Plant Biotechnology Journal

Plant Biotechnology Journal (2014) **12**, pp. 1154–1162 doi: 10.1111/pbi.12250

Review article

Tailoring lignin biosynthesis for efficient and sustainable biofuel production

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Received 7 June 2014;
revised 30 July 2014;
accepted 2 August 2014.
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Keywords: lignin, cell wall, biofuels.

Summary

Increased global interest in a bio-based economy has reinvigorated the research on the cell wall structure and composition in plants. In particular, the study of plant lignification has become a central focus, with respect to its intractability and negative impact on the utilization of the cell wall biomass for producing biofuels and bio-based chemicals. Striking progress has been achieved in the last few years both on our fundamental understanding of lignin biosynthesis, deposition and assembly, and on the interplay of lignin synthesis with the plant growth and development. With the knowledge gleaned from basic studies, researchers are now able to invent and develop elegant biotechnological strategies to sophisticatedly manipulate the quantity and structure of lignin and thus to create economically viable bioenergy feedstocks. These concerted efforts open an avenue for the commercial production of cost-competitive biofuel to meet our energy needs.

Introduction

The rapid depletion of fossil-fuel resources worldwide and the tangible climate change we are experiencing have triggered perceivable global interest in a bio-based economy that will secure our energy future and sustain livable environments. In the transition from a fossil-based energy economy to a bio-based one, biomass seems poised to provide a major alternative to fossil fuels. With both agronomic and societal concerns on the increased use of grains and oilseeds for biofuel production, the lignocellulosic biomass of dedicated crops, such as poplar, switchgrass and Miscanthus, a genus of perennial grasses, or crop residues will be the primary source for producing liquid fuels. The species for cellulosic biomass production can grow on marginal lands using little fertilizer over multiple annual cycles; therefore, the energy output and carbon savings are expected to be much higher than those of first-generation biofuel crops (Somerville *et al.*, 2010). In 2007, the US Energy Independence and Security Act (P.L. 110-140, H.R. 6) set an aggressive standard for renewable fuels, mandating the use of 9 billion gallons of renewable fuels in 2008 and stepping up to 36 billion gallons by 2022. Under this renewable fuel standard, the production of corn-based ethanol (conventional biofuel) essentially is capped at 15 billion gallons by 2015, while 21 of the 36 billion gallons in 2022 must be derived from advanced biofuels, such as cellulosic and noncorn-based ethanol. However, since the inception of cellulosic programme in 2010, no system for generating cellulosic biofuel has been scaled-up to commercial production. The US Environmental Protection Agency (EPA) had to waive the mandate for cellulosic fuel every year. The major obstacle to meeting the goal of commercial production is the higher cost of producing cellulosic ethanol than that of the conventional corn ethanol. This drawback primarily is due to the recalcitrance to hydrolysis of the cellulosic biomass. Plant secondary cell walls, the primary source of cellulosic feedstock, comprise a cellulose/hemicellulose network impregnated with lignin (Carpita and McCann, 2000). In the wood of *Populus* spp., these polymers occur in approximate proportions of 45% cellulose, 25% hemicelluloses and 20% lignin (Geisler-Lee *et al.*, 2006). Lignin in the cell wall forms a rigid physical barrier effectively preventing the digestive enzymes' access to the polysaccharides; furthermore, the hydrophobicity of this aromatic polymer effectively adsorbs and inactivates exogenous hydrolytic enzymes (Sammond *et al.*, 2014; Weng *et al.*, 2008); therefore, the presence of lignin is the main factor contributing to the recalcitrance of cell wall biomass to hydrolysis and impeding the enzymatic release of simple sugars for subsequent fermentation. Structurally, lignin is a complex, irregular biopolymer composed of hydroxylated and methylated phenylpropane units. It is derived mainly from the oxidative coupling of three classical monolignols, that is *p*-coumaryl, coniferyl and sinapyl alcohols. These three monolignols, incorporated into lignin polymer, produce *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits. The G unit is methylated singly on the 3-hydroxyl group, whereas the S unit is methylated on both the 3- and 5-hydroxyl moieties. The ratio of S-to-G subunits dictates the degree of lignin condensation by allowing different types of polymeric linkages. A higher G content creates a more highly condensed lignin composed of a greater portion of biphenyl and other carbon–carbon linkages, whereas S subunits commonly are linked through more labile ether bonds (Ralph *et al.*, 2004; Vanholme *et al.*, 2010a). To efficiently degrade the cell wall biomass for agro-industrial applications, the lignin content should be lowered, or the amount of the chemically labile lignin increased (Boerjan *et al.*, 2003; Boudet *et al.*, 2003). Over the decades, a plethora of biotechnological efforts have been made towards manipulating lignin biosynthesis to improve

the degradability of cell wall biomass (Boudet *et al.*, 2003; Vanholme *et al.*, 2010a). In particular, the increased interest in renewable bioenergy has become a major impetus for studies of the process of cell wall lignification. Many breakthroughs have been achieved in recent years towards understanding lignin biosynthesis, deposition, assembly and its metabolic interplay with other biological processes. Even more successfully, several novel biotechnological strategies, beyond the conventional genetic approach, have been developed to more effectively manipulate lignin quantity or structure geared towards increasing the bioconversion efficiency of cellulosic biomass for producing liquid biofuels or bioproducts. This review summarizes some recent advances focusing on these research aspects.

