among plant taxa (Espineira *et al.*, 2011; Martone *et al.*, 2009). The discovery of a novel ferulate 5-hydroxylase (*F5H*) in *Selaginella*, which is structurally distinct from the angiosperm *F5H*, supports the hypothesis that the enzyme evolved independently at least twice (Weng *et al.*, 2008a). It is interesting to note that *Ginkgo biloba* cell suspension cultures have the ability to synthesize S-lignin, a trait not observed in its woody tissues (Uzal *et al.*, 2009).

Lignin, the second most abundant polymer in nature, functions as inter- and intramolecular adhesive, providing rigidity to secondary cell walls and the plant body (Boerjan *et al.*, 2003; Jones *et al.*, 2001). Lignin is formed via oxidative coupling of three p-hydroxyphenylpropanoids, namely *p*-coumaryl, coniferyl,

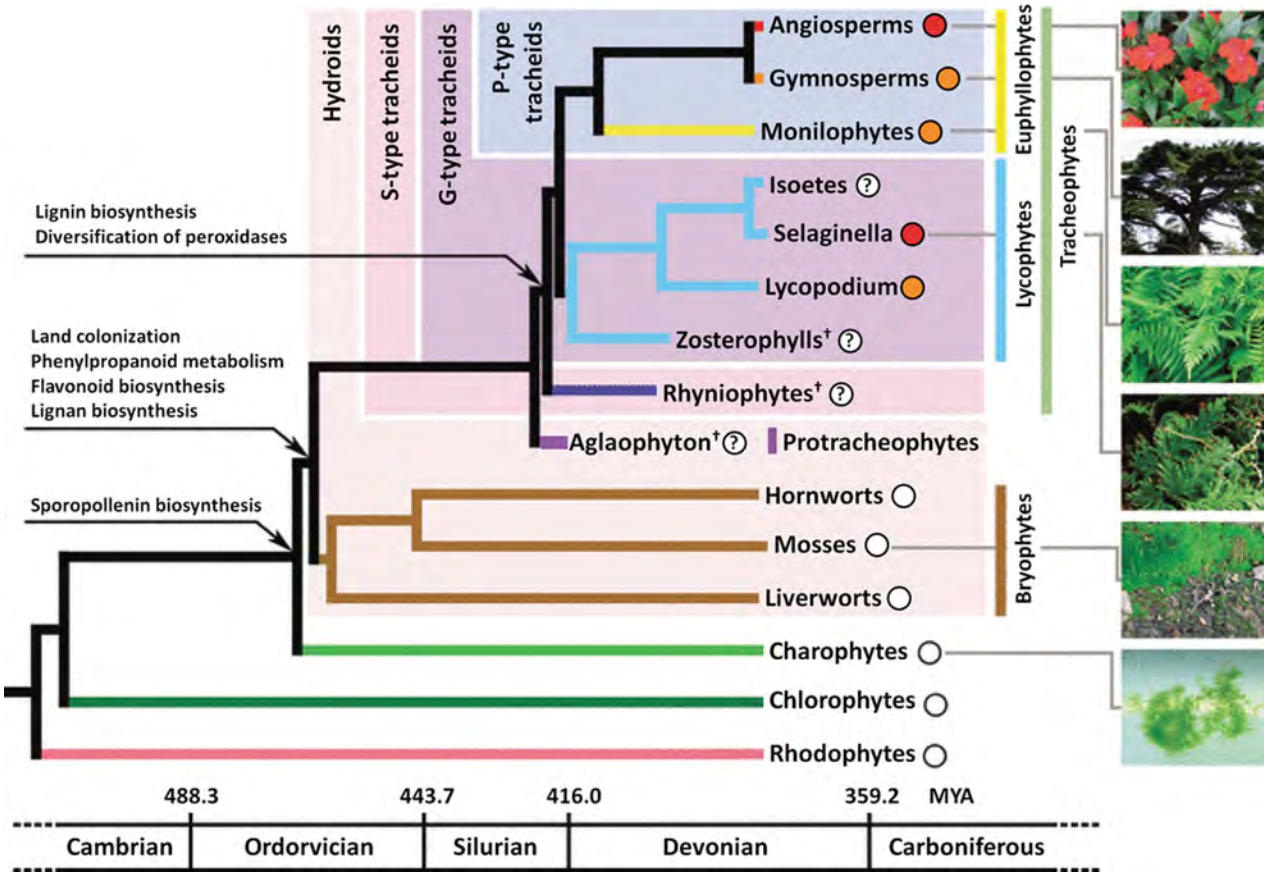

Figure 1 Evolution of lignin biosynthesis. Phylogenetic tree depicting the evolution of monolignols across major plant phyla (Weng and Chapple, 2010). Hollow circles indicate absence of lignin, orange circles indicate plants with H-lignin and G-lignin, and red circles indicate plants with S-lignin, G-lignin, and H-lignin. The presence of red circles more than once in the evolutionary tree indicates convergent evolution of S-lignin in *Selaginella* and angiosperms. Image used with permission from John Wiley & Sons, Inc.

