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Review article

Plant biotechnology for lignocellulosic biofuel production

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Summary

Lignocelluloses from plant cell walls are attractive resources for sustainable biofuel production. However, conversion of lignocellulose to biofuel is more expensive than other current technologies, due to the costs of chemical pretreatment and enzyme hydrolysis for cell wall deconstruction. Recalcitrance of cell walls to deconstruction has been reduced in many plant species by modifying plant cell walls through biotechnology. These results have been achieved by reducing lignin content and altering its composition and structure. Reduction of recalcitrance has also been achieved by manipulating hemicellulose biosynthesis and by overexpression of bacterial enzymes in plants to disrupt linkages in the lignin–carbohydrate complexes. These modified plants often have improved saccharification yield and higher ethanol production. Cell wall-degrading (CWD) enzymes from bacteria and fungi have been expressed at high levels in plants to increase the efficiency of saccharification compared with exogenous addition of cellulolytic enzymes. *In planta* expression of heat-stable CWD enzymes from bacterial thermophiles has made autohydrolysis possible. Transgenic plants can be engineered to reduce recalcitrance without any yield penalty, indicating that successful cell wall modification can be achieved without impacting cell wall integrity or plant development. A more complete understanding of cell wall formation and structure should greatly improve lignocellulosic feedstocks and reduce the cost of biofuel production.

Introduction

Biomass is biological material derived from living organisms. It is an important renewable energy resource, either for direct production of heat or for indirect conversion to biofuels. With increased demand for energy and the need for cost-effective sustainable production of alternative fuels to reduce dependence on fossil fuel, a great effort has been made to create more cost-effective biofuels. The ideal biofuel feedstocks would have high-energy content, could be produced at high yield on a large scale, and could grow on marginal land that would not compete with agriculture.

Biofuels are classified into different categories depending on the source of biomass. First-generation biofuels are derived from sugar or starch, which is easily extracted and fermented to ethanol. Second-generation biofuels are produced from lignocellulosic non-food plants, or from agricultural and municipal waste, converting cellulose to ethanol. Currently, the dominant biomass-based liquid biofuel is ethanol from maize kernels and sugarcane stalks (Sticklen, 2006). However, the production of first-generation biofuel often competes with food crops for land and can only meet a limited fraction of the global fuel requirement (Field *et al.*, 2008). Plant lignocelluloses, as the most abundant organic raw materials, are regarded as the best feedstock for ethanol biofuel production (Carroll and Somerville, 2009).

Lignocelluloses consist predominantly of lignin, carbohydrates, pectin and proteins. Lignocellulosic biofuel production is the process of removing lignin and converting cellulose to ethanol. It includes five main steps: feedstock collection and transport to the processing plant, pretreatment to reduce lignin, enzyme hydrolysis for saccharification, fermentation, and distillation (Figure 1). Cell wall polymers first need to be hydrolysed to sugars for fermentation to ethanol, but lignocelluloses are highly recalcitrant to microbial and enzymatic degradation. Cellulose is highly crystalline and cross-linked with lignin and hemicelluloses, limiting the accessibility of cellulases to cellulose (Himmel *et al.*, 2007). To achieve effective hydrolysis of cellulose, pretreatment is necessary to remove lignin and hemicelluloses or to disrupt their linkages with cellulose and to reduce its crystallinity. Technologies for pretreatment include physical pretreatment (mechanical reduction to small particle size and pyrolysis), physicochemical pretreatment (steam, carbon dioxide, and ammonia fibre explosion), chemical pretreatment (acid, alkaline, ionic liquids, and ozonolysis) and biological pretreatment (using biomass-degrading micro-organisms) (Kumar *et al.*, 2009). Chemical and thermochemical pretreatments are currently the most effective for industrial application (Alvira *et al.*, 2010). After pretreatment, more cellulose can be hydrolysed to glucose by polysaccharide hydrolases. Glucose is then fermented to ethanol, which is recovered by distillation.

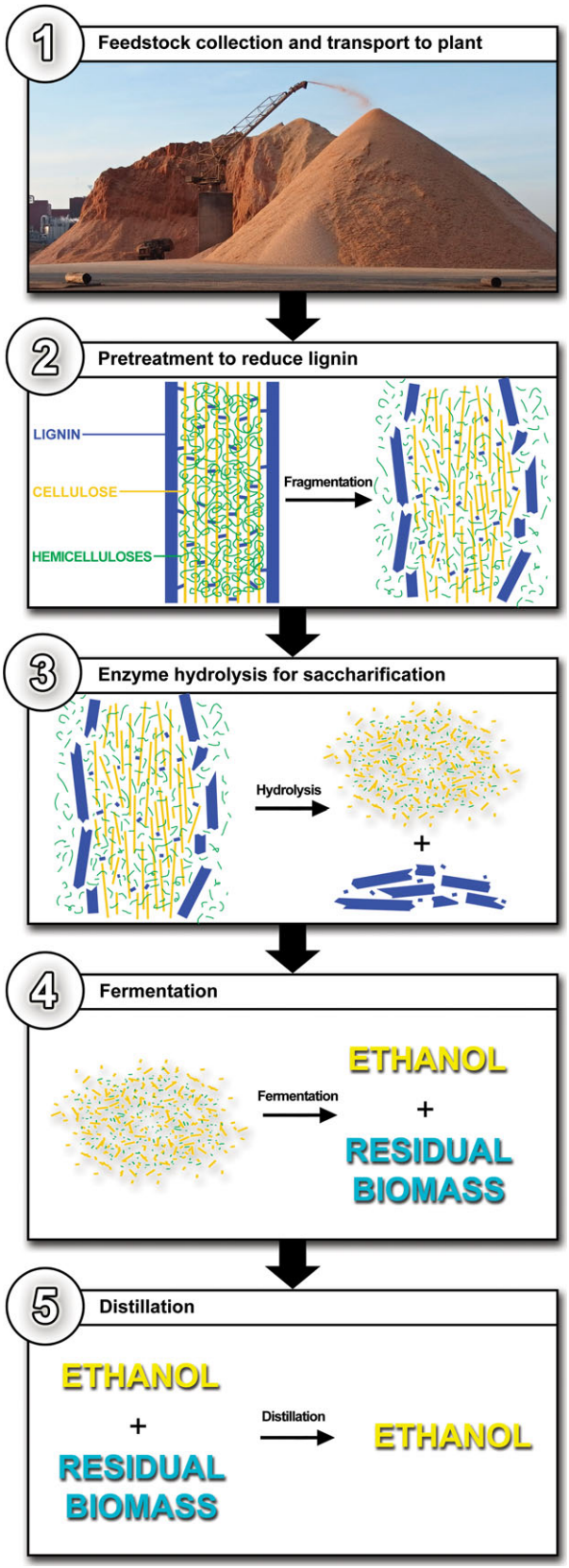

Figure 1 Biofuel production.

The cost of conversion of lignocellulose to sugar is high, due to the costs of biomass cultivation, collection, transport, storage, chemical pretreatment and the price of exogenous enzymes. Pretreatment accounts for 20% of the total cost (Mosier *et al.*, 2005). To decrease the cost of biofuel production, research has focused on the optimization of pretreatment, hydrolysis and fermentation. With the growing understanding of the biosynthesis of the cell wall components, cellulose, hemicelluloses and lignin, genetic modification of the plant cell wall has generated plant materials with higher saccharification efficiency. Polysaccharide hydrolases from bacteria and fungi have been expressed in plants to achieve a high-level accumulation of enzymes to provide an alternative to production of enzymes by microbial fermentation (Sticklen, 2006). Recently, transgenic plants with *in planta* expression of polysaccharide hydrolases have been evaluated for saccharification efficiency (Shen *et al.*, 2012). In this review, we describe the progress of biotechnology studies towards conquering the cell wall recalcitrance to biomass deconstruction. It includes modifications of plant cell walls and the engineering of polysaccharide hydrolases in plants for biofuel production.

Plant cell wall modifications for biofuel production

Cell wall formation is a complex plant-specific process, involving the deposition of polysaccharides and the building of a polysaccharide network (Cosgrove, 2005). When new cell plates are formed after cytokinesis, primary cell walls are continuously built during cell growth, assembling the polysaccharides cellulose, hemicelluloses and pectin. After cells cease enlargement, secondary cell walls (SCWs) are formed in specialized cells to provide mechanical strength and to form sclerenchyma cells. These terminally differentiated cells include vessels and fibres in angiosperms and tracheids in gymnosperms (Evert, 2006).

The SCW is rich in cellulose, hemicelluloses and lignin, and is a major biomass resource for biofuel. Efforts have focused on the biosynthesis of cellulose, hemicelluloses and lignin to understand how SCWs are formed. Of these three components in the SCW, the lignin biosynthetic pathway is best understood. Many aspects of the route of metabolic flux through the monolignol biosynthesis pathway were identified by characterizations of mutants and antisense suppression transgenics, combined with biochemical enzyme assays (Boerjan *et al.*, 2003; Campbell and Sederoff, 1996).

Engineering of monolignol biosynthesis pathway genes

Lignin is a phenolic polymer covalently linked with cellulose and hemicelluloses in the SCW of vascular plants (Balakshin *et al.*, 2008; Sarkanen and Ludwig, 1971). It plays a critical role in vascular transport and mechanical support (Harada and Cote, 1985). It also plays a unique role in the adaptation of plants to their environment and in resistance to biotic and abiotic stresses (Harada and Cote, 1985).

Lignin surrounds the preformed hemicelluloses and cellulose, reducing the accessibility of cellulose-degrading enzymes. Lignin is the major barrier for the accessibility of cellulolytic enzymes to cellulose to hydrolyse plant fibres. Several lignin characteristics, such as content, subunit composition and the degree of ester linkages between lignin and carbohydrates, affect the recalci-

trance (Chen and Dixon, 2007; Grabber *et al.*, 2009). Lower lignin content and a higher ratio of syringyl/guaiacyl units (S/G ratio) increase sugar release (Chen and Dixon, 2007; Chiang, 2002; Davison *et al.*, 2006; DeMartini *et al.*, 2013; Min *et al.*, 2012; Studer *et al.*, 2011).