Advances in fundamental understanding of lignin biosynthesis

New enzyme for monolignol biosynthesis

As a concerted cellular process, lignin biosynthesis encompasses three biochemical events: the synthesis of monolignols within the cytosol, the transport of monomeric precursors across plasma membrane and the oxidative polymerization of monolignols to form macromolecules within the cell wall (Liu, 2012). Over the decades, the monolignol biosynthesis pathway has been extensively studied. The pathway leading to three conventional hydroxycinnamyl alcohols (monolignols) has been simplified from a complex metabolic grid to a linear pathway (Humphreys and Chapple, 2002), in which hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT) is defined as the first committed enzyme branching the general phenylpropanoid carbon flux to the synthesis of the guaiacyl and syringyl lignin precursors (Figure 1). HCT is demonstrated to reversibly shuttle hydroxycinnamoyl units between its CoA- and shikimate/quinate-esterified forms (Hoffmann *et al.*, 2003; Niggeweg *et al.*, 2004). It accepts *p*-coumaroyl-CoA (and caffeoyl-CoA) as the acyl group donor and catalyses the efficient acyl group transfer to shikimate or quinate so to form *p*-coumaroyl shikimate or quinate ester, which is the substrate of *p*-coumarate 3′-hydroxylase (C3′H) (Figure 1). In the presence of CoA cofactor, HCT also catalyses the reverse reaction *in vitro*, forming caffeoyl-CoA from the caffeoyl quinate/shikimate ester. Therefore, it is proposed to control two catalytic reactions on monolignol biosynthesis pathway: the formation of *p*-coumaroyl shikimate ester from *p*-coumaroyl CoA and then the conversion of the C3′H-produced caffeoyl shikimate ester to caffeoyl-CoA (Hoffmann *et al.*, 2003). Genetically manipulating HCT expression severely affects lignin biosynthesis (Hoffmann *et al.*, 2004; Wagner *et al.*, 2007), manifesting the importance of HCT in the formation of monolignols. However, this proposed linear and sequential transesterification route has been amended recently with characterization of a caffeoyl shikimate esterase (CSE; Vanholme *et al.*, 2014). By intensive gene co-expression analysis, the Arabidopsis gene,

