Genetic manipulation of lignocellulosic biomass for bioenergy

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Abstract

Lignocellulosic biomass represents an abundant and sustainable raw material for biofuel production. The recalcitrance of biomass to degradation increases the estimated cost of biofuel production and limits its competitiveness in the market. Genetic engineering of lignin, a major recalcitrance factor, improves saccharification and thus the potential yield of biofuels. Recently, our understanding of lignification and its regulation has been advanced by new studies in various systems, all of which further enhances our ability to manipulate the biosynthesis and deposition of lignin in energy crops for producing cost-effective second generation biofuels.

Introduction

Lignocellulose accounts for 70% of the carbon fixed by photosynthesis in land plants every year and a substantial amount of this biomass is available for use by humans [1]. Conversion of lignocellulosic feedstocks to biofuels has become increasingly attractive as a partial replacement for petroleum [2,3]. In contrast to bioethanol derived from starch, proposed lignocellulosic biofuel production facilities would use forest or crop residues and perennial grasses as feedstocks [1,2]. Biochemical and genetic investigations of lignocellulose in energy crops such as poplar, sorghum, sugarcane, miscanthus, and switchgrass are motivated by improving biofuel yield and providing a carbon-neutral source of transportation fuel.

Plant secondary cell walls are largely composed of cellulose, hemicelluloses and lignin. Cellulose, a β -1,4-linked polymer of glucose, is the most abundant component in secondary cell walls and hence the major source of sugar units used in biofuel production. Hemicelluloses include mannans, xylans, xyloglucans, or mixed-linkage glucans, which interact with cellulose to form a semi-rigid matrix [4]. Lignin is a heterogeneous polymer predominantly constituted of three subunits, namely *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignols. These monomers are derived from phenylalanine through the phenylpropanoid pathway, the structure of which is now well established (Figure 1) [5]. Lignin imparts mechanical strength and hydrophobicity to cell walls, but decreases saccharification efficiency and thereby increases the cost of lignocellulosic biofuel production. Fortunately, genetic modification of lignification results in changes in lignin content and/or composition, which in many cases leads to improved enzymatic digestion of the polysaccharide components of the cell wall [6-8]. Here, we summarize recent advances in the genetic manipulation of model plants and energy crops for enhanced fermentable sugar release.

Identification of new genes for lignin manipulation

Lignification is the consequence of coordinated monolignol biosynthesis and oxidative polymerization, both of which are tightly regulated transcriptionally and post-translationally [5,9]. The identification and functional characterization of the genes involved in these processes provide potential targets for modifying lignin and improving saccharification. For example, the recent discovery of caffeoyl shikimate esterase (CSE) revealed that this enzyme catalyzes the release of caffeate from caffeoyl shikimate which can then be utilized by 4-coumarate CoA ligase (4CL) to form caffeoyl CoA, an important intermediate for monolignol biosynthesis (Figure 1) [10]. In effect, the CSE/4CL route bypasses the transfer of the caffeoyl moiety from caffeoyl

shikimate back to CoA by hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT). The *cse* mutant accumulates less total lignin but over 30-fold more H units compared with wild-type *Arabidopsis*. Although the *cse* mutant produces less biomass, it releases 75% more glucose on a per plant basis during saccharification without pretreatment. As another example, cytochrome P450 reductases (CPRs) are also important for lignification because they transfer electrons from NADPH to the three cytochrome P450 hydroxylases required for monolignol synthesis, cinnamate 4-hydroxylase (C4H), *p*-coumarate 3'-hydroxylase (C3'H), and ferulate 5-hydroxylase (F5H) [11]. Although it had been assumed that the CPRs in *Arabidopsis* were functionally redundant, it was recently demonstrated that the *Arabidopsis atr2* mutant, defective in CPR2, produces less total lignin with a 10-fold enhancement in H unit content, and also exhibits improved glucose yields when alkaline-pretreated stems are saccharified [12].

Disruption of genes required for lignin polymerization also results in reduced lignin content and altered composition. Laccases (LACs) has been proposed to activate monolignols for radical coupling in the apoplast [5]. The first *in vivo* evidence came from the *Arabidopsis lac4 lac17* double mutant, which displays an irregular xylem phenotype and has 40% less lignin with an increased S/G ratio. These phenotypes led to higher glucose release when the mutant's cell wall residue was hydrolyzed with commercial cellulase [13]. The *Brachypodium lac5* mutant also deposits less lignin and exhibits enhanced saccharification, further demonstrating that LACs are potential genetic targets for improving biofuel production in monocot energy crops [14]. Like LACs, peroxidases have been shown to oxidize monolignols [5] and participate in lignin deposition in the Casparian strip [15], but more *in planta* data are necessary to define the biological functions of different peroxidases in vascular lignification [16,17]. Clearly, enzymes involved in both monomer synthesis and polymerization hold promise as tools for the improvement of biofuel crops.