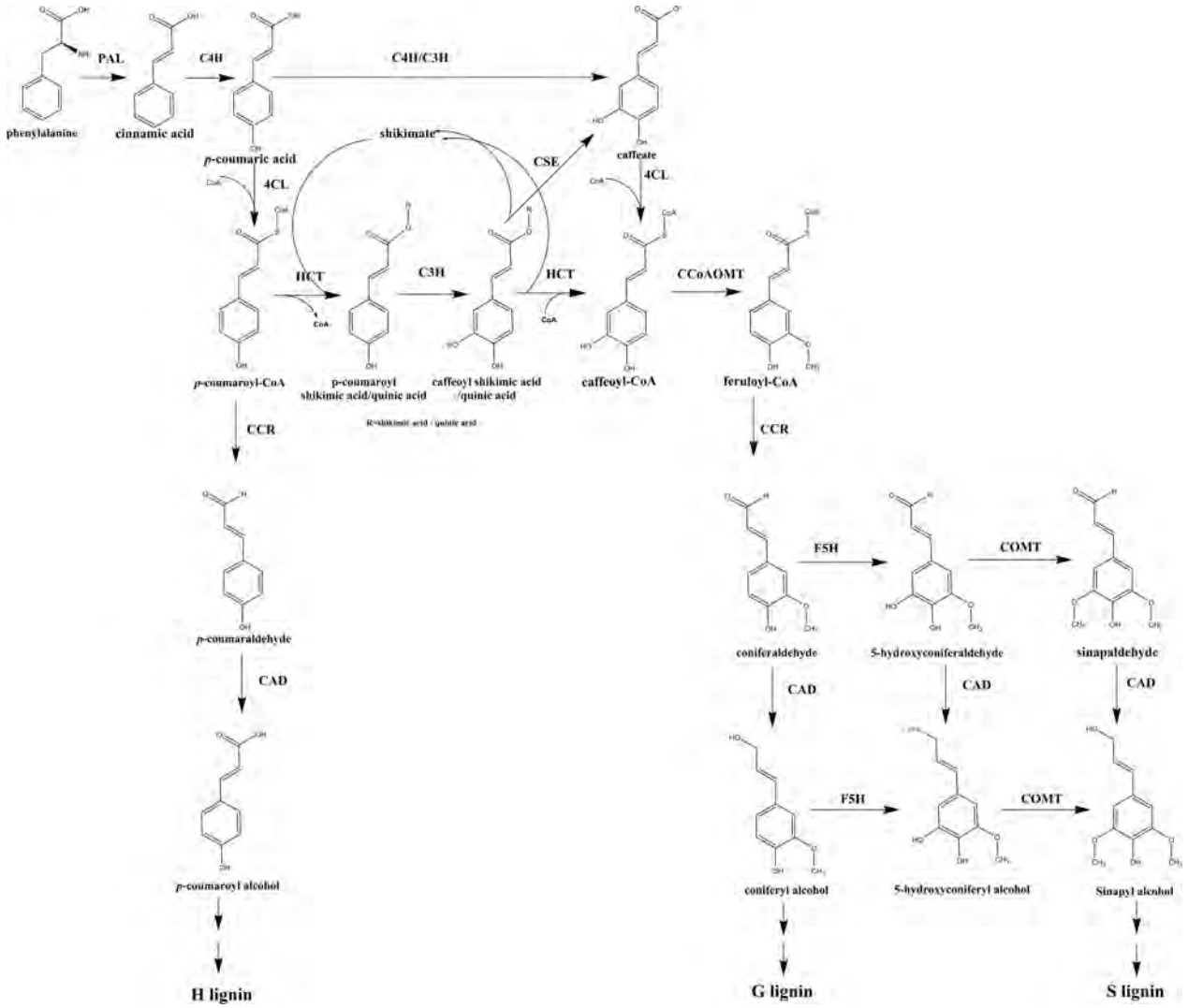

Figure 2 Diagram of the current accepted model of monolignol biosynthesis for *p*-coumaryl, coniferyl, and sinapyl alcohol in angiosperms including the newly identified enzyme caffeoyl shikimate esterase (Vanholme *et al.*, 2013b). The abbreviations: *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamate 4-hydroxylase; *CSE*, caffeoyl shikimate esterase; *4CL*, 4-coumarate:CoA ligase; *C3H*, *p*-coumarate 3-hydroxylase; *HCT*, *p*-hydroxycinnamoyl-CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase; *CCoAOMT*, caffeoyl-CoA *O*-methyltransferase; *CCR*, cinnamoyl-CoA reductase; *F5H*, ferulate 5-hydroxylase; *COMT*, caffeic acid *O*-methyltransferase; and *CAD*, cinnamyl alcohol dehydrogenase.

and sinapyl alcohols, which produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Figure 2) (Nove-Uzal *et al.*, 2012). The relative quantity of each component varies by species, organ, and growth stage (Hisano *et al.*, 2009). The lignin polymer in dicots is primarily composed of G and S monolignol units with trace amounts of *H* units. Similar to dicot lignin, G and S units are the main components of monocot lignin; however, monocot lignin tends to incorporate a higher proportion of H units than dicot lignin. Gymnosperm lignin is made up of almost entirely G units (Boerjan *et al.*, 2003; Gross, 1980). Biosynthesis of monolignols followed by their polymerization are the two main steps in lignin biosynthesis (Figure 2). Monolignol biosynthesis is thought to be highly conserved among angiosperm taxa, at least in its basic layout. The deamination of phenylalanine to produce cinnamic acid in the presence of phenylalanine ammonia-lyase (*PAL*) followed by the conversion of cinnamic acid to *p*-coumaric acid by cinnamate 4-hydroxylase (*C4H*) are the initial steps in the monolignol biosynthesis pathway. Following this conversion, *p*-coumaric acid undergoes a series of hydroxylation and methylation reactions that lead to the downstream production of the monolignols (Humphreys and Chapple, 2002) (Figure 2). Characterization of most of the enzymes in the monolignol biosynthesis pathway, along with the identification of various intermediates and precursors, has led to the discovery of pathways for further hydroxylation and methylation steps (Humphreys and Chapple, 2002; Ye *et al.*, 1994). The quantity of various monolignols produced in the earlier steps of lignin biosynthesis is the main determinant of the lignin polymer structure and is dependent on the plant species, cell type, and developmental stage.