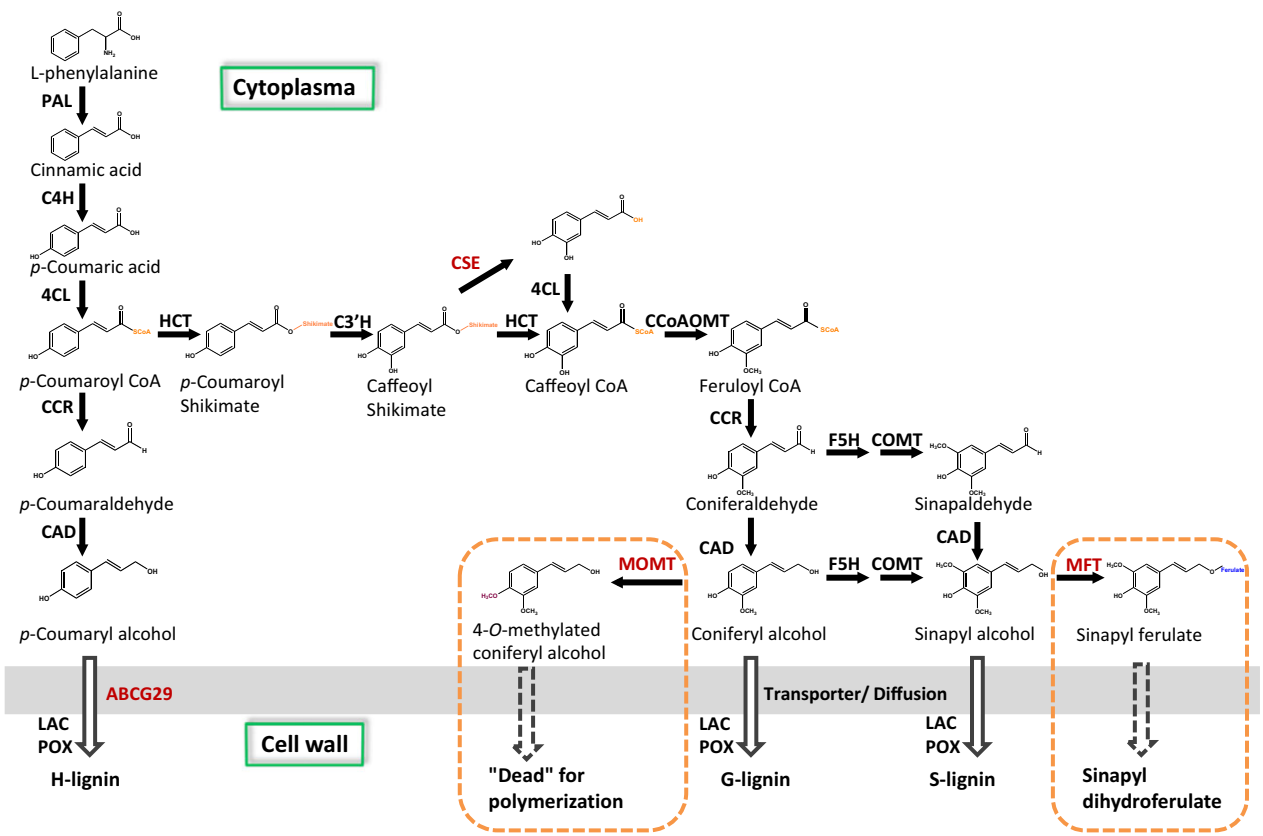

Figure 1 The phenylpropanoid-lignin biosynthesis pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase; HCT, hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase; C3′H, *p*-coumaroyl shikimate 3′-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, coniferaldehyde/ferulate 5-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; CAD, (hydroxy)cinnamyl alcohol dehydrogenase; LAC, laccase; POX, peroxidase. The boxes with dashed line indicate the engineered steps; MOMT, monolignol 4-*O*-methyltransferase; MFT, monolignol ferulate transferase.

At1G52760, coding for a putative lysophospholipase, is found to be expressed predominantly in lignifying vascular tissues and highly co-expressed with a set of lignin biosynthetic genes. Compared with the wild-type control, the deficiency of this gene in the T-DNA insertion mutant line results in the reduction of 17%–36% of acetyl bromide lignin content in the cell wall and causes more than 100-fold increase in the accumulation of caffeoyl shikimate, the product of HCT-catalysed transesterification and C3′H-catalysed hydroxylation reactions (Figure 1). The recombinant enzyme of the putative lysophospholipase displays a preferential esterase activity converting caffeoyl shikimate to the caffeate *in vitro* (Vanholme *et al.*, 2014). These data suggest that the identified lysophospholipase functions as CSE and is an additional key enzyme central to the lignin biosynthetic pathway. Together with *p*-coumaroyl/caffeoyl-CoA ligase (4CL), the activities of CSE and 4-CL enable the bypassing of the reversion reaction catalysed by HCT, leading to the formation of caffeoyl-CoA (Figure 1). *CSE* orthologs can be found in a range of plants, such as poplar, eucalyptus and switchgrass, suggesting its conserved role in lignin biosynthesis. This gene could potentially be a new target for modulating the recalcitrance of lignocellulosic biomass to hydrolysis. It remains of interest to determine why land plants co-evolve different mechanisms leading to the synthesis of central precursor caffeoyl-CoA for G and S lignin synthesis, and whether the CSE-4CL- and HCT-mediated pathways have distinct tissue or cell-type specific biological roles in plant lignification.

Monolignol deposition

After their synthesis in the cytosol, monolignols must be exported across the plasma membrane and deposited into the cell wall. The mechanism underlying their translocation is still debatable. Some transcriptomics, proteomics and modern autoradiographic studies suggest the likelihood of the involvement of ATP-binding cassette (ABC) transporters in plant lignification (Ehlting *et al.*, 2005; Kaneda *et al.*, 2008; Nilsson *et al.*, 2010; Smith *et al.*, 2013). An *in vitro* biochemical analysis using membrane preparations from Arabidopsis leaves and poplar roots reveals the involvement of an active transportation mechanism for exporting lignin monomeric precursors across the plasma membrane (Miao and Liu, 2010). While Arabidopsis leaves contain primarily mesophyll cells that do not lignify the cell wall, and therefore, the detected monolignol transport activity may not be simply implied to the vascular lignin deposition, an Arabidopsis full-size ABCG subfamily member, ABCG29/PDR1, has been recently characterized as a particular monolignol transporter responsible for lignin biosynthesis (Alejandro *et al.*, 2012; Sibout and Hofte, 2012). AtABCG29 is found from analysis of the gene co-expression network, in which *ABCG29*/*PDR1* shows a relatively high co-expression ratio to three phenylpropanoid-lignin pathway genes, that is *4CL2*, *5* and *CCoAOMT* (Alejandro *et al.*, 2012). In concordance with these results, Ehlting *et al.* (2005) have also reported that *AtABCG29* shows an expression pattern consistent with those of monolignol biosynthetic genes and the increased lignin content in developing stem (Ehlting *et al.*, 2005). *AtABCG29* is found to express primarily in the endodermis and vascular tissue of roots. The mutant plants deficient in *AtABCG29* are hypersensitive to treatment with *p*-coumaryl alcohol, but not with coniferyl and sinapyl alcohols; correspondingly, microsomal preparations from yeasts harbouring ABCG29 exhibit specific uptake activity to *p*-coumaryl alcohol but not to coniferyl alcohol, implying that ABCG29 may be specific for transporting *p*-coumaryl alcohol, and participate in depositing H lignin (Alejandro *et al.*, 2012). Interestingly, the cell walls of root tissues from *atabcg29* deficient lines show altered lignin composition, with a reduction in release of all of the H, G, and S lignin units. The authors deduce that altered export of *p*-coumaryl alcohol via a deficiency of *ABCG29* may have affected the synthesis and/or the deposition of G and S lignin units, thus resulting in an overall effect on lignin synthesis (Alejandro *et al.*, 2012). The characterization of ABCG29 seemingly substantiates the concept of the involvement of ABC transporters in plant lignification and suggests that ABC-type transporters may have functional specification for particular monolignol deposition. Nevertheless, the prevalent recognition of the high degree of plasticity in lignification, and the facile deposition and incorporation of many nontraditional lignin monomers or pathway intermediates into the cell wall lignin apparently challenge the notion of a transporter-mediated specific-deposition of lignin monomeric precursors (Kim *et al.*, 2000; Ralph *et al.*, 1997; Vanholme *et al.*, 2010b; Wilkerson *et al.*, 2014; Zhao *et al.*, 2013). In dicotyledonous species, lignified vascular tissues in the normal condition consist predominantly of G and S units. The H lignin subunit accounts only a very small portion (~2%) of the lignin polymer (Boerjan *et al.*, 2003). We need to explore whether there are any additional transporter proteins participating in the lignification of vascular tissues, and responsible for the deposition of the G and S lignin monomers. Currently, underpinning the roles of ABC transporters in the lignification of xylem cells is difficult. Different cell types of developing xylem tissues demonstrably adopt distinct mechanisms of lignification; for example, it is shown that autonomous lignification process occurs in extraxylary fibre cells, whereas the neighbouring cell-contributed lignin deposition takes place in xylary cells (Smith *et al.*, 2013); moreover, lignification likely can occur prior to and after programmed cell death (postmortem) (Smith *et al.*, 2013; Terashima *et al.*, 1993). It is not clear whether the mechanism of lignin deposition and the required molecular machineries are same in different cell types and/or at different periods of the cellular programming process. Comprehensively understanding the biochemical and molecular events for transporting lignin precursors potentially could yield a novel biotechnological solution for better manipulating lignification of the cell wall.