Lignin is typically polymerized from three phenylpropanoid monomers, *p*-coumaryl, coniferyl and sinapyl alcohols, also known as the H, G and S monolignols. Members of ten enzyme families convert phenylalanine to monolignols through a metabolic grid, including serial reactions of hydroxylation and methylation (Figure 2) (Dixon *et al.*, 2001; Higuchi, 1997, 2003; Humphreys *et al.*, 1999; Shi *et al.*, 2010; Vanholme *et al.*, 2008). These ten enzyme families are phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), *p*-hydroxycinnamoyl-CoA: shikimate *p*-hydroxycinnamoyltransferase (HCT), *p*-coumaroyl shikimate 3′-hydroxylase/coumarate 3-hydroxylase (C3H), caffeoyl-CoA-*O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), coniferaldehyde 5-hydroxylase/ferulate 5-hydroxylase (CAld5H/F5H), caffeic acid/5-hydroxyconiferaldehyde 3-*O*-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD) respectively. Monolignols are exported to the cell wall (Higuchi, 1997; Liu, 2012; Miao and Liu, 2010; Smith *et al.*, 2013) and oxidized by peroxidases (POs) and laccases (LACs) to phenoxy radicals to form lignin polymers (Berthet *et al.*, 2011; Higuchi, 1997; Lu *et al.*, 2013; Zhao *et al.*, 2013b). G and S subunits polymerize typically through β-O-4, β-5 and β-β linkages (Figure 3a) (Ralph *et al.*, 2004).

The supply and ratios of monolignols determine lignin composition, content and structure. These lignin properties are key

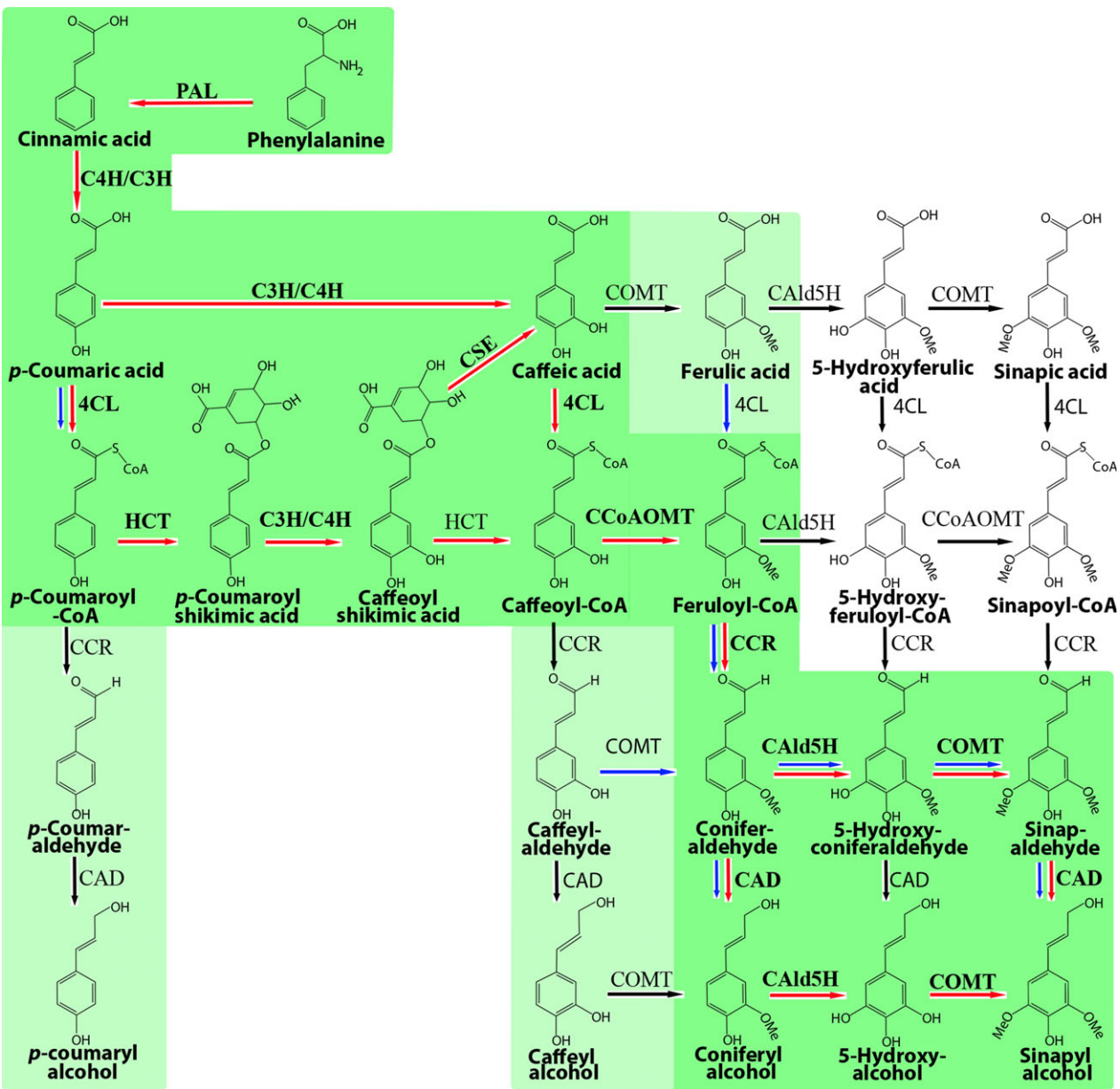

Figure 2 Monolignol biosynthetic pathway. The principal path in Arabidopsis and poplar is shown in the heavy green colour and red arrows. The pathway in switchgrass that is supported by biochemical and genetic studies is shown by blue arrows.

factors affecting the efficiency of lignin removal during conversion of SCWs into biofuels. Engineering of the lignin pathway has been carried out in many species, including poplars, eucalyptus, switchgrass, alfalfa and maize.

Monolignol pathway mutation in the model plant Arabidopsis

Because of the negative impact of lignin on the accessibility of polysaccharide-degrading enzymes, engineering of monolignol pathway genes to decrease lignin content has been a strategy for producing crops with improved digestibility (Chen and Dixon, 2007). The Arabidopsis *ref8* mutant showed that mutation of *C3H* caused cell walls to be more completely degraded by polysaccharide hydrolases than in the wildtype (Franke *et al.*, 2002a).

Conversion efficiency of cellulose was estimated using a set of 20 Arabidopsis mutants in 8 families of 10 different genes involved in the monolignol biosynthesis pathway (Van Acker *et al.*, 2013). These mutants alter lignin content and S/G ratios at different levels. In wildtype Arabidopsis, only 18% of cellulose can be converted to glucose after pretreatment. In most Arabidopsis SCW mutants, cellulose conversion efficiency was increased, with the highest being 88% in the *ccr* mutant (Van Acker *et al.*, 2013).

The hydrolysis of Arabidopsis mutants shows that increases of cellulose saccharification by engineering monolignol biosynthesis can be the basis for monolignol pathway engineering in other species. Different species may have alternative routes for monolignol biosynthesis (Weng *et al.*, 2010; Zhou *et al.*, 2010), and down-regulation of specific monolignol pathway genes in different species may not have the same effects. For example, down-regulation of *C3H* in Arabidopsis caused reduction of both G and S monolignols (Ralph *et al.*, 2006). However, in a hybrid poplar (*Populus alba* × *grandidentata*), the lignin content reduction was mainly due to the decrease of G monolignols, and S monolignols remained at about the same level (Coleman *et al.*, 2008).

Recently, the *caffeoyl shikimate esterase* (*CSE*) gene was identified converting caffeoyl shikimic acid to caffeic acid in Arabidopsis (Vanholme *et al.*, 2013) (Figure 2). The *cse* mutants had reduced lignin content with enriched H units. The saccharification efficiency of the *cse-2* mutant was increased to 75%; fourfold higher than that in the wildtype (Vanholme *et al.*, 2013). However, the involvement of CSE in other species has not been verified, although other species also possess *CSE* genes that are highly expressed in the lignification tissues. For example, switchgrass *CSE* gene expression was not