Lignin manipulation in monocot and dicot species

Plants that have received considerable interest as potential biofuel feedstocks include monocot perennial grasses such as switchgrass

Miscanthus, and sugarcane (*Saccharum* spp.), as well as the dicot species, poplar (*Populus* spp.), and alfalfa (*Medicago sativa* L.). Monocot and dicot species each have distinct cell walls which differ from each other in the types and amounts of structural polysaccharides present, how the polysaccharides are linked, and in the abundance of proteins and phenolics (Vogel, 2008). In particular, the relative abundance and composition of the noncellulosic components, specifically hemicellulose and pectin, tend to differ between the two. Dicot and noncommelinoid monocot cell walls have type I cell walls, in which the cellulose microfibrils are cross-linked with xyloglucans and surrounded by a matrix of pectins and proteins. Type II cell walls, which are found in grasses and other commelinoid monocot species, are characterized by cross-links with glucoarabinoxylans and contain higher levels of phenolic acids such as hydroxycinnamates (McCann and Carpita, 2008; Shedletzky *et al.*, 1992; Vogel, 2008). Despite these compositional and structure differences, the lignin pathway is considered to be highly conserved across plant species, and similar trends resulting from lignin modification can be observed among monocots and dicots. For example, approaches for manipulating lignin in alfalfa (Chen and Dixon, 2007; Guo *et al.*, 2001) have been transferable to the monocot species, switchgrass, and sugarcane (Fu *et al.*, 2011a; Jung *et al.*, 2012). The next section will discuss various biotechnological methods for manipulating lignin biosynthesis in important model species and potential bioenergy crops, specifically focusing on how these approaches could be utilized for improving cell wall saccharification and biofuel yields in commercially relevant monocot and dicot species.

Cornucopia of choices for lignin modifications

The genes in the lignin biosynthesis pathway have been identified (Bonawitz and Chapple, 2010; Vanholme *et al.*, 2012; Weng and Chapple, 2010). More than ten enzymatic conversions are involved in monolignol synthesis, each step representing a potential target for lignin manipulation by down-regulation or overexpression of the enzyme involved in its catalysation. Polymerization of the monolignols is catalysed by peroxidases and laccases (Berthet *et al.*, 2011; Herrero *et al.*, 2013; Lee *et al.*, 2013); manipulating this process of monolignol polymerization is also considered to be a possible route for engineering lignin (Weng *et al.*, 2008b). Another approach for altering lignin lies in the manipulation of regulators of lignin biosynthetic genes. The expression of monolignol biosynthesis genes are regulated by a series of transcription factors during secondary cell wall formation (Zhong *et al.*, 2008, 2010), some of which can be manipulated to reduce carbon flow into lignin biosynthesis (Fornalé *et al.*, 2010; Shen *et al.*, 2012). MicroRNAs (miRNAs) that have been found to regulate certain aspects of plant development and lignification can also be manipulated to improve bioenergy-related traits (Rubinelli *et al.*, 2013). It is clear that there are many biotechnological approaches that can be used to change lignin in cell walls given the large number of biosynthetic genes and regulators that are involved in lignin biosynthesis. Currently, we know more about the identity and functions of the lignin biosynthetic enzymes than the regulators of lignin biosynthesis. Several monolignol biosynthetic genes have been down-regulated in commercially relevant bioenergy crops to better understand their effect on lignin biosynthesis and saccharification (Table 1). Progress is being made towards identifying and characterizing the regulatory genes that might influence important bioenergy characteristics such as biomass yield and saccharification (Fu *et al.*, 2012; Rubinelli *et al.*, 2013; Weng *et al.*, 2008b).

Manipulation of monolignol biosynthetic genes

Manipulation of genes in the monolignol biosynthesis pathway has a significant effect on total lignin and lignin composition. Depending on genes chosen for modification, part or all of the metabolite flux can be restricted for monolignol synthesis (Vanholme *et al.*, 2012). A number of genes have been overexpressed or down-regulated in transgenic plants to understand their effect on lignin biosynthesis. The first enzyme of the phenylpropanoid pathway is PAL, and the general pathway ends with caffeoyl-CoA *O*-methyltransferase (CCoAOMT). Monolignol-specific biosynthesis starts with cinnamoyl-CoA reductase (CCR) (Figure 2). Down-regulation of genes that occur early in the phenylpropanoid pathway can reduce lignin content, but can also cause pleiotropic effects, such as stunted growth and collapsed vasculature (Bonawitz and Chapple, 2013). For example, in *PAL*-down-regulated tobacco, the number and size of the vessels were reduced, which compromised vascular integrity of the transgenic plants (Korth *et al.*, 2001). Allelic mutants of cinnamate 4-hydroxylase (C4H) had discrete effects on plant growth, lignin content, and the S : G monolignol ratio. The weakest *Arabidopsis thaliana* mutant not only had less lignin content, but also had the least yield penalty and was similar to wild-type plants (Schilmiller *et al.*, 2009).