Enzymes and proteins for oxidative dehydrogenation and lignin deposition

After deposition into the cell wall, monolignols undergo oxidative dehydrogenation, yielding chemically activated, resonance-stabilized radicals. The resulting phenoxy radicals couple with each other or to the growing lignin polymer, if the polymer is also oxidized, forming different types of cross-linkages (Ralph *et al.*, 2004). Oxidative dehydrogenation of monolignols is catalysed by peroxidases, laccases or other phenol oxidases (Boerjan *et al.*, 2003; Ralph *et al.*, 2004). Those enzymes widely occur in lignin-forming tissues and can oxidize a variety of phenolics, including monolignols *in vitro* (Davin *et al.*, 2008; Freudenberg, 1959; Ralph *et al.*, 2004).

Peroxidase uses hydrogen peroxide as its electron receptor to oxidize a variety of phenolics (Harkin and Obst, 1973). Different peroxidases have distinct substrate preferences in oxidizing the conventional monolignols. Most peroxidases readily oxidize coniferyl alcohol, whereas sinapyl alcohol itself is a poor substrate for oxidation; and it is catalysed only by specific peroxidases. Alternatively, the resulting radicals from coniferyl alcohol seemingly can transfer to sinapyl alcohol and thus oxidize it (Sasaki *et al.*, 2004, 2006, 2008).

Peroxidases in plant are encoded by a multigene family. These secretory proteins show high degree of sequence similarity and potential function redundancy; therefore, definitive identification of lignin-specific peroxidases is difficult. Down-regulation of the gene coding for a tobacco cationic peroxidase, NtPrx60 (TP60), results in up to a 50% reduction in total lignin content (Blee *et al.*, 2003), and the down-regulation of an anionic peroxidase PkPrx03 (PrxA3a) in aspen causes a decline of up to 20% of total lignin in the wood tissue of transgenic lines (Li *et al.*, 2003). In Arabidopsis, 73 class-III peroxidase genes have been identified (Tognolli *et al.*, 2002); and a couple of peroxidase homologues are implicated in xylem lignin deposition (Herrero *et al.*, 2013; Ostergaard *et al.*, 2000). However, the involvement of peroxidases in lignin polymerization in vascular tissues still requires a more definitive assessment.

Nevertheless, an endodermis-specific type III peroxidase, PER64, has been found critical for lignin deposition and the formation of the Casparian strip (Lee *et al.*, 2013). The Casparian strip is a belt-like cell wall thickening of endodermic cells. It generates a *para*-cellular diffusion barrier controlling the selective uptake of nutrients and stress resistance. The strip is composed of archetypal lignin, but not suberin (Naseer *et al.*, 2012). Recently, a small group of plasma membrane-localized proteins in endodermis cells, denoted the Casparian strip membrane-domain proteins (CASPs), are found able to form polymeric lattices on the defined plasma membrane domains, which is critical for Casparian strip formation (Roppolo *et al.*, 2011). PER64 most likely physically associates with or is proximal to CASP1, by which it subcellularly localizes in the Casparian strip domain of the endodermis, therefore conferring spatial specificity of lignin polymerization (Lee *et al.*, 2013). In conjunction with the CASP1-mediated PER64 localization, an NADPH oxidase, viz, respiratory burst oxidase homolog F (RBOHF), is also found to spatially distribute in the Casparian strip domain, likely through its interaction with the CASP scaffold protein (Lee *et al.*, 2013). This subcellular precision engenders a locally restricted production of reactive oxygen species (ROS) at the Casparian strip that could further convert to H_2O_2 as the substrate for a PER64-mediated oxidative reaction to generate phenoxy radicals. These findings suggest that CASP might serve as a central physical scaffold on the plasma membrane, organizing and positioning lignin polymerization machinery, by which it forms an efficient metabolic channelling of the produced ROS/H_2O_2 towards peroxidase and, more importantly, guides spatial localization of lignin deposition for the formation of Casparian strips (Lee *et al.*, 2013). Intriguingly, a dirigent domain-containing protein, *Enhanced suberin 1* (*ESB1*), is also revealed as one of the structural components of the CASP-nucleated biochemical machinery to control the correct patterning of lignin deposition in the Casparian strip of the endodermis (Hosmani *et al.*, 2013). Dirigent protein has been characterized to act as a nonenzymatic template, guiding bond formation between monolignols and, therefore, defining the stereochemistry of the resulting dimeric lignans (Davin *et al.*, 1997). It is also argued to guide the synthesis of lignin by providing a template for monomer cross-coupling (Davin *et al.*, 2008). *ESB1* is uniquely expressed in the endodermis and localizes to Casparian strip, where CASP1 is required for ESB1's localization; conversely, disruption of *ESB1* also affects CASP1's localization. The *ESB1* mutant line phenocopies the deficiency of *CASP* and changes the pattern of the formation of Casparian strip; meanwhile, deficiency of *ESB1* enhances the deposition of lignin in a nonspatially controlled manner, suggesting that ESB1 is critical for the patterning of lignin deposition, but not for the synthesis of lignin polymer (Hosmani *et al.*, 2013).