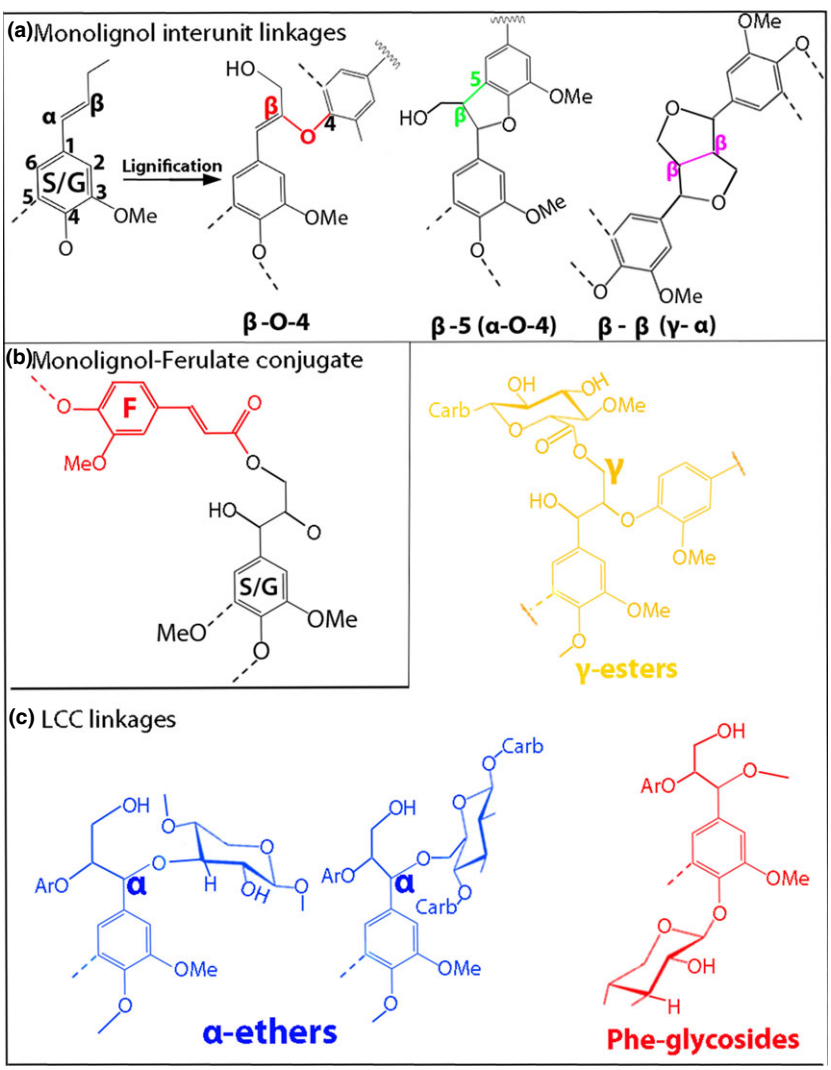

Figure 3 Linkages. (a) Some typical G and S interunit linkages. (b) Monllignol–Ferulate Conjugate. F: Ferulate. (c) LCC linkages.

induced in the lignified cell cultures (Shen *et al.*, 2013). Nevertheless, engineering of the monolignol pathway in species with industrial potential could lead to better biofuel production.

Monolignol pathway engineering in woody plants

Woody plants highly specialized in lignin biosynthesis in wood formation also provide fundamental information on the genetic and biochemical basis of lignification (Li *et al.*, 2006). Wood formation is a process of xylem differentiation derived from continuous cell division in the vascular cambium, which gives rise to xylem mother cells inside and phloem mother cells outside. The cells derived from xylem mother cells undergo further cell division, followed by cell expansion, cell wall thickening and programmed cell death. In angiosperm, xylem differentiation produces vessels, ray parenchyma and fibre cells. The thick-walled fibre cells are the major components of the secondary xylem (wood). In gymnosperms, there is only one major type of cell with thick SCWs, the tracheids, which provide both mechanical support and water transport (Evert, 2006).

As a major SCW component, lignin accounts for more than 20% of the dry weight of wood. In the wood of *Populus*, the S/G ratio is approximately 2 (Higuchi, 1997; Hu *et al.*, 1999; Sarkanen and Ludwig, 1971). Many studies of lignin biosynthesis were carried out on woody species (softwoods or hardwoods) because they have economic importance as raw materials or for biofuel. Engineering of monolignol pathway genes has enabled the alteration of lignin content and composition in trees (Lu *et al.*, 2010).

4CL mediates the activation of hydroxycinnamates for monolignol biosynthesis (Figure 2). Down-regulation of *4CL* in aspen (*Populus tremuloides*) resulted in a 45% reduction of lignin content, with both G and S subunits decreased, and accordingly cellulose content increased by 15% (Hu *et al.*, 1999). Repression of *4CL* by RNAi in the gymnosperm *Pinus radiata* also caused a lignin content reduction, up to 36%, but an increase of polysaccharide was only observed for galactan (Wagner *et al.*, 2009). The *4CL* gene was silenced by antisense in *Populus trichocarpa*, and enzymatic hydrolysis showed an increase of up to 65% of the saccharification efficiency in the *4CL*-down-regulated transgenic wood (Min *et al.*, 2012).

CAld5H catalyses hydroxylation at the 5-position of the aromatic ring of cinnamic intermediates and is a key enzyme for S monolignol biosynthesis (Figure 2) (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999). Overexpression of *CAld5H* in *P. tremuloides* and in a hybrid poplar (*Populus tremula* × *alba*) increased the lignin S/G ratio (Franke *et al.*, 2000; Stewart *et al.*, 2009). Antisense silencing of *4CL* and overexpression of *CAld5H* in quaking aspen caused a 52% reduction of lignin content and a 64% increase of lignin S/G ratio (Li *et al.*, 2003).

Cinnamoyl-CoA reductase catalyses the conversion of cinnamoyl-CoA esters to their corresponding cinnamaldehydes in monolignol biosynthesis (Figure 2). In the transgenic hybrid poplar (*P. tremula* × *alba*) when *CCR* was down-regulated by antisense, lignin content was reduced to 50%, and this transgenic had improved pulping efficiency (Leplé *et al.*, 2007). The cellulose content in the CCR-down-regulated poplar was not increased, and the saccharification yield is improved due to higher cellulose conversion (Van Acker *et al.*, 2014). Field-grown *CCR*-down-regulated poplar had a 161% higher ethanol yield than the wildtype. In *P. radiata*, a 46% lignin content reduction was obtained in a *CCR* RNAi line, and an 8% lignin content reduction was obtained in a Norway spruce *CCR* antisense line (Wadenbäck *et al.*, 2008; Wagner *et al.*, 2013).

Cinnamyl alcohol dehydrogenase catalyses the last step of monolignol biosynthesis, converting hydroxycinnamyl aldehydes to alcohols (Figure 2). Transgenic poplar (*P. tremula* × *alba*) down-regulating *CAD* had normal growth in the field, and its wood was more easily extracted (Baucher *et al.,* 1996; Lapierre *et al.*, 1999; Pilate *et al.*, 2002).

Monolignol pathway engineering in grass species

Grasses, such as switchgrass (*Panicum virgatum*), *Miscanthus sinensis*, rice (*Oryza sativa*) and maize (*Zea mays*), are also potential cellulosic bioenergy crops (Heaton *et al.*, 2008; Schmer *et al.*, 2008). Some grasses, such as switchgrass, can grow well on marginal land and therefore do not necessarily compete with crop plants for land (Parrish and Fike, 2005). In switchgrass, 4CL, COMT, CCR and CAD have been characterized (Escamilla-Treviño *et al.*, 2010; Fu *et al.*, 2011a,b; Saathoff *et al.*, 2011; Xu *et al.*, 2011), and recently the genes involved in the monolignol biosynthesis were identified in a systematic genome-wide study (Shen *et al.*, 2013).

In transgenic switchgrass lines down-regulating *COMT* by RNAi, lignin content was reduced as much as 24% and the lignin S/G ratio was decreased from 0.71 to as low as 0.37 (Fu *et al.*, 2011b). Ethanol yield from *COMT*-down-regulated switchgrass was 38% higher than wildtype. The same amount of ethanol was produced from this transgenic switchgrass with less severe pretreatment and much lower cellulose dosages as the control (Fu *et al.*, 2011b). Field-grown 2-year-old *COMT* RNAi switchgrass plants had similar sugar release and ethanol yield as those grown in a greenhouse and did not show any negative effects on biomass yield or disease susceptibility, indicating that *COMT* RNAi switchgrass can be used for biofuel production at lower cost (Baxter *et al.*, 2014). RNAi silencing of the *4CL* gene in switchgrass caused an 80% decrease of plant protein extract 4CL activity, leading to a reduction of lignin content up to 32% in a severe knockdown line. The dilute acid-pretreated transgenic biomass yielded 57.2% more fermentable sugar than pretreated wildtype wood (Xu *et al.*, 2011).

In *Brachypodium distachyon*, *COMT* and *CAD* genes were silenced by artificial microRNA. Fermentation of *CAD1*-down-regulated and *COMT*-down-regulated plants yielded 9% and 17% higher ethanol than the control respectively (Trabucco *et al.*, 2013). Saccharification was also analysed in two *B. distachyon* mutants that have point mutations at different locations in the *CAD* gene and were found to have 46% and 44% increases of saccharification efficiency (d'Yvoire *et al.*, 2013). In ryegrass (*Lolium perenne*), modifications of lignin biosynthesis by RNAi-mediated down-regulation of *COMT* and *CCR* also enhanced the enzyme digestibility (Tu *et al.*, 2010).

The leaves and stalks of maize and rice (stover), or bagasse (stalks and leaves after crushing to remove sugars) of sorghum or sugarcane, can be feedstocks for biofuel. Stalks contain vascular bundles, composed of many thick-walled vessels and fibre cells. Several cases of lignin reduction in these species showed positive effects on saccharification efficiency. Five *brown midrib* (*bm*) maize mutants and four *bm* sorghum mutants have reduced lignin. Some have been characterized as *CAD* or *COMT* mutations. These bm mutants showed increased digestibility (Sattler *et al.*, 2010). Mutation of sorghum *CAD* and *COMT* caused 22% and 21% increases, respectively, of the conversion of cellulose to ethanol. The increase was 43% in the double

mutant (Dien *et al.*, 2009; Saballos *et al.*, 2008). Saccharification efficiency was increased by 28%–32% in sugarcane *COMT* RNAi transgenic plants, grown either in a greenhouse or in the field (Jung *et al.*, 2012, 2013).

Grass lignins contain substantial amounts of *p*-coumarate (*p*CA) (Ralph, 2010). Grass-specific *p*-coumaroyl-CoA: monolignol transferases (PMTs) have been identified as enzymes acylating monolignols with *p*-CAs in rice and *B. distachyon* (Petrik *et al.*, 2014; Withers *et al.*, 2012). Disruption of *BdPMT* in *B. distachyon* caused a reduction of *p*-coumarate content (Petrik *et al.*, 2014). In *Z. mays*, RNAi suppression of *p*-coumaroyl CoA: hydroxycinnamyl alcohol transferase (*p*CAT), which is responsible for formation of *p*CA-monolignol conjugates, also resulted in a decrease of *p*CA levels (Marita *et al.*, 2014). Because *p*CA does not participate in the radical coupling reactions of lignification, engineering of the acyltransferases that specifically acylate monolignols could generate lignin with lower amounts of *p*CA and more monolignol conjugates, which could be cleaved by alkaline or acidolytic processes, and provides a potential strategy for improving saccharification efficiency specifically in grasses.