The lignin biosynthesis pathway enzyme 4-coumarate:CoA ligase (4CL) has been extensively studied. 4CL catalyses the conversion of *p*-coumaric and caffeic acids to their thio ester form in the phenylpropanoid pathway and takes part in the regulation of monolignol precursors, thereby controlling lignin content and composition (Saballos *et al.*, 2012). 4CL down-regulation has been shown to have a positive, negative, or neutral effect on biomass production and can vary depending on the gene expression level and host species. For example, when 4CL was down-regulated in switchgrass, there was no change in biomass yield (Xu *et al.*, 2011), whereas a negative relationship between the level of 4CL down-regulation and biomass production was observed in poplar (Voelker *et al.*, 2010). In *Populus tremuloides*, the decrease in lignin as a result of 4CL down-regulation resulted in a relative increase in cellulose and enhanced growth (Hu *et al.*, 1999). Decreases in lignin content caused by down-regulation of 4CL can be associated with increased saccharification efficiency in some species. For instance, in sorghum brown midrib2 mutants with mutations in the 4CL gene, an increase in glucose release efficiency of up to 17% was observed (Saballos *et al.*, 2008, 2012). In 4CL-down-regulated switchgrass, transgenic plants yielded 57% more fermentable sugars than their controls following a dilute acid pretreatment (Xu *et al.*, 2011).

Another series of conversions are catalysed by *p*-coumarate 3-hydroxylase (C3H) and *p*-hydroxycinnamoyl-CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase (*HCT*). C3H catalyses the addition of hydroxyl groups at the meta-position of *p*-coumarate and is involved in the conversion of *p*-coumarate coA to caffeoyl coA. Hydroxycinnamoyltransferase catalyses the reactions before and after the addition of the 3-hydroxyl group (Hoffmann *et al.*, 2004). Hydroxycinnamoyltransferase down-regulation in alfalfa produced plants with stunted growth and decreased biomass production relative to their controls. Transgenic plants had lower lignin levels and altered lignin composition characterized by a decrease in both S and G units and an increase in *H* units (Shadle *et al.*, 2007). The recent identification of a rare natural mutant in

Table 1 A list of current and potential bioenergy crops in which lignin biosynthesis has been altered, leading to modifications to lignin content and/or composition and saccharification efficiency

		Lignin manipulation			Saccharification by enzymatic hydrolysis		
Gene	Species	Method of manipulation	Lignin content	S : G ratio	Pretreatment	Saccharification efficiency	References
4CL	*Populus*	AS	↓ 10%	↓	HW	NC	Voelker *et al.* (2010)
	Switchgrass	RNAi	↓ 17%–32%	↓	DA	↑57%	Xu *et al.* (2011)
C4H	Alfalfa	AS	↓	↓	None	↑	Chen and Dixon (2007)
					DA	↑	
HCT	Alfalfa	AS	↓	↑	None	↑	Chen and Dixon (2007)
					DA	↑	
C3H	Alfalfa	AS	↓	↑	None	↑	Chen and Dixon (2007)
					DA	↑	
CCo-AOMT	Alfalfa	AS	↓	↑	None	NC	Chen and Dixon (2007)
					DA	↑	
CCR	*Populus*	AS	↓ 5%–24%	NA	None	↑ 14%–139%	Van Acker *et al.* (2014)
					DA	↑ 19%–95%	
					A	↑ 11%–78%	
F5H	Alfalfa	AS	NC	↓	None	NC	Chen and Dixon (2007)
					DA	NC	
COMT	Alfalfa	AS	↓	↓	None	↑	Chen and Dixon (2007)
					DA	↑	
	Switchgrass (greenhouse-grown)	RNAi	↓ 6%–14%	↓	None	↑ 29%–38%	Fu *et al.* (2011a)
					DA	↑ 17%–22%	
	Switchgrass (field-grown)	RNAi	↓ 8%–15%	↓	HW	↑ 6%–34%	Baxter *et al.* (2014)
	Sugarcane (greenhouse-grown)	RNAi	↓ 4%–14%	↓	None	↑ up to 29%	Jung *et al.* (2012)
					DA	↑ 20%–34%	
	Sugarcane (field-grown)	RNAi	↓ 6%–12%	↓	DA	↑ 19%–32%	Jung *et al.* (2013)
CAD	Switchgrass	RNAi	↓ 14%–22%	↓	None	↑ 19%–89%	Fu *et al.* (2011b)
					DA	↑ 19%–44%	
	Switchgrass	RNAi	↓	NA	A	↑ 2%–11%	Saathoff *et al.* (2011)
	Maize	RNAi	NC in stems, ↓ 6.4% in midribs	↓	None	↑ 25% in stems, ↑ 16% in midribs	Fornalé *et al.* (2012)
MYB4	Switchgrass	OE	↓ 40%–50%	NC	None	↑ up to 250%	Shen *et al.* (2012)

Values represent per cent change relative to the control. ↑, increase and decrease.