It remains unclear whether a similar biochemical machinery for lignin deposition and assembly is adopted in the lignification of xylem tissues of the vasculature. CASP represents a membrane protein family of 38 members in Arabidopsis (Roppolo *et al.*, 2011). It will be interesting to see whether the assembly of lignin polymerization complex by CASPs or CASP-like proteins observed in the formation of Casparian strip is a general feature that applies to other types of lignifying cells.

Similar to peroxidase, laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) also oxidizes monolignols *in vitro* (Bao *et al.*, 1993; Freudenberg, 1959; Richardson *et al.*, 1997; Sterjiades *et al.*, 1992; Takahama, 1995), and laccase-like activities have been detected in the lignifying cell walls of differentiating xylem (Bao *et al.*, 1993; Driouich *et al.*, 1992; Liu *et al.*, 1994; Richardson *et al.*, 2000). Laccases are copper-containing extracellular glycoproteins that require O_2 as secondary substrate to oxidize various phenolic, inorganic, and/or aromatic amine substrates (Reinhammar and Malmstroem, 1981). Arabidopsis contains 17 putative laccase homologous genes, and their expressions concur across a wide variety of tissues/development stages (Turlapati *et al.*, 2011). Simultaneously disrupting Arabidopsis *LAC4* and *LAC17* results in about 30%–40% reduction in lignin content in the stem of mutant lines grown under continuous illumination (Berthet *et al.*, 2011), which provides the first definitive genetic evidence of laccases functioning in lignin polymerization. Recently, Zhao *et al.* (2014) have discovered that knockingout an additional member *LAC11* gene in *lac4/lac17* mutant nearly completely diminishes lignin deposition, and consequently, the *lac4/lac11/lac17* triple mutant exhibits severe growth and development defects, including weak roots, nonadhesive anthers and defective vascular development. Most strikingly, the expressions of peroxidases that are implicated in lignifying xylem tissues and Casparian strip appear at a normal level or even higher in the triple mutant line; and the endodermic Casparian strip is still functional in controlling ion permeability (Zhao *et al.*, 2014). These findings imply that either peroxidases and laccases have strictly separated, nonredundant tissue- or cell-type specific functions in lignin polymerization or they might function at different times of xylem lignification with laccases probably dominating the early events of lignification and the peroxidases functioning in the latter stages. In the latter case, the laccase-mediated initiation of lignin polymerization should be critically important to prime the subsequent lignification process involving peroxidases. The nonredundant function of laccase and peroxidase in lignification has been implied in an early biochemical study, in which Sterjiades *et al.* (1993) find that the sycamore maple's laccase and peroxidase have differential substrate specificity with the laccases being less active on phenolic substrates containing multiple aromatic rings (Sterjiades *et al.*, 1993); therefore, it is assumed that laccases may polymerize monolignols into oligo-lignols during the early stages of lignification, whereas the cell wall peroxidases may function when H_2O_2 is generated during the later stages of the development of xylem cells or in response to environmental stresses (Sterjiades *et al.*, 1993). More detailed studies are required to delineate the nonredundant functions of peroxidases and laccases in lignin polymerization. It is intriguing to explore whether and how oxidative enzymes initiate lignifi-

cation in a discrete region of the cell wall of differentiating xylem tissues.