Monolignol pathway engineering in herbaceous plants

Perennial herbaceous plants have high biomass yield and can be grown on marginal land (Bhattarai *et al.*, 2013). Alfalfa is regarded as one potential plant for biofuel. In alfalfa, the functions of four enzymes C4H, COMT, CAD and CCR involved in the monolignol pathway have been characterized (Guo *et al.*, 2001; Reddy *et al.*, 2005; Shadle *et al.*, 2007; Zhao *et al.*, 2013c).

The relationships between lignin content/composition and chemical/enzymatic saccharification for biofuel production were investigated using transgenic alfalfa lines independently down-regulated in each of six lignin biosynthetic enzymes C4H, HCT, C3H, CCoAOMT, F5H and COMT (Chen and Dixon, 2007). A strong negative correlation between lignin content and sugar release by enzymatic hydrolysis was observed. The highest saccharification efficiency was 79% in the *HCT* line that had the lowest lignin content (Chen and Dixon, 2007). More sugars were released from xylan in transgenics than in the wildtype, indicating that lignin modification also increases the accessibility of residual hemicellulose to degradative enzymes (Chen and Dixon, 2007). Further, the alfalfa *COMT* transgenic that has reduced S lignin was examined for ethanol production. Ethanol yield was as much as 277 L/ton, 19.7% higher than wildtype (Dien *et al.*, 2011).

Growth of lignin transgenics

Because lignin is essential for normal growth and development, strong reduction of lignin content could affect plant growth (Novaes *et al.*, 2010). Except *CAD*, down-regulation of other monolignol pathway genes, such as *C3H*, *CCR*, *HCT*, at severe levels (i.e. >40% lignin reduction) resulted in a severe dwarf phenotype (Franke *et al.*, 2002b; Shadle *et al.*, 2007; Voelker *et al.*, 2010). The decreased biomass yield will have adverse effect on the ethanol production. For example, *CCR* down-regulation in hybrid poplar (*P. tremula* × *alba*) had 161% higher ethanol production than the wildtype. If the yield penalty is taken into account, the ethanol produced by CCR transgenic poplar was only 57% higher than that produced by wildtype (Van Acker *et al.*, 2014).

The stunted growth may be due to the perturbation of the auxin signal or alteration of the salicylic acid (SA) level, but not by lignin content reduction, because bringing the SA level back down to normal restored growth (Besseau *et al.*, 2007; Gallego-Giraldo *et al.*, 2011). The HCT-down-regulated Arabidopsis maintained reduced lignin independent of the level of SA (Gallego-Giraldo *et al.*, 2011).

In the case of low-lignin maize *bm2* mutant, time to flowering is longer than for wildtype (Vermerris and Mclntrye 1999). Recently map-based cloning showed that *bm2* gene encodes a methylenetetrahydrofolate reductase that is involved in the biosynthesis of SAM (Tang *et al.*, 2014). This work provides an example of avoiding lignin pathway genes for generating transgenic plants with low lignin, but not affecting agronomic performance.

MED5a and MED5b, subunits of the transcriptional coregulatory complex Mediator, are required for homeostatic repression of phenylpropanoid biosynthesis in Arabidopsis (Bonawitz *et al.*, 2012). Disruption of MED5a and MED5b in the Arabidopsis *C3H* missense mutant *reduced epidermal fluorescence 8-1* (*ref8-1*) rescued the stunted growth and lignin deficiency and produced a novel lignin consisting almost exclusively of *p*-hydroxyphenyl lignin subunits (Bonawitz *et al.*, 2014). The *med5a/med5b/ref8-1* triple mutant substantially increased the conversion of cellulose to glucose, with more than twice the glucose yield than the wildtype or *med5a/5b*. The increase of saccharification efficiency may be due to the altered lignin composition and structure. Engineering of the Mediator in the transcription machinery and signalling pathways responding to cell wall defects provides another potential mechanism for reducing biomass recalcitrance.

Modification of lignin structures

Although lignin reduction by silencing/knocking-down monolignol pathway genes in all the species tested is a promising approach to improve biofuels, increased saccharification efficiency can be compromised by decreased biomass. Strategies for altering lignin structure without affecting plant growth have been studied to provide materials that are more easily deconstructed. Incorporating a new linkage in lignin is an effective way to generate more easily digested plants without affecting plant growth (Wilkerson *et al.*, 2014).

Incorporating new linkages in lignin

Lignin can be polymerized using isolated maize cell walls and added lignin precursors, including subunits for generating novel linkages. For example, the incorporation of ferulate is compatible with normal lignification reactions, allowing its integration into lignin polymers (Figure 3b) (Ralph, 2010). Cell walls with coniferyl ferulate esters produce ester-interunit linkages that enhance alkaline delignification and enzymatic degradation, because ester linkages are readily cleaved by alkali (Grabber *et al.*, 2008). Enhanced delignification was also observed when epigallocatechin gallate (EGCG) was incorporated into lignin. The EGCG-lignified walls yielded 34% more glucose and total sugars than the control (Elumalai *et al.*, 2012).

Chemically labile ester linkages were introduced into the lignin backbone in hybrid poplar (*P. alba* × *grandidentata*) by overexpressing an *Angelica sinensis* (dong quai) gene encoding feruloyl-coenzyme A (CoA) monolignol transferases (FMT), which catalyse the formation of the conjugate (Wilkerson *et al.*, 2014). Both coniferyl ferulate and sinapyl ferulate were incorporated into the lignin of the transgenic poplar. This transgenic poplar wood was more easily deconstructed, with as much as a twofold increase of the saccharification efficiency (Wilkerson *et al.*, 2014). These transgenic plants did not show any growth reduction and

demonstrate a new approach to improve plant biomass for biofuel.

Reducing the degree of lignin polymerization

Hydroxycinnamoyl-CoA hydratase-lyase (HCHL) can cleave the propanoid side chain of hydroxycinnamoyl-CoA lignin precursors. The resulting monomers, when incorporated into lignin polymers, lack propanoid side chains and conjugated double bonds, disabling them from further polymerization with other monomers. Overexpression of *Pseudomonas fluorescens HCHL* in plants caused the accumulation of a non-conventional side-chain-truncated monomer, resulting in a reduced degree of lignin polymerization (reduced molecular weight) (Eudes *et al.*, 2012; Mayer *et al.*, 2001). Arabidopsis plants overexpressing a codon-optimized *HCHL* gene driven by a SCW-specific promoter were normal in phenotype in contrast to tobacco plants expressing CaMV 35S promoter-driven *HCHL* that were dwarfed and sterile. The *HCHL*-overexpressing Arabidopsis, with a reduced degree of lignin polymerization, had a higher saccharification efficiency, up to a ~70% increase over wildtype (Eudes *et al.*, 2012).

Engineering artificial enzymes

Through iterative saturation mutagenesis, Zhang *et al.* (2012) engineered a monolignol 4-*O*-methyltransferase (MOMT4) that can regiospecifically methylate the *p*-hydroxyl of monolignols, important for coupling during lignin polymerization. Overexpression of *MOMT4* in Arabidopsis caused etherification of the *p*-hydroxyl of lignin monomeric precursors, which blocked coupling/polymerization of linkages between side chain carbons and conventional lignin monomers, and subsequently caused lignin content reduction. Plant growth was not severely affected in the *MOMT4*-overexpressing Arabidopsis that has lignin content reduction up to 34%. Overexpression of *MOMT4* enhanced the hydrolysis of cell wall carbohydrates, producing 22% more sugars than the wildtype.

Cellulose biosynthesis and engineering

Cellulose is the world's most abundant polymer. It accounts for 40%–50% of plant SCW. It is a linear polymer composed of (1→4)-linked β-D-glucose residues (Doblin *et al.*, 2002) (Figure 4). Thirty to thirty-six parallel cellulose chains, each containing up to 14 000 glucose molecules, form microfibrils through hydrogen bonds and Van der Waals forces (Somerville, 2006). At the base of the growing microfibril there is a rosette structure; a large multimeric plasma membrane-bound complex, responsible for cellulose synthesis (Brown, 1996; Eckardt, 2003). The high crystallinity of cellulose makes it recalcitrant to enzymatic hydrolysis by cellulases into glucose subunits.

Cellulose synthase (CesA) is the catalytic subunit of the cellulose synthase (CESA) complex. Thirty-six CesA proteins may be required for the CESA complex (Somerville, 2006). A CESA complex requires at least three different interacting CesA proteins (Endler and Persson, 2011; Timmers *et al.*, 2009). Ten *CesA* genes have been identified in Arabidopsis (Richmond and Somerville, 2000; Somerville, 2006). *AtCesA4*, *AtCesA7* and *AtCesA8* encode the three protein subunits necessary for cellulose biosynthesis in SCWs (Atanassov *et al.*, 2009; Endler and Persson, 2011; Taylor *et al.*, 2000, 2003). Their functions are not interchangeable (Gardiner *et al.*, 2003). Of the 17 *CesA* genes identified in *P. trichocarpa*, the *PtrCesA4* gene, the *PtrCesA7/17* gene pair and the *PtrCesA8/18* gene pair encode interacting proteins in the CESA complex for cellulose synthesis in wood. These genes have the highest protein sequence identities with Arabidopsis *AtCesA4*, *AtCesA7* and *AtCesA8* respectively (Song *et al.*, 2010; Suzuki *et al.*, 2006). There are no reports of increasing cellulose content by overexpressing one or more *CesA* genes.