AS, antisense-mediated down-regulation; RNAi, RNA interference-mediated down-regulation; and OE, overexpression; NA: not available; NC: no change; HW, hot water; DA, dilute acid; and A, alkaline; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; *C4H*, cinnamate 4-hydroxylase; *4CL*, 4-coumarate:CoA ligase; *C3H*, p-coumarate 3-hydroxylase; *CCo-AOMT*, caffeoyl-CoA *O*-methyltransferase; *F5H*, ferulate 5-hydroxylase; *HCT*, p-hydroxycinnamoyl-CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase; MYB4, myeloblastosis.

poplar with a premature stop codon in the HCT gene had a 17-fold increase in H units over the wild type and showed no growth penalty. The G unit pool was significantly reduced in dormant, but not in actively growing, tissues (Vanholme *et al.*, 2013a). However, it is debatable whether these natural mutants can be utilized for modifying lignin without growth penalty, as the overall *H* unit content did not change due to the presence of the catalytic site leading to residual HCT activity.

The recent identification of an otherwise uncharacterized lignin biosynthesis gene, caffeoyl shikimate esterase (CSE), adds another step to the lignin biosynthesis pathway that can be manipulated (Vanholme *et al.*, 2013b). Studies with Arabidopsis *cse* T-DNA mutants suggest that CSE functions in the lignin biosynthetic pathway by catalysing the hydrolysis of caffeoyl shikimate to caffeate. Arabidopsis *cse* mutants had lower lignin levels and a higher proportion of *H* units which was associated with an increased saccharification efficiency of up to 75% over the wild type. One mutant exhibited a slower growth rate and reduced dry weight yield, while the other performed similar to the wild type. While manipulation of some genes in the general phenylpropanoid pathway can be associated with severe growth defects, identification and characterization of natural mutants can be useful in identifying novel genes that can be manipulated to reduce lignin content without significantly impacting biomass production. It is also important to consider that large increases in saccharification efficiency can compensate some losses in biomass production (Chen and Dixon, 2008).

Monolignol-specific pathway enzymes include CCR, F5H, caffeic acid *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD). Changes in the expression level of these genes directly affect the amount of lignin and/or the lignin composition (Boerjan *et al.*, 2003). The saccharification and/or ethanol yield of lignocellulosic biomass can be increased by down-regulating CCR, COMT, and CAD. For instance, down-regulation of CCR in poplar doubled the amount of cell wall sugars released by *Clostridium cellulolyticum* relative to the

nontransgenic parent (Boudet *et al.*, 2003). Another study with poplar found that CCR down-regulation resulted in an ethanol yield increase of up to 161% in one line; however, a yield penalty was also observed, hypothesized to be a result of collapsed xylem vessels. Taking the yield decline into account, the increase in ethanol yield in this line was still 57% higher than the control (Van Acker *et al.*, 2014). COMT is primarily involved in the synthesis of S-lignin, and its down-regulation is characterized by a decrease in total lignin content and a reduction in S units (Guo *et al.*, 2001; Humphreys *et al.*, 1999; Osakabe *et al.*, 1999). Brown midrib mutant and transgenic grasses with suppressed COMT activity had improved saccharification efficiencies relative to controls, and in the case of switchgrass and bmr12 sorghum, higher ethanol yields (Dien *et al.*, 2009; Fu *et al.*, 2011a; Jung *et al.*, 2012). Interestingly, COMT down-regulation can result in an incorporation of a novel monomer, derived from the COMT substrate 5-hydroxyconiferaldehyde, into the lignin polymer. In addition to the reduced lignin content and decreased S/G ratio, it is hypothesized that the incorporation of this monomer into the lignin structure may also contribute to the improved digestibility and/or saccharification that is often observed in COMT-down-regulated plants through its effect on lignin cross-linking (Simmons *et al.*, 2010). While yield reductions have been reported in COMT-deficient brown midrib mutants of maize (bm3) and sorghum (bmr12) (Lee and Brewbaker, 1984; Miller *et al.*, 1983; Oliver *et al.*, 2005), COMT down-regulation in transgenic switchgrass, maize, and perennial ryegrass (*Lolium perenne*) had no effect on growth or development (Fu *et al.*, 2011a; Piquemal *et al.*, 2002; Tu *et al.*, 2010). In COMT-down-regulated sugarcane, the transgenic line with the strongest reduction in lignin content (13.7%) exhibited a biomass yield reduction and thin stalks, whereas the transgenic lines with a moderate lignin reduction (3.9%–8.4%) showed normal growth and development compared with nontransgenic controls (Jung *et al.*, 2012). The final step in the biosynthesis of monolignols is catalysed by CAD, which, when down-regulated, leads to an assimilation of hydroxycinnamaldehydes into the lignin polymer (Boerjan *et al.*, 2003; Li *et al.*, 2008). The involvement of CAD in lignin biosynthesis has been studied in several species including poplar, eucalyptus, switchgrass, maize, and *Brachypodium* (Bouvier d'Yvoire *et al.*, 2013; Chabannes *et al.*, 2001; Fornalé *et al.*, 2012; Jackson *et al.*, 2008; Lapierre *et al.*, 2004; Ralph *et al.*, 2001; Saathoff *et al.*, 2011; Valerio *et al.*, 2003). A reduction in CAD activity in transgenic and mutant lignocellulosic grasses resulted in reduced lignin content and modified lignin structure, which translated to improved saccharification efficiency in switchgrass and *Brachypodium*, and higher ethanol yield in maize (Bouvier d'Yvoire *et al.*, 2013; Fornalé *et al.*, 2012; Fu *et al.*, 2011b; Saathoff *et al.*, 2011). In the plants studied for down-regulation of CAD, biomass production was not significantly affected. These studies signify the importance of down-regulating monolignol biosynthetic genes for reducing lignin content and improving saccharification efficiency. Unlike CCR, COMT, and CAD, manipulating the expression of F5H causes changes to the lignin composition without affecting the total lignin content. Some studies suggest that the lignin composition, specifically the S/G ratio, may be as or more influential than lignin content on the saccharification of pretreated biomass (Corredor *et al.*, 2009; Li *et al.*, 2010). F5H is a particularly important target for manipulating the S/G ratio as it controls partitioning between the synthesis of coniferyl and sinapyl alcohols and is the main enzyme responsible for S-lignin production. Consequently, overexpression of F5H in poplar led to an increase in S-lignin and no change in total lignin content (Stewart *et al.*, 2009), while the lignin of Arabidopsis mutants deficient in F5H was found to be comprised almost entirely of G units (Marita *et al.*, 1999; Meyer *et al.*, 1998). Although no change in total lignin content was observed, transgenic poplar with overexpression of F5H were more digestible and had improved pulping performance relative to control, which was attributed to the increased S monomer content (Huntley *et al.*, 2003). Similarly, Arabidopsis plants with overexpression of F5H driven by a C4H promoter had lignin that was comprised of over 90% S units, resulting in an improved saccharification efficiency relative to the control following a hot water pretreatment. In alfalfa, down-regulation of F5H decreased the S/G ratio without affecting the lignin content, and no change in saccharification efficiency of pretreated biomass was observed (Chen and Dixon, 2007).