Engineering lignin synthesis towards the industrial exploitation of biofuels

Develop energy crops with low lignin but significantly improved ethanol yield

Due to the negative effect of presence of lignin in cell wall on the efficient utilization of cellulosic fibres, there have been numerous efforts towards engineering lignin biosynthesis (Coleman *et al.*, 2008a; Huntley *et al.*, 2003; Stewart *et al.*, 2009; Wagner *et al.*, 2007, 2009). Those efforts were often undertaken in line with genetic studies on gene functions in lignin biosynthesis pathway. It is clear that the quantity of lignin is the major factor contributing to the recalcitrance of cell wall biomass, and it is negatively correlated with the release of fermentable sugars. This reciprocal correlation is firmly upheld via analyses in a set of lignin down-regulation alfalfa transgenic lines (Chen and Dixon, 2007) and a set of 20 knockingout Arabidopsis mutant alleles representing 10 genes of the phenylpropanoid-lignin biosynthesis pathway (Van Acker *et al.*, 2013b). Whereas, although the S/G ratio often is regarded as a parameter indicating lignin condensation and reactivity (Davison *et al.*, 2006), the effect of S/G ratio on cell wall digestibility is not obvious, or is somewhat inconsistent. The saccharification efficiency of cell wall biomass from a set of lignin down-regulation alfalfa plants shows no obvious correlation with the changes of S and G lignins, irrespective of whether the samples being used have been pretreated or not (Chen and Dixon, 2007), whereas the digestibility study on Arabidopsis cell wall biomass reveals that the S/G ratio of the lignin has a negative influence on the saccharification yield when the samples are not pretreated (Van Acker *et al.*, 2013b).

To project the laboratory-based genetic study to the industrial exploitation of biofuel, two promising studies have been carried out using dedicated bioenergy crops (Fu *et al.*, 2011; Van Acker *et al.*, 2013a). Caffeic acid *O*-methyltransferase (COMT) catalyses predominantly the *O*-methylation at the C5 position of the phenolic ring in monolignol biosynthetic pathway (Figure 1). It is responsible for the synthesis of sinapyl alcohol, the precursor of syringyl lignin units. Arabidopsis *comt1* mutant yields lignins lacking S units and containing 5-hydroxyguaiacyl units derived from 5-hydroxyconiferyl alcohol (Goujon *et al.*, 2003b; Vanholme *et al.*, 2010b). To evaluate the effect of reducing lignin content on ethanol production, Fu *et al.* (2011) have down-regulated the *COMT* gene in perennial prairie grass, switchgrass, which results in a moderate level (~13%) of lignin reduction and a decrease of the S/G ratio compared with the control plants. When assessing the bioconversion efficiency of the generated transgenic biomass with the simultaneous saccharification and fermentation (SSF) procedure, the ethanol yield is surprisingly increased by up to 38% of the level of controls. Moreover, the study reveals that the COMT down-regulation lines require far less severe pretreatment and lower amount of digestive enzymes than do the control lines for the equivalent ethanol yield during the SSF test (Fu *et al.*, 2011). Such improvement in biomass conversion and ethanol yield is expected to render a ~33% improved efficiency in land use, or to produce significantly more fermentation product per hectare, which could substantially lower the cost of producing bioethanol.

Another promising study is the field trials of transgenic poplar with down-regulated cinnamoyl-CoA reductase (CCR; Van Acker *et al.*, 2013a). CCR catalyses the conversion of feruloyl-CoA to coniferaldehyde. It is considered the first enzyme in the monolignol-specific branch of the phenylpropanoid pathway (Figure 1). Down-regulation of the *CCR* gene in annual model plants significantly reduces lignin content (Goujon *et al.*, 2003a; Jones *et al.*, 2001; Piquemal *et al.*, 1998). Similarly, down-regulation of *CCR* in woody plant poplar (*Populus tremula* × *Populus alba*) also results in up to 50% lignin reduction in greenhouse-grown plants; and the lignin reduction is associated with a patchy, red-brown coloration of the outer xylem of the transgenic plants (Leple *et al.*, 2007). To translate the knowledge gained in the laboratory (greenhouse) to conditions closer to industrial exploitation, field trial becomes a necessary step. When transgenic poplars are grown in the field, the lignin reduction in CCR down-regulation plants is confirmed up to 19% in the scraped red xylem compared with that of the wild type (Van Acker *et al.*, 2013a). With or without acid and/or alkaline pretreatments, wood biomass from field-grown trees shows a considerable increase in the sugar yield from saccharification, calculated on per gram dry wood biomass. Evaluation by SSF shows that the increased efficiency of saccharification can translate into a higher yield of ethanol of up to 160%, compared with the wild-type biomass (Van Acker *et al.*, 2013a). However, down-regulation of *CCR* has severely affected plant growth (Thevenin *et al.*, 2011; Van Acker *et al.*, 2013a). The yield of wood biomass of CCR transgenic poplar is decreased considerably. Consequently, the penalty in biomass yield largely outweighs the improvement in ethanol yield per gram biomass for most field-grown CCR transgenic trees.

Overcoming the growth penalty

Although a study on Arabidopsis mutant lines has revealed that reduction in lignin content down to the 64% of the wild-type level is tolerated without an obvious growth penalty (Van Acker *et al.*, 2013b), the morphological defects in fact are common to many genetically modified plants with decreased lignin (Bonawitz and Chapple, 2013). The morphological abnormalities often appear as dwarf stature in plants, collapsed xylem, reduced fertility and increased susceptibility to fungal and other pathogens. As lignin is a structural component, providing vascular integrity and mechanical strength, defects of plant growth and development with lignin reduction are often linked to the loss of vessel integrity and, thus, the malfunctioning of the vascular conduit system for transporting water and nutrients (Coleman *et al.*, 2008b; Kim *et al.*, 2014). However, a recent study of Arabidopsis and alfalfa HCT down-regulation lines, both of which show severely stunted growth, has proposed a different mechanism; the hyperaccumulation of salicylic acid (SA) in such lignin defective plants is suggested to be the reason for the growth defect (Gallego-Giraldo *et al.*, 2011). The study reveals that *HCT* gene-silencing lines, as well as *ccr1* mutant lines, accumulate a higher level of SA than do the controls, which is inversely correlated with the height of the stem and the extractable amount of pectin in cell walls. Blocking SA synthesis or converting SA to catechol by a SA hydroxylase, NahG (Gaffney *et al.*, 1993), restores the growth of HCT down-regulation plants. Based on these data, authors hypothesize that a reduction in lignin affects the programming of the secondary cell wall, which could result in the production of more extractable pectin. Some pectic oligosaccharides then might serve as signal molecules triggering SA synthesis and/or SA-mediated signalling and, consequently, impairing plant growth (Gallego-Giraldo *et al.*, 2011). However, this hypothesis is refuted by the studies on Arabidopsis C3′H