Other proteins are associated with the CESA complex. Sucrose synthase (SuSy) and KOR can be immunoprecipitated from protein extract of *P. deltoides* × *trichocarpa* stem-differentiating xylem (SDX) by CesA-specific antibodies (Song *et al.*, 2010). Immunogold labelling with anti-SuSy antibodies showed that SuSy is an integral component of the catalytic unit of cellulose biosynthesis (Fujii *et al.*, 2010). SuSy catalyses the formation of UDP-Glc from sucrose. Overexpression of SuSy in hybrid poplar (*P. alba* × *grandidentata*) resulted in increased cellulose synthesis (Coleman *et al.*, 2009). Overexpression of KOR, a membrane-bound endo-β-1,4-glucanase, decreased cellulose crystallinity (Takahashi *et al.*, 2009).

Hemicellulose biosynthesis and engineering

Hemicelluloses are short abundant branched polysaccharides, ranging from 500 to 3000 units, with β-(1→4)-linked backbones in plant cell walls. Hemicelluloses include xyloglucan (XyG), xylan, mannan, and glucomannan, and β-(1→3,1→4)-glucan (Scheller and Ulvskov, 2010). XyG is mainly deposited in the primary cell wall of seed plants except the grasses, constituting 20%–25% and 10% of the wall in dicot and conifer respectively (Scheller and Ulvskov, 2010). XyG is composed of backbone of repeated (1→4)-linked β-D-Glc units, substituted by α-D-xylose, and the xylosyl residue can be substituted with additional residues, such as galactose, arabinose and fucose (Figure 4). Xylan, the most abundant hemicellulose in SCWs, is a linear polymer with a backbone composed entirely of (1→4)-linked β-D-xylose units (Figure 4). In dicot SCWs, the major hemicellulose is glucuronoxylan (GX), in which some Xyl residues are attached to Glucuronic acid (GlcA) or 4-*O*-methyl GlcA (MeGlcA). Some Xyl residues can also be substituted with arabinosyl or acetyl groups (Ebringerova and Heinze, 2000). In wood of angiosperms, the xylose backbone is partially substituted by MeGlcA groups through α-(1-2)-glycosidic linkages (Fengel and Wegener, 1983). Xylan in angiosperm wood is also referred to as 4-*O*-methylglucuronoxylan. The backbone terminates with a tetrasaccharide containing an α-L-rhamnopyranosyl-1,2-α-D-galactopyranosyl uronic acid unit linked to the reducing end of the polymer (Andersson *et al.*, 1983; Johansson and Samuelson, 1977). Glucuronoarabinoxylan (GAX) represents the major hemicellulose in both the primary and secondary walls of grasses (Scheller and Ulvskov, 2010).

Currently, hemicelluloses are commonly removed during the initial stage of biomass processing to increase the efficiency of enzymatic cellulose hydrolysis. Manipulations of hemicellulose biosynthesis to reduce hemicellulose content or to break its cross-links with cellulose and lignin could improve the accessibility of cellulases and hemicellulases to their substrates.

Engineering of xylan

Information on the involvement of glycosyltransferases (GTs) in xylan biosynthesis comes from work on Arabidopsis. *IRX9*, *IRX9-L*, *IRX10*, *IRX-10L, IRX14* and *IRX14-L* are involved in xylan backbone synthesis (Brown *et al.*, 2007, 2009; Lee *et al.*, 2010, 2012; Pena *et al.*, 2007; Wu *et al.*, 2009, 2010). *IRX7/FRA8*, *F8H*, *IRX8* and *PARVUS* are implicated in synthesis of the tetrameric end group (Figure 4) (Brown *et al.*, 2007; Lee *et al.*, 2007, 2009a; Pena *et al.*, 2007).

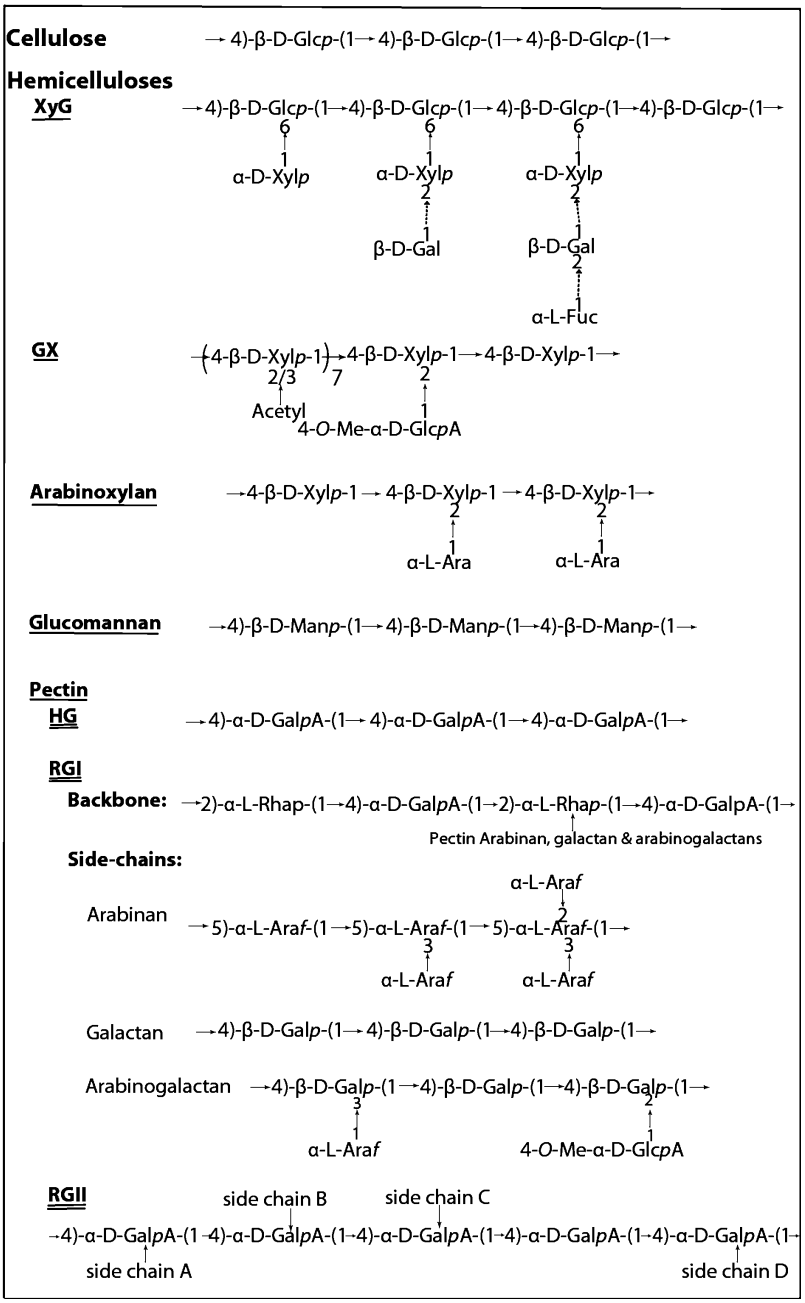

Figure 4 Structures of cellulose and the hemicelluloses xyloglucan (XyG), glucuronoxylan (GX), arabinoxylan, glucomannan and pectin. GX contains the tetrameric end group 4-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*A-(1→4)-D-Xyl*p* at its reducing end.

The GTs of poplars involved in xylan biosynthesis have been characterized for their functions during wood formation (Kong *et al.*, 2009; Lee *et al.*, 2009b, 2011; Li *et al.*, 2011; Zhou *et al.*, 2006, 2007). Down-regulation of family GT47 *PoGT47C* in *P. alba* × *tremula* showed an up to 48% increase in the conversion of cellulose to glucose compared to wildtype (Lee *et al.*, 2009b).

Xylan is covalently attached to lignin through ester linkages to branch MeGlcA, which is also involved in the xylan–cellulose and xylan–xylan interactions (Kabel *et al.*, 2007; Watanabe and Koshijima, 1988). Glucuronic acid substitution of xylan 1 (GUX1) and GUX2 are the xylan glucuronyltransferases adding GlcA and MeGlcA branches to xylan in Arabidopsis (Bromley *et al.*, 2013; Mortimer *et al.*, 2010). The α-(1,2)-glycosidic bond between MeGlcA and Xyl is difficult to break and thus α-glucuronidases are added into the enzyme mixture containing xylanases and xylosidases for complete xylan hydrolysis. Xylan that lacks branches in the *gux1 gux2* double mutants showed more enhanced xylan extraction from the cell walls than the xylan with branches in the wildtype. All xylan in the *gux1gux2* double mutant could be hydrolysed to monosaccharide sugars by mixtures of xylanases and xylosidases, while only ~40% of the xylan was hydrolysed in the wildtype and in the *irx9* and *irx14* mutants (Mortimer *et al.*, 2010).

Arabinoxylan is a major hemicellulose in the primary and SCWs of grasses. It has β-1,4-D-xylopyranosyl backbones and 1,3-or 1,4-α-L-arabinofuranosyl residues (Figure 4). Arabinofuranosidase was overexpressed in rice to reduce arabinose, which forms a cross-link between arabinoxylan and lignin. The transgenic rice had more than a 28% increase of cellulose content and a

46% increase of saccharification efficiency (Sumiyoshi *et al.*, 2013). Overexpression of OsAt10, a *p*-coumaroyl coenzyme A transferase involved in glucuronoarabinoxylan modification, increased matrix polysaccharide-associated ester-linked *p*-CA and decreased matrix polysaccharide-associated ferulic acid. A 20%–40% increase of saccharification yield was observed in the *OsAt10* overexpression plants, providing an attractive target for improving grass cell wall quality for biofuel (Bartley *et al.*, 2013).