After monolignols are produced, they are oxidized and combined by coupling to form lignin polymers (Vanholme *et al.*, 2008). Laccases have been suggested to play a role in this oxidation process. To better understand the role of laccases in lignification, Berthet *et al.* (2011) analysed lignin profiles of laccase T-DNA insertion mutants of Arabidopsis. Two genes, *LAC4* and *LAC17*, were identified as playing a role in lignification in Arabidopsis. They observed that *LAC4* and *LAC17* mutants had lower lignin levels and that the deposition of G-lignin was influenced by a *lac17* mutation. Interestingly, double mutants of *lac4* and *lac17* had significantly reduced lignin levels and no growth defects under normal growth conditions. The double mutants also had increased saccharification, even though saccharification efficiency of *lac17* plants was similar to that of controls. Sugarcane laccase *SofLAC* was able to complement the Arabidopsis *lac17* mutation and restore lignin levels to those comparable to wild-type plants (Cesarino *et al.*, 2013). The results of this study suggest that manipulating the expression of laccases involved in lignification could provide another route for increasing cell wall saccharification (Berthet *et al.*, 2011).

Manipulation of transcription factors of lignin biosynthetic genes

Expression of genes in the monolignol biosynthetic pathway is regulated by R2R3-type transcription factors (TFs) which have the *MYB* DNA binding domain (Rogers and Campbell, 2004; Stracke *et al.*, 2001). Manipulation of these transcription factors can have a dramatic effect on lignin content and composition as these TFs can regulate several genes at once. *MYB* TFs bind to AC elements in promoters, which are rich in adenosine and cytosine residues. Most monolignol biosynthetic gene promoters contain these AC elements, which allows for xylem-specific expression (Bell-Lelong *et al.*, 1997; Bevan *et al.*, 1989; Mizutani *et al.*, 1997). The Arabidopsis *F5H* promoter does not contain AC elements and is not regulated by SND1 (Öhman *et al.*, 2013), while *Medicago F5H* promoter contains AC elements and is regulated by SND1 (Raes *et al.*, 2003; Zhao *et al.*, 2010). Secondary cell wall biosynthesis is regulated by master switches early in the monolignol biosynthetic pathway (Figure 3). *SND1* and *SND2* are NAC domain proteins which act to initiate monolignol biosynthesis (Hussey *et al.*, 2011; Zhong *et al.*, 2006, 2007, 2010). *Eucalyptus EgMYB2, Pinus PtMYB1, PtMYB,* and *PtMYB8,* and *Poplar PtrMYB3,* and *PtrMYB20* act as transcriptional activators, while *Eucalyptus EgMYB1,* maize *ZmMYB31 ZmMYB42,* poplar *PttMYB21a,* and switchgrass *PvMYB4* act as transcriptional repressors (Zhao and Dixon, 2011). Besides these biofuel crops,