mutant line (*ref8*) (Bonawitz *et al.*, 2014; Kim *et al.*, 2014). *ref8* line contains C3′H gene with a mis-sense mutation and, therefore, has a reduced content of G and S lignin in cell wall and a dwarf phenotype even severer than that of HCT down-regulation lines (Franke *et al.*, 2002a,b). Disruption of the genes encoding subunits of the transcriptional mediator complex, MED5a and MED5b, in *ref8* mutant mostly restores the stunted growth and leads to an overaccumulation of H lignin subunit with level higher than that detected in *ref8* mutant line; while the G and S lignin units remains similar to that of *ref8* line (Bonawitz *et al.*, 2014). Interestingly, the growth-rescued *med5a/5b/ref8* triple mutant line still contains a higher level of SA compared with that of *ref8* plants, and disruption of SA biosynthesis in *ref8* does not alleviate plant dwarfism (Bonawitz *et al.*, 2014). These data are in contrast to those observed in HCT down-regulation and *ccr1* mutant lines. Apparently, the molecular mechanisms underlying growth defects of the lignin biosynthetic mutant are far from well understood at present.

Novel strategies in controlling lignin quantity and/or structure

Concerning the deleterious effects of a lignin defect on the integrity and conduit function of vessel cells in vasculature, a study has exploited the possibility of tissue- or cell-type specific rescuing lignification (Yang *et al.*, 2012). The VASCULAR NAC-DOMAIN6 (VND) transcription factor shows a vessel-specific expression (Kubo *et al.*, 2005). The promoter of *VND6* is used to drive the expression of a *C4H* gene in *c4h* mutant background. Plants harbouring *ProVND6:C4H* nearly completely restore the growth defect caused by a deficiency of C4H, but lignin deposition in the interfascicular fibres appears lower than the wild type (Yang *et al.*, 2012). This operation, together with tailoring activity of endogenous transcriptional regulation network towards up-regulation of cellulose synthesis, increases the efficiency of saccharification of the cell wall biomass of transgenic plants (Yang *et al.*, 2012). Using similar methodology, another recent study has employed a promoter from *CESA7*, a cellulose synthase gene with high specific expression in the xylem cells with secondary cell walls, to drive microRNA targeting on *CCR1*, which results in a defect of lignification specifically in interfascicular fibre cells, but not in xylary cells (Smith *et al.*, 2013). This observation intriguingly suggests that xylary and extraxylary cells adopt two distinct mechanisms of lignin deposition, that is, autonomous in extraxylary cells and neighbouring cells contribution in xylary cells (Smith *et al.*, 2013). The transgenic plants have overall reduced lignin levels yet no collapsed xylary cells; the plants are fertile and grow to the heights typical of wild-type ones. Therefore, this study opens opportunity for independently manipulating two lignin pools, xylary and extraxylary, to create plants with intact xylem but overall lower lignin levels. This would expect to overcome the growth penalty that accompanies lignin engineering.

Plant lignification displays inherent plasticity allowing the incorporation of a range of nonconventional monolignols or pathway intermediates into the polymer (Sederoff *et al.*, 1999; Vanholme *et al.*, 2010b). The plasticity of lignification opens a potential avenue to change lignin composition, and thus structure, thereby creating more cleavable lignin for improving cell wall digestibility (Ralph, 2007; Vanholme *et al.*, 2008). *In vitro* studies have demonstrated that co-polymerization of hydroxycinnamate conjugates such as coniferyl ferulate, rosmarinic acid and epigallocatechin gallate with normal monolignols improves the alkali extractability of lignin and the subsequent enzymatic hydrolysis of fibres (Elumalai *et al.*, 2012; Grabber *et al.*, 2008, 2012). This is because incorporating such conjugates results in readily cleavable ester linkages in the backbone of the polymer, which permits lignin depolymerization under mild alkaline conditions (Grabber *et al.*, 2008). Producing monolignol ferulate conjugate requires BAHD acyltransferase that transfers the ferulate moiety from its CoA ester donor to the receptor monolignols (Liu, 2010; Ralph, 2010). Monolignol ferulate transferase has been recently identified using a deep sequencing and sequence-mining strategy from Chinese angelica (*Angelica sinensis*) that contains ~2% coniferyl ferulate in root tissues (Wilkerson *et al.*, 2014). The identified BAHD acyltransferase shows a kinetic preference for conjugating monolignols using feruloyl-CoA. Expressing this BAHD acyltransferase gene in hybrid poplar, driven by a CESA xylem specific promoter, results in the incorporation of approximately 7%–23% coniferyl ferulate conjugate into the backbone of the polymers. While the total lignin level of transgenic poplar remains similar to that of wild type, incorporation of the ester linkage has substantially improved the efficiency of saccharification of the mild alkaline-pretreated wood biomass (Wilkerson *et al.*, 2014). This study offers an example on how to redesign lignin property, by tailoring the inherent metabolic plasticity of lignification, thus to reduce energy and/or chemical inputs for deconstructing cell walls.