Engineering of pectin

Pectin is a major component of primary cell walls in dicots and nongraminaceous monocots, accounting for 30%–35% of dry weight. It is also present in SCWs (in the middle lamella) and in grasses (Mohnen, 2008). Pectin is a galacturonic acid (GalA)-rich linear polymer. Homogalacturonan (HG) accounts for ~65% of pectin and has more than 100 (1→4)-linked GalA residues (Figure 4) (Mohnen, 2008; Thibault *et al.*, 1993). HG can be modified by linkage of methyl esters to some C6 positions. Other major pectins are rhamnogalacturonan I (RG-I) and RG-II, comprising 20%–35% and 10% of pectin respectively (Mohnen, 2008).

Overexpression of a fungal gene encoding polygalacturonase (PG) or an Arabidopsis gene encoding an inhibitor of pectin methylesterase (PMEI) reduced the content of de-esterified HG (Capodicasa *et al.*, 2004; Lionetti *et al.*, 2007). Transgenic Arabidopsis, tobacco and wheat leaves overexpressing PG or PMEI had up to three times the saccharification efficiency, due to modification of pectin composition or architecture (Lionetti *et al.*, 2010). In addition, transgenic stems also gave a higher efficiency of enzymatic saccharification (Lionetti *et al.*, 2010). In poplar, degradation of pectin by overexpression of pectate lyase (that degrades HG) improved saccharification of wood, due to a greater release of pentoses and hexoses (Biswal *et al.*, 2014).

Engineering of mannan

Glucomannan is a minor hemicellulose (3%–5% in weight) in SCWs of angiosperms. It is a linear polymer of (1→4)-linked β-D-glucose and β-D-mannose, with glucose: mannose ratios of 1:1 to 1:3 (Figure 4) (Aspinall, 1973). The Arabidopsis cellulose synthase-like (*Csl*) gene *AtCslA9* and the *P. trichocarpa PtrCslA3* encode glucomannan synthases (Dhugga *et al.*, 2004; Goubet *et al.*, 2009; Liepman *et al.*, 2005; Suzuki *et al.*, 2006). *Populus trichocarpa* endo-1,4-β-mannanase PtrMAN6 not only has a hydrolysis function on mannan but also suppresses the thickening of the SCW in xylem tissue (Zhao *et al.*, 2013a). *PtrMAN6* may be a new target for modification of the SCW to weaken cross-linking between lignin and hemicelluloses.

Disruption of linkages in the lignin–carbohydrate complex

Lignin-carbohydrate complexes (LCCs) may be formed by the same coupling mechanisms as lignin polymerization. Benzyl ether, benzyl ester and phenyl glycosides (Figure 3c) are the three major linkages of cellulose and xylan to lignin. In *P. trichocarpa* wood, approximately 15% of the monomers in lignin are attached to polysaccharides through benzyl ether (Figure 3c, blue contours), γ-ester (orange contours) or phenyl glycosidic linkages (red contours) (Balakshin *et al.*, 2007; Capanema *et al.*, 2004). LCCs link the cellulose and xylan polymers to lignin in the SCW of angiosperms to form a rigid matrix of the three polymers. The resulting SCW structure is a multilayered composite of adjacent cell walls (Schniewind and Berndt, 1991) and has very high tensile and compressive strength, acting as a major barrier to enzymatic hydrolysis of cellulose in production of fermentable sugars for biofuel.

Esters are unique linkages between lignin and xylan through the primary hydroxyl group at the C γ of the lignin side chain and the uronic acid of the MeGlcA in xylan (Watanabe and Koshijima, 1988). The lack of MeGlcA caused by *GUX1* and *GUX2* mutations reduces cross-linking between xylan and lignin (Bromley *et al.*, 2013; Mortimer *et al.*, 2010). Overexpression of the *Phanerochaete carnosa* protein family 15 carbohydrate esterase (PcGCE), which targets ester linkages between xylan and lignin, disrupts the cross-links between xylan and lignin in SCWs of Arabidopsis. The *PcGCE*-overexpressing Arabidopsis had an increase of xylose extractability up to 15% (Tsai *et al.*, 2012).

Ferulate is esterified to arabinoxylan and is also incorporated into lignin, forming lignin–ferulate–arabinoxylan complexes. Disruption of the ester bond of the lignin–ferulate–arabinoxylan complex showed enhanced cell wall deconstruction. Overexpression of an *Aspergillus niger* ferulic acid esterase in the apoplast, ER or golgi of tall fescue (*Festuca arundinacea*) enhanced sugar release, to more than twice that of the wildtype (Buanafina Marcia *et al.*, 2010).

Modifications of the genetic regulatory network controlling SCW formation

Plant SCW formation is controlled by a genetic regulatory network of transcription factors (TFs) (Zhao and Dixon, 2011; Zhong *et al.*, 2010). Many *NAC* (*NAM*, *ATAF1/2* and *CUC2*) and *MYB* genes directly or indirectly regulate the biosynthesis of lignin, cellulose and xylan (Figure 5). In Arabidopsis, SECONDARY WALL-ASSOCIATED NAC DOMAIN (SND) and VASCULAR-RELATED NAC DOMAIN (VND) TFs are activators of SCW synthesis in fibre and vessel cells respectively. SND1, VND6 and VND7 activate a cascade of TFs, including the direct targets *MYB46* and *MYB83* (Kubo *et al.*, 2005; Mitsuda *et al.*, 2007; Ohashi-Ito *et al.*, 2010; Yamaguchi *et al.*, 2008, 2010, 2011;

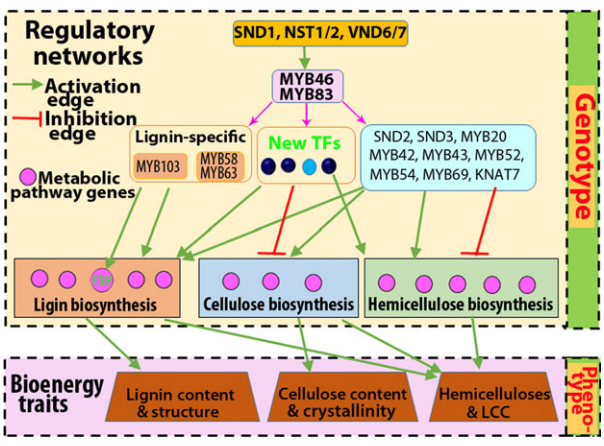

Figure 5 A model of a genetic regulatory network controlling secondary cell wall (SCW) formation in Arabidopsis. It shows part of genome-wide regulatory nodes and their directed (activation ↑, positive or inhibition ⊥, negative) hierarchical regulatory interaction (genotype) that determine the characteristics of the bioenergy traits (phenotype). Pink circles represent the genes involved in lignin, cellulose and hemicellulose biosynthesis. New TFs represent unidentified transcription factors in the network controlling SCW formation.

Zhong *et al.*, 2006, 2008). When overexpressed, *MYB46* and *MYB83* can induce ectopic SCW formation (Ko *et al.*, 2009; McCarthy *et al.*, 2009; Zhong *et al.*, 2007). *MYB103*, a direct target of SND1, is required for *F5H* expression and S lignin biosynthesis in Arabidopsis (Öhman *et al.*, 2013).

Overexpression of *SND1* and *VND* genes did not enhance cellulose deposition as expected, but inhibited plant growth (Kubo *et al.*, 2005; Zhong *et al.*, 2006). In the *c4h* mutant, lignin biosynthesis was reduced and vessel collapsed. Overexpression of a *VND6* promoter-driven *C4H* in the *c4h* mutant (i.e. *c4h*+*p*VND6::*C4H*) rescued lignin biosynthesis in vessels, but not in fibre cells. In addition, *NST1,* a TF that is functionally redundant to *SND1*, was overexpressed in the *c4h*+*p*VND6::*C4H* background under the *IRX8* promoter. Because the *IRX8* promoter is self-induced by NST1, it creates positive feedback for NST1 overexpression to enhance cell wall deposition in fibre cells. The *c4H*+*p*VND6::*C4H*+*p*IRX8::*NST1* Arabidopsis grew normally, due to the normal vessel integrity. The lignin reduction and enhanced cell wall deposition in fibre cells may be the reason resulting in improved sugar release of two times higher than the wildtype (Yang *et al.*, 2013). These results provide an example of modification of the regulatory network to enhance SCW deposition for reducing recalcitrance.

Engineering of cell wall-degrading enzymes in plants

Plant cell wall biopolymer hydrolysis requires polysaccharide hydrolases [cell wall-degrading (CWD) enzymes] to break down the glucosidic bonds or xylosidic bonds in cellulose and hemicelluloses. Polysaccharide hydrolyases are mainly produced by bacteria and fungi (Tomme *et al.*, 1995). The individual fungal hydrolases are different from the multiple hydrolases in bacteria, which include the cellulases and hemicellulases that form a complex called the cellulosome (Bayer *et al.*, 2004). Ding *et al.* (2012) found that bacterial and fungal enzymes use different mechanisms to deconstruct plant cell walls. Bacterial cellulosomes first digested the compound middle lamella (CML), while the fungal cellulases digested the cell wall, leaving the CML intact.

A complete hydrolysis of polysaccharides needs exo- and endo-hydrolyases together. Hydrolysis of cellulose into glucose monomers requires three types of enzymes: exo-1,4-β-glucanases, endo-1,4-β-glucanases and β-D-glucosidases. Exo- and endo-glucanases can hydrolyse cellulose to cellobiose (a β(1,4)-linked disaccharide of glucose), which can be converted to glucose by β-D-glucosidases (Singhania *et al.*, 2013). Two enzymes involved in xylan backbone hydrolysis are endo-1,4-β-D-xylanase (xynA) and β-xylosidase (xyD). α-glucuronidase (aguA) cleaves the glycosidic linkage between MeGlcA and backbone xylose units. Cellulase and xylanase enzymes act synergistically to improve cellulose hydrolysis and produce a secondary stream of fermentable pentose sugars (Gottschalk *et al.*, 2010; Murashima *et al.*, 2003).