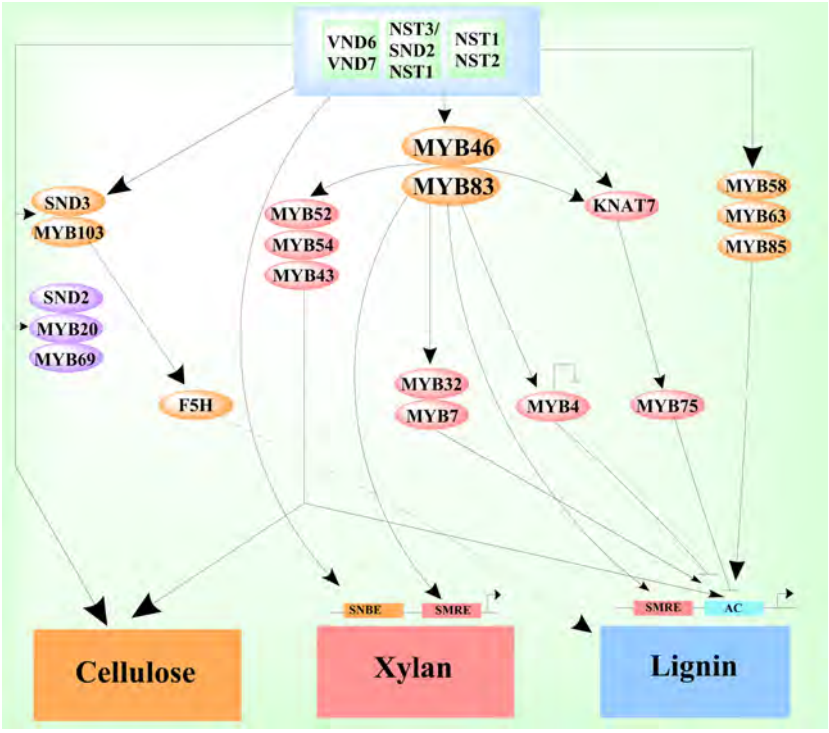

Figure 3 Lignin and secondary cell wall biosynthesis transcriptional regulatory network. *SND*, *VND*, *NST*, and its homologous act as master switches of secondary cell wall biosynthesis in different tissues. *MYB* transcription factors bind to AC elements in lignin biosynthesis gene promoters to regulate lignin biosynthesis and also bind to SMRE elements. The secondary cell wall master switches also bind to SNBE elements to regulate xylan synthesis. Cellulose synthesis is regulated by the *SND* and *MYB* homologous. Abbreviations: *SND*, secondary wall associated NAC domain protein; *NST*, *NAC* secondary wall thickening promoting factor; *VND*, vascular-related *NAC* domain; *F5H*, ferulate-5-hydroxylase; *SNBE*, secondary wall *NAC* binding element; and *SMRE*, secondary wall *MYB* responsive element. This figure is an expanded version of a published model (Zhong and Ye, 2012).

transcriptional activators and repressors have been identified in other species. For instance, in *Antirrhinum, AmMYB308* and *AmMYB330* repress the activity of *4CL*, *CAD,* and *C4H* genes, and Arabidopsis *AtMYB46*, *AtMYB83*, *AtMYB58/63*, and *AtMYB85*, *AtMYB61* act as activators (Zhao and Dixon, 2011). Overexpression of repressors can increase saccharification efficiency, but can have negative consequences on growth similar to that of monolignol gene down-regulation. For example, transgenic switchgrass plants with overexpression of PvMYB4 had reduced lignin content and up to a 250% increase in saccharification efficiency and biofuel production compared with the nontransgenic parent, but had a 40% reduction in plant height (Shen *et al.*, 2012, 2013).

Other known transcription factors involved in monolignol biosynthesis are basic helix-loop-helix (bHLH) and LIM (Lin11, Isl-1 and Mec-3 homeodomain proteins) transcription factors. Yan *et al.* (2013) analysed 13 different *Sorghum bicolor* bmr mutants and found a bHLH transcription factor, SbbHLH1, to be associated with the low-lignin brown midrib phenotype. The expression of SbbHLH1 in Arabidopsis resulted in significantly lower lignin content, a reduction in the expression of multiple lignin biosynthetic genes, and an up-regulation of several *MYB* transcription factors: *MYB46*, *MYB83*, *MYB*58, and *MYB*63. It was hypothesized that the repression of the monolignol biosynthetic genes in the presence of *SbbHLH1* could activate the expression of the *MYB* TFs. Alternatively, *SbbHLH1* may have combined with the *MYB* activator to repress lignin synthesis (Yan *et al.*, 2013). The LIM family of TF members have been known to regulate some genes in the monolignol pathway (Kawaoka *et al.*, 2000). LIM TFs have a consensus cysteine-rich protein sequence known as the LIM domain which binds to AC elements of monolignol biosynthetic gene promoters. Studies with tobacco by Kawaoka *et al.* (2000) have shown that *NtLIM1* acts as weak activator of lignin genes to maintain a basal level of gene expression in the monolignol biosynthetic pathway. Suppression of *NtLIM1* did not produce any abnormal growth phenotype, and even with a 27% decrease in lignin content, vessels did not collapse. One of the most desirable phenotypes for lignocellulosic biomass has been demonstrated in transgenic rice by the overexpression of Arabidopsis TF *SHINE* under the *CaMV 35S* promoter, which increased the cellulose content by 34%, decreased the total lignin content by 45%, and reduced the G-lignin content by 54%, without decreasing the biomass of the transformed plants (Ambavaram *et al.*, 2011). Although many studies have been conducted with constitutive promoters, TFs can be powerful tools to perturb the lignin biosynthetic pathway, but the TFs must be carefully regulated themselves, say, by the use of tissue-specific promoters, or they could lead to undesirable pleiotropic effects in transgenic plants.