While many efforts are focused on perturbing single or multiple catalytic steps in the monolignol pathway to change lignin content or composition, a strategy is developed to prevent monolignols from the processes of dehydrogenation and oxidative coupling, thus more directly and effectively modulating lignin polymerization, whilst preventing perturbing the pathway of endogenous lignin biosynthesis and thus avoiding unexpected detrimental effect on plant growth (Zhang *et al.*, 2012). Lignin polymerization occurs via one-electron oxidation of phenols by oxidative enzymes (peroxidases/laccases) to yield resonance-stabilized phenolic radicals. In this process, an unsubstituted phenol, the free *para*-hydroxyl of the monolignol, is critically important in generating phenoxy radical intermediates and in forming different types of lignin subunits (Harkin, 1967; Ralph *et al.*, 2004). Therefore, a chemical modification, for example, methylation of the phenol (the *para*-hydroxyl), would prevent radical generation and deny the derived monolignols from participating in the subsequent coupling process. This action will either lower the quantity of lignin being produced or change its structure if particular monolignol is modified and blocked from further incorporation into lignin. To obtain the enzyme that can modify the 4-hydroxyl of monolignol, which does not commonly exist in plant kingdom, we alter the substrate specificity of a phenylpropene 4-*O*-methyltransferase (IEMT) by iterative saturation mutagenesis (Bhuiya and Liu, 2010). The resulting variants accommodate monolignol as the substrate, meanwhile retaining the 4-*O*-methylation property. Expression of the resulting enzyme variants in Arabidopsis results in a 24% reduction in total lignin content in the cell wall of transgenic lines and concomitantly yields novel 4-*O*-methylated phenolic esters in both the soluble- and wall-bound fractions. When stem biomass is assessed for its digestibility, the efficiency of the release of simple sugars increases by up to 22% compared with wild-type controls; more attractively, the reduction of lignin in cell wall does not generate discernible growth abnormalities (Zhang *et al.*, 2012). This study in experimental model plant offers a promising strategy to engineer lignin in energy crops to reduce cost of producing cellulosic biofuels.

Conclusion and perspectives

Study of lignin biosynthesis has been one of the central research themes on plant cell wall in recent years because of its importance to the plant structure and function and also because of the major influence on agro-industrial processing for a range of bio-based products, including wood pulp and biofuels from plant tissues. A set of new genes for lignin biosynthesis including those coding for CSE, ABC transporter, laccases and peroxidases have been discovered. In particular, the identification of CSE leads to the further revision of the monolignol biosynthesis pathway. The enigma of lignin deposition and assembly in apoplasts is being revealed; there are intriguing discoveries on the deposition and precision of the Casparian strip lignin along with discovery of a family of interesting scaffold proteins, the CASPs. The structural diversity of lignin exemplified by the discovery of catechol lignin in seed coats (Chen *et al.*, 2012) and the plasticity of lignin polymerization open a door to design the synthesis of desirable lignin. In addition, the metabolic regulation of the synthesis has been unveiled beyond the transcriptional level; microRNA-mediated regulation of key gene expression (Lu *et al.*, 2013), and post-translational modifications of the key enzymes have been found to control pathway activity (Zhang *et al.*, 2013). Along with fundamental studies, many elegant biotechnological strategies have been devised and developed for sophisticated manipulation of lignin synthesis. In the future, our knowledge on plant lignification will continually be refined. Many currently puzzling yet intriguing questions centring on plant lignification are waiting to be defined, for instance,

I. What is the dominant molecular mechanism underlying the lignin precursors' apoplastic deposition during the xylogenesis of vascular tissues?
II. Are the inherent processes of lignin deposition and assembly distinct among different cell types of vascular tissue and require particular biochemical machinery?
III. How is lignin polymerization initiated in the cell wall compartment in terms of its timing and spatial precision? Does the process interconnect and/or coordinate with polysaccharide deposition in the cell wall? If so, how?
IV. Does lignin deposition/defect affect cell wall polymers' organization and the ultimate ultrastructure, property and function? If so, how?
V. Does deficiency of lignin in the cell wall trigger cellular signals that cross-talk with events of plant growth and development? If so, how?

Furthermore, many engineered energy crops with low lignin or altered lignin structures need to go beyond laboratory studies and to be evaluated continuously in the field environment to assess and further improve their fitness in growth and tolerance to stresses. These researches will ultimately enable to transform the developed technologies into industrial exploitation to produce renewable, sustainable fuels and bio-based materials using lignocellulosic feedstocks on a commercial scale.

Acknowledgements

This work was supported by the Division of Chemical Sciences, Geosciences and Biosciences, Office of Basic Energy Sciences of the US Department of Energy through Grant DEAC0298CH10886; the National Science Foundation through grant MCB-1051675 to C.J.L.

Conflict of interest

No conflict of interest is declared.

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