Polysaccharide hydrolases from bacteria are now commercially available. For example, CellicCTec2 (Novozymes) is a blended mixture of cellulases, β-glucosidases and hemicellulases. Several crop plants (Table 1) have been used to express bacterial and fungal CWD enzymes as exogenous reagents for lignocellulosic ethanol production. Recently, researchers have been begun to take advantage of thermostable CWD xylanases to produce self-digesting materials, which can be processed for cellulose hydrolysis by exogenous cellulases alone or with less hemicellulases added (Shen *et al.*, 2012).

Overexpression of CWD hydrolases as exogenous enzymes

Production of heterologous recombinant proteins in plants is well established for research and industrial applications (Ma *et al.*, 2003). Great effort has been made to increase transgenic protein yield and stability, two major factors that determine the feasibility and efficiency of the production system (Ma *et al.*, 2003). Table 1 lists the major studies of recombinant polysaccharide hydrolases in transgenic plants.

The yield of transgene products is affected by the genetic characteristics of the transgenes, in particular (i) codon usage in host plants, (ii) the promoter used for expression and (iii) the encoded signal peptides for subcellular localization (Hood, 2004; Hood *et al.*, 2003, 2007; Streatfield, 2007). Cellulases from bacteria, such as *Acidothermus cellulolyticus* and *Trichoderma reesei*, have been expressed in many plant species, including Arabidopsis, tobacco and alfalfa (Table 1). Because codon usage differs between prokaryotes and eukaryotes, the coding sequences of prokaryotic genes may require modification to enhance translational efficiency in plants (Grantham *et al.*, 1981; Hood *et al.*, 2007; Murray *et al.*, 1989; Perlak *et al.*, 1991). Hood *et al.* (2007) optimized the first 40 codons of a *A. cellulolyticus* endo-1,4-β-glucanase (E1, AcE1) gene and a *T. reesei* cellobiohydrolase 1 (TrCBH 1) gene for expression in maize seeds and recovered both transgenic proteins with yields up to 18% of the total soluble protein (TSP). This production level of transgenic TrCBH 1 protein was nearly 1000-fold higher than the level of this protein in all other studies (Hood *et al.*, 2007) (Table 1).

Hood *et al.* (2007) also ascribed the high accumulation of AcE1 and TrCBH1 to a maize embryo-preferred promoter and signal peptides, allowing cytoplasmically translated products to target specific subcellular locations. In general, tissue-specific promoters result in higher protein production (Table 1), but in many cases the resulting protein yields were considerably lower than the 18% level reported for AcE1 and TrCBH1 (Hood *et al.*, 2007). In Hood *et al.* (2007), the tissue-specific promoter-driven expression was carried out in a homologous system (i.e. a maize promoter in transgenic maize), where the promoter specificity as well as the strength had been established (Clough *et al.*, 2006). In another case, driving grain-specific expression of *Clostridium stercorarium XynB* under the rice glutelin 4 promoter (GluB-4) resulted in a higher accumulation, reaching 16% of maize-grain TSP (Gray *et al.*, 2011). Many studies (Table 1) have used the constitutive cauliflower mosaic virus promoter (CaMV 35S) to drive protein accumulation in plant leaves.

Signal peptides may also improve yield of specific expressed proteins. Although general yields of CWD enzymes are 0.1%–5% TSP in plant tissues, Ziegler *et al.* (2000) reported high AcE1 expression in transgenic Arabidopsis leaf cell walls, up to 26% of the TSP. A signal sequence that targeted the protein to the apoplast (cell walls and the intercellular spaces) was incorporated into the transgene. In many other cases, transgenes with an encoded signal peptide for targeting gene products through the ER pathway also led to high levels of products in specific subcellular locations, such as cell wall, ER and chloroplast (Dai *et al.*, 2000a,b; Dai *et al.*, 2005; Harrison *et al.*, 2011; Hood *et al.*, 2007; Ziegelhoffer *et al.*, 2001; Ziegler *et al.*, 2000) (Table 1). Subcellular targeting signals in ER, vacuole and chloroplast also had substantial impact on the accumulation of fungal cellobiohydrolase I (CBH1), CBH2, and bacterial endoglucanase (EG) in sugarcane (Harrison *et al.*, 2011). Targeting products into

Table 1 Production of saccharification hydrolases in transgenic plants

Cellulolytic enzyme	Host plant	Transgene				Putative transgenic protein location	Protein yield	References
		Native signal peptide	Promoter	Enhancer	Plant targeting signal			
AcE1	Maize	Removed	Glob-1	None	Three types	CW, ER or Vac	>17% TSP in single seed	Hood *et al.* (2007)
	Tobacco	Retained or removed	Mac	TMV Ω	Native, KDEL, sporamin A,	Cyt, Chl, Apo, ER or Vac	Up to 1.35% TSP in leaves	Dai *et al.* (2000a,b, 2005)
			RbcS-3C	None	PR-S or rbcS-2A			
			CaMV 35S	None	Native,VSPβ or rbcS			Ziegelhoffer *et al.* (2001)
AcE1-cat	Arabidopsis	Removed	CaMV 35S	TMV Ω	Prla	Apo	Up to 26% TSP in leaves	Ziegler *et al.* (2000)
	Rice	Removed	CaMV 35S	TMV Ω	Prla	Apo	Up to 4.9% TSP in leaves	Oraby *et al.* (2007)
	Tobacco	Removed or retained	CaMV 35S	None	Native,VSPβ or rbcS	Cyt,Chl,Apo	Up to 1.6% TSP in leaves	Ziegelhoffer *et al.* (2001)
psbA-AcE1	Tobacco	Removed	None	None	Chloroplast transformation		Up to 12% TSP in leaves	Ziegelhoffer *et al.* (2009)
TrCBH1	Maize	Removed	Glob-1	None	Three types	CW, ER, Vac	>16% TSP in single seed	Hood *et al.* (2007)
TfCel6A	Tobacco	Removed	psbA	None	Chloroplast transformation		Up to 2%–3% TSP	Yu *et al.* (2007)
TfCel6B							Up to 3%–4% TSP	
DB-TfCel6A	Tobacco	Removed	None	None	Chloroplast transformation		Up to 10.7% TSP in leaves	Gray *et al.* (2009)
CtxynZ	Tobacco	Removed	CaMV 35S	None	prII	Apo	Up to 4.1% TSP in leaves	Herbers *et al.* (1995)
CsxynB	Maize	Removed	GluB-4 and rubi3	None	BAASS and GluB-4	CW and grain	Up to 16.4% TSP	Gray *et al.* (2011)
SoxynB	Potato	Removed	2× CaMV 35S	None	With and without prII	Cyt or Apo	Up to 5% TSP in leaves	Yang *et al.* (2007)
TrXylII	Arabidopsis	Removed	RbcSK-1A	None	RA or SKL	Chl, Per	1.2%–4.8% TSP in leaves	Hyunjong *et al.* (2006)
Zera-Xyl	Tobacco	Removed	CaMV 35S	TEV	Spg	PB	Up to 9% TP	Llop-Tous *et al.* (2011)
BSX	Maize	Removed	GluB-4 and rubi3	None	BAASS and GluB4SP	CW and grain	Up to 4% TSP	Gray *et al.* (2011)
	Tobacco	Retained	prrn	psbA	Chloroplast transformation		Up to 6% TSP	Leelavathi *et al.* (2003)
TmXyl10B	Tobacco	Retained	prrn	None	Chlorophyll transformation		11%–15% TSP	Kim *et al.* (2011)

Cellulolytic enzymes: *AcE1*: the *Acidothermus cellulolyticus* gene encoding endo-1,4-β-glucanase (E1); *AcE1-cat*: catalytic domain of the *A. cellulolyticus* gene encoding endo-1,4-β-glucanase (E1); *psbA-AcE1*: catalytic domain of the *A. cellulolyticus* E1 (AcE1) fused with N-terminus of the psbA gene product; *TrCBH1*: the *T. reesei* gene encoding cellobiohydrolase I (CBH I); *TfCel6A* and *TfCel6B*: *T. fusca* genes encoding thermostable cellulases Cel6A and Cel6B; *DB-TfCel6A*: the *T. fusca* cel6A fused with downstream box (DB); *CtxynZ:* the *C. thermocellum gene encoding xylanase Z*; *CsxynB*: the *C. stercorarium* gene encoding xylanase B; *SoxynB*: the *Streptomyces olivaeoviridis* gene encoding xylanse; *TrXylII*: the *T. reesei* gene encoding endo-β-1,4-xylanase Xyl II; Zera-Xyl: A codon-optimized xynTB synthetic gene from *S. olivaceoviridis* fused to a proline-rich domain (Zera) of maize storage protein γ-zein; *BSX*: the Bacillus sp. NG-27 gene encoding xylanse; *TmXyl10B*: the *Thermotoga maritima* gene encoding GH10 xylanase Xyl10B. Promoters: Glob-1: maize embryo-preferred globulin-1 promoter; CaMV 35S: the constitutive cauliflower mosaic virus 35S promoter; Mac: a hybrid promoter of mannopine synthase promoter and the CaMV 35S promoter enhancer region (TMV-Ω); RbcS-3C: the tomato leaf-specific Rubisco small subunit promoter; psbA: the tobacco psbA promoter; GluB-4: the rice glutelin 4 promoter; rubi3: the rice ubiquitin 3 promoter; RbcSK-1A: the promoter of alfalfa RbcS gene; prrn: the rice prrn promoter. Enhancers: TEV: the tobacco etch virus translation enhancer; psbA: the rice psbA signal. Plant targeting signals: KDEL: an ER retention signal; Sporamin A: a sweet potato signal peptide targeting vacuole; PR-S: a signal peptide targeting apoplast; RbcS-2A: the signal peptide of tomato Rubisco small subunit 2A targeting chloroplast; VSPβ: a plant apoplast signal peptide; rbcS: a plant chloroplast signal peptide; BAASS: barley α-amylase signal sequences for cell wall targeting; GluB4SP: the rice glutelin B-4 gene signal peptide for grain-specific expression; prII: signal peptide of the proteinase inhibitor II protein; RA:N-terminal transit peptide of the Rubisco activase for chloroplast targeting; SKL: a peroxisome targeting signal; Spg: the γ-zein signal peptide. Putative protein locations: CW: cell wall; ER: endoplasmic reticulum; Vac: vacuole; Cyt: Cytosol; Chl: chloroplast; Apo: apoplast; Per: peroxisome; PB: protein bodies. Note: Protein yields lower than 1% TSP or not quantified are not listed.

protein bodies could also result in high-level accumulation: for example, up to 9% of total protein for *Thermobifida fusca* xylanase cel6A (Llop-Tous *et al.*, 2011). Many bacterial and fungal CWD enzymes were highly expressed when targeted to chloroplasts (Verma *et al.*, 2010). AcE1 and Cel6A accumulated to very high levels in the transplastomic plants when they were fused with other protein domains that increase translation of cellulolytic enzymes. The expressed enzymes can account for up to 12% of TSP, a 200-fold increase compared to the unfused form (Gray *et al.*, 2009; Ziegelhoffer *et al.*, 2009).