Manipulation of regulatory factors that affect lignin biosynthesis

MicroRNAs (miRNAs) regulate various developmental programmes of plants. They have been found to regulate the expression of transcription factors that influence biomass yield, lignin content and composition, cell wall recalcitrance, and other characteristics important for biofuel production (Fu *et al.*, 2012; Rubinelli *et al.*, 2013). One such miRNA encoded by the maize

Corngrass1 (*Cg1*) gene that belongs to the *miR156* class, targets the *SQUAMOSA PROMOTER BINDING LIKE* (*SPL*) family of transcription factors, and supports the development of juvenile morphology with reduced lignification of cell walls (Rhoades *et al.*, 2002). The *Cg1* gene has been constitutively expressed in poplar and switchgrass, where it reduced the lignin content and also had significant effects on plant architecture, including increased branching and reduced stature, which correlated with increasing expression of *Cg1* (Chuck *et al.*, 2011; Rubinelli *et al.*, 2013). In switchgrass, *Cg1* overexpression produced plants that had 250% more starch than controls, resulting in a significantly higher glucose release (Chuck *et al.*, 2011). Similar to *Cg1* overexpression, the overexpression of the rice *OsmiR156b* precursor in switchgrass had dramatic effects on growth phenotype (Fu *et al.*, 2012). Three classes of transgenic plant phenotypes were obtained from this study: high transgene overexpression resulted in severely dwarf plants, medium expressers had delayed flowering, less recalcitrance and more biomass, and low expressers of *OsmiR156b* had increased tillering but were otherwise similar to the nontransgenic parent switchgrass. Recently, an miRNA that targets laccases, *miR397,* has been identified in poplar. Twenty-nine *PtrLACs* were identified to be the *PtrmiR397a*. Overexpression of *PtrmiR397a* reduced lignin content without affecting the expression level of monolignol biosynthetic genes, suggesting that PtrmiR397 is involved in lignin polymerization (Lu *et al.*, 2013).

To date, most bioenergy feedstock research targeting lignin has employed a single gene strategy, whereby genes were expressed or silenced using constitutive promoters. This research has been valuable to down select gene targets that might ultimately provide desired phenotypes, but no single experiment has yielded perfect phenotypes. It is clear that spatial and temporal regulation of multiple transgenes at 'sweet spot' expression levels will be needed to decrease or modify lignin in aboveground tissues for increased biofuel without negative pleiotropic effects. That said, constitutive promoter-driven transgenes have been heuristic for our increased understanding of the consequences of modifying lignin with regards to recalcitrance and plant performance.

Consequences of lignin modification

As shown above, there are numerous gene targets that can be manipulated to modify lignin biosynthesis and increase the saccharification efficiency, thereby producing plants that yield more biofuel per mass of feedstock. However, there can be negative effects associated with perturbing lignin in plants (Vermerris *et al.*, 2010). For example, the high overexpression of *PvMYB4* in transgenic switchgrass resulted in lower lignin and increased saccharification efficiency, but also led to shorter plant stature (Shen *et al.*, 2012). Similarly, plants with reduced activity of C4H, C3H, HCT, or CCR are often dwarfed (Hoffmann *et al.*, 2004; Reddy *et al.*, 2005). Cell wall collapse in vasculature, which can lead to water conductance problems, might be one cause of dwarfism in lignin-altered plants. When linked with a significant enhancement in saccharification efficiency, moderate growth penalties might be tolerable, but extreme dwarfism or increased pest susceptibility would not be useful in agricultural fields.

The effect of lignin down-regulation on plant stress, such as response to pathogens, is just beginning to be understood. It is known that plants accumulate lignin and other related phenolics as a defence mechanism against pathogens (Hammond-Kosack and Jones, 1996) and that its biosynthesis is strongly influenced by several biotic and abioic stresses (Moura *et al.*, 2010). Recently, the role of specific monolignol genes in plant defence responses has been studied and several are hypothesized to be involved in disease resistance (Bi *et al.*, 2011). For example, Tronchet *et al.* (2010) demonstrated that CAD-C and CAD-D, the primary CAD genes involved in Arabidopsis lignification, play an important role in the response of Arabidopsis to infection by the bacterial pathogen *Pseudomonas syringae* pv. *tomato*. While these studies suggest that lignin gene silencing could negatively impact disease resistance, a recent study demonstrated that this is not always the case. The down-regulation of the gene encoding HCT was associated with an increased tolerance to fungal infection and drought in alfalfa, which could possibly be due to crosstalk between lignin and salicylic acid biosynthesis (Gallego-Giraldo *et al.*, 2011).

Clearly, we need to gain a better understanding of lignin's role in whole plant physiology in field settings. The generation of transgenic plants with a wide variety of lignin-associated transgenes will allow for transgene stacking using plant hybridization to better understand synergistic transgene effects, as will crossing transgenic plants into a variety of genetic backgrounds. Novel approaches are being developed to overcome some of the undesirable downstream effects of lignin modification, as demonstrated by a recent study in which the yield penalty was avoided using a tissue-specific promoter designed to decrease lignin content in fibre cells only, thus preventing vessel collapse (Yang *et al.*, 2013). Tissue-specific promoters could also be used to regulate lignin production in tissues crucial for biofuel production, such as aboveground tissue in perennial grasses, while not perturbing lignin biosynthesis in roots. We also envisage the utilization of high-biomass germplasm which can be hybridized with low-lignin germplasm to decrease yield penalties associated with altering certain lignin biosynthesis genes. Certainly, biotechnology holds much promise to increase biofuel yield per hectare, but the fine-tuning of lignin biosynthesis will be needed to realize this promise.

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