High-level accumulation of CWD enzymes has been achieved in many plants, which is important for industrial hydrolysis of lignocellulose (Table 1). Using crude extracts directly for enzyme reactions and omitting enzyme purification can lower costs. All the plant systems in Table 1 produced the target proteins with the expected enzymatic function. Plant crude extracts, containing chloroplast-derived bacterial and fungal CWD enzymes, displayed higher activities compared with *Escherichia coli* crude extracts. For example, the activity of *T. reesei* exoglucanase (TrCelO) from plants was 24-fold higher than that from *E. coli* (Verma *et al.*, 2010). Cellulose in chemically pretreated tobacco, maize and rice leaves could be converted into glucose by a total protein extract containing AcE1 from transgenic tobacco, maize or rice leaves (Biswas *et al.*, 2006; Oraby *et al.*, 2007; Verma *et al.*, 2010). Plant crude extracts from transgenic leaves overexpressing bacterial and fungal xylanases completely hydrolysed xylan (Kim *et al.*, 2011).

Currently, commercial enzyme production by bacteria and fungi is still costly and has low capacity. Comparisons of plant systems for producing cellulolytic enzymes with the bacterial systems indicate that plants provide a less expensive platform for enzyme production. In the production of cellulolytic enzymes in chloroplasts, such as *C. thermocellum* endoglucanase (CelD) and exoglucanase (CelO), the cost of this enzyme from plants is 100- to 3000-fold lower than from *E. coli* (Verma *et al.*, 2010). Enzyme cocktails prepared from these chloroplast-derived enzymes exhibited significantly higher ability to digest filter paper, pinewood or citrus peel. The glucose produced by the digestion of filter paper using chloroplast-derived enzyme cocktails was 3625% and 396% more than the Novozyme 188 cocktail or the Celluclast 1.5L cocktail respectively (Verma *et al.*, 2010).

In planta expression of CWD enzymes for autohydrolysis

Conversion of lignocellulose to fermentable sugars normally requires chemical pretreatment to help overcome barriers to enzymatic saccharification of polysaccharides, particularly lignin and lignin carbohydrate complexes (LCCs). The main effect of mild pretreatment is removal of xylan (Mosier *et al.*, 2005). Xylanases, which hydrolyse hemicelluloses and make cellulose more accessible to enzymatic hydrolysis, are required for complete hydrolysis of plant cell walls into fermentable sugars, such as glucose and xylose.

Among the CWD enzymes (Table 1) engineered in plants, most are from extremophile bacteria, including *Thermotoga maritima*, *A. cellulolyticus, T. fusca*, *Clostridium thermocellum* and *Streptomyces olivaceoviridis* that thrive at temperatures over 70 °C. Most enzymes characterized from hyperthermophiles are optimally active at temperatures close to the host organism's optimal growth temperature, usually 70–125 °C, but are not active at plant growth temperature. Transgenic plants tolerate a high accumulation of these enzymes in the cell wall with no deleterious effects on phenotype (Kawazu *et al.*, 1999; Leelavathi *et al.*, 2003; Ziegler *et al.*, 2000). Normal growth of transgenic alfalfa and maize has also been observed in field tests, a key step towards commercial production (Hood *et al.*, 2007; Ziegelhoffer *et al.*, 1999). Thermopohilic enzymes retain thermostability and are stable in plants (Table 1). Engineered xylanases remain stable in corn stover dried for 2 weeks (Shen *et al.*, 2012), demonstrating an essential feature for the application of 'built-in' systems to reduce the quantity of enzyme required for feedstock hydrolysis.

There are only a few studies on the autohydrolysis of cell walls by engineered CWD enzymes (Borkhardt *et al.*, 2010; Brunecky *et al.*, 2011; Chou *et al.*, 2011; Kawazu *et al.*, 1999; Shen *et al.*, 2012). Two endo-xylanases *DtXynA* and *DtXynB* from *Dictyoglomus thermophilum* were codon-modified and expressed in Arabidopsis under the CaMV 35S promoter and targeted to the apoplast (Borkhardt *et al.*, 2010). DtXynA and DtXynB retained high activities in dry stems and were able to hydrolyse plant xylan (Borkhardt *et al.*, 2010). After pretreatment, transgenic plants overexpressing *A. cellulolyticus* endocellulase Cel5A or endoglucanse AcE1 were more digestible than the wildtype, reducing cell wall recalcitrance (Brunecky *et al.*, 2011; Chou *et al.*, 2011). Consolidated pretreatment and hydrolysis of transgenic maize overexpressing two or more CWD enzymes showed a higher efficiency of cell wall autohydrolysis compared with the control, with a 141% higher glucose yield and a 172% higher xylose yield (Zhang *et al.*, 2011). Transgenic maize overexpressing native *DtXynB* had shrivelled seeds and low fertility (Shen *et al.*, 2012). Shen *et al.* modified DtXynB by adding a self-splicing peptide, an intein (Perler *et al.*, 1994), combined with mutation screening and generated iXynB, whose enzymatic activity was induced at high temperature. The *iXynB* maize had normal seeds and fertility. Under mild heat pretreatment, >90% of the theoretical glucose and 63% of the theoretical xylose yields were produced in the *iXynB* stover, with a 20% increase over the wildtype (Shen *et al.*, 2012).

Future perspective

Recent investigations in plant biotechnology have demonstrated successful engineering to reduce the recalcitrance of plant cell walls for lignocellulosic biofuel production. The most efficient manipulations targeting the monolignol pathway or reducing the cross-linking between lignin and carbohydrates are based on the current understanding of lignin biosynthesis. However, the progress of enhanced ethanol production by technology studies has come mainly from laboratories under highly controlled conditions. New procedures need to be tested as industrial processes; other impacts, such as environmental impacts, gene stability, etc. should be considered. Our understanding of cellulose, hemicelluloses, and lignin biosynthesis during plant cell wall formation is still limited. Plant cell wall formation is a more complex process than we anticipated. Reducing the capital cost of lignocellulosic biofuel production depends on the further elucidation and modification of plant cell wall formation and structure.

TF-mediated genetic regulatory networks control SCW formation. Our understanding of the SCW regulatory network in biofuel species is rudimentary. Various strategies, including large-scale co-expression and computational analysis, are being used to identify network regulatory genes (Ruprecht and Persson, 2012; Wang *et al.*, 2012). A genome-wide high-throughput system for discovery and validation of specific TF-directed hierarchical gene regulatory networks has been developed for studying wood

formation (Lin *et al.*, 2013). This system includes a high-throughput protoplast system, high-throughput deep RNA sequencing, computation by top-down Graphical Gaussian Modelling (GGM)-based algorithms, and validation by chromatin immunoprecipitation (ChIP)-sequencing (Lin *et al.*, 2013). A bottom-up GGM algorithm was used to build a three-layer network controlling wood formation (Lu *et al.*, 2013). This approach, combining RNA-seq data and computational prediction, can be used to reveal the hierarchy of transcriptional regulatory networks controlling SCW formation in other species.

Systems biology, which is the integration of genomics and related sciences with computational strategies for analysis and mathematical modelling, has been applied to study the biosynthesis of cell wall components (Yuan *et al.*, 2008). Our systems biology study of the regulation of lignin biosynthesis in wood formation includes advanced quantitative methods of genomics, proteomics, metabolomics, biochemistry, organic synthesis and structural chemistry. Protein–protein interactions affect the direction and rate of metabolic flux for monolignol biosynthesis (Chen *et al.*, 2011, 2014). Lignin biosynthesis is more complex than expected. Using a systems biology approach, we have obtained complete kinetic parameters for enzymes in the basic monolignol biosynthetic pathway. Using this biochemical information combined with absolute quantitation of each enzyme, and the lignin phenotypes of transgenic plants, we have constructed a predictive metabolic-flux model of this pathway. Such models provide a comprehensive mathematical description of monolignol biosynthesis, to guide future experiments leading to more desirable lignin quantity and structure (Chen *et al.*, 2014; Wang *et al.*, 2014). The results on modification of lignin content and composition show a high level of plasticity where dramatic modification is possible. The recent introduction of ferulate coniferyl esters into lignin, which permits more efficient mild alkaline hydrolysis (Wilkerson *et al.*, 2014), demonstrates the potential for designing novel plants for improved biofuel feedstocks.

Normal growth of transgenic poplars, alfalfa and maize in field tests is a key step towards commercial production of new biofuel feedstocks. Complete understanding of plant cell wall formation and elucidation of the genetic regulatory network controlling cell wall formation will lead to more sustainable and affordable feedstocks for biofuel.

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Conflict of interest

There are no conflicts of interest to be declared.

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