The introduction of genes from other organisms is an alternative strategy to perturb lignin biosynthesis that is independent of the modification of endogenous plant genes. For example, the plastidial expression of 3-dehydroshikimate dehydratase from *Corynebacterium glutamicum* in *Arabidopsis* diverts flux from the shikimate pathway to synthesize protocatechuate, causes a 50% reduction in total lignin content and results in enhanced sugar release after enzymatic hydrolysis [18]. An interesting observation is that diverting carbon flux away from the shikimate pathway affects downstream lignification but not the pools of aromatic amino acids or salicylic acid [18]. These findings demonstrate that expression of foreign genes can also be used to redirect flux away from lignin and to reduce the recalcitrance of biomass for bioenergy production.

Modification of lignin monomers

In addition to the three primary *p*-hydroxycinnamyl alcohols, native lignins also contain numerous additional phenolic metabolites such as hydroxycinnamaldehydes, hydroxycinnamic acids, and hydroxybenzaldehydes [19]. When lignin biosynthesis is genetically perturbed, phenylpropanoid intermediates that accumulate as a result can be incorporated into the polymer (Figure 2) [5-7]. Indeed, overexpression of F5H in caffeic acid O-methyltransferase (COMT) - deficient *Arabidopsis* mutant resulted in an increased deposition of 5-hydroxyguaiacyl units in

lignin (Figure 1) [20,21]. Similarly, a catechyl lignin polymer derived from caffeoyl alcohol was identified in *CAFFEYOYL CoA O-METHYLTRANSFERASE (CCoAOMT)* suppressed pine cell culture (Figure 1) like that recently characterized in the seed coat of a variety of members of the Orchidaceae, Cactaceae, Euphorbiaceae, and Cleomaceae [22-24]. The abundance of benzodioxane structures in these two novel lignin types can minimize potential cross-linking between lignin α -carbons and hemicellulosic alcohol or acid groups (Figure 3) [19]. This suggests that the engineering of plants that deposit catechyl lignin might lead to improved cellulose conversion, similar to the COMT-deficient switchgrass and alfalfa that have been developed recently that deposit lignin with increased 5-hydroxyconiferyl alcohol monomer content [25-27].

The plasticity of lignin biosynthesis and polymerization makes it possible to incorporate new monomers and thus new functionality into the lignin polymer or to interfere with polymerization. An array of phenolic compounds has been proposed as promising candidates to improve saccharification through these mechanisms [19]. The first example successfully realized *in vivo* involved heterologous expression of an *Angelica sinensis* feruloyl CoA monolignol transferase in poplar, which resulted in the incorporation of ferulate conjugates into the lignin backbone (Figure 3) [28]. The novel lignins formed did not impact the growth of the poplar trees but the ester bonds within these novel lignin building blocks could be easily cleaved by alkaline pretreatment post-harvest, thus enhancing the release of sugar [28]. Instead of “editing” monolignols before polymerization, enzymatic modification of the lignin polymer itself can also result in readily cleavable bonds. A C- α dehydrogenase from *Sphingobium* was shown to oxidize the hydroxyl group of the α -carbon in β -O-4 linked dilignols and oligolignols *in vitro* or when expressed in Arabidopsis (Figure 3) [29]. Lignin of the transgenic lines showed only a small increase in α -keto β -O-4 linkages presumably because most of the C- α dehydrogenase appeared to localize to the cytoplasm even though it had been targeted for secretion [29]. It might be possible to attain higher levels of α -keto β -O-4 linkages in lignin in the future if the C- α dehydrogenase can be successfully targeted to the apoplast of lignifying cells.

The elongation of the lignin polymer is mainly the result of β -O-4 coupling between the β -carbon of monomers and the 4-O position of oligomers [7,19]. Monomers that lack a β -carbon or in which the *para*-hydroxyl group is blocked prevent the polymer from growing. For example, hydroxybenzaldehydes are known as minor non-conventional monomers in a variety of species and form end groups in natural lignins (Figure 3) [30]. To increase these end units and alter lignin architecture, a *Pseudomonas fluorescens* hydroxycinnamoyl CoA hydratase-lyase was expressed in Arabidopsis [31]. Although the transgenics displayed normal growth, they contained more reduced-length lignins and provided an increased yield of sugars after various pretreatments [31]. Methylation of the *para*-hydroxyl group of monolignols is another way to reduce polymerization by preventing β -O-4 linkages with the free monomers. The *Clarkia breweri* isoeugenol O-methyltransferase was engineered to catalyze the 4-O-methylation of monolignols, and expression of this enzyme in Arabidopsis reduced lignin content and led to elevated glucose yield without pretreatment [32]. Taken together, these results show that genetic manipulation of lignification can result in the incorporation of non-conventional monomers with positive impacts on biomass utility.

Regulation of lignocellulose biosynthesis

A hierarchical transcriptional network regulates secondary cell wall biosynthesis in plants, and coordinates cell elongation, differentiation, synthesis of polysaccharides and lignin, and programmed cell death [9]. The top-level master switches include a series of tissue-specific NAM, ATAF1,2, and CUC2 (NAC) transcription factors, which activate the secondary-level MYB master switches, such as MYB46 and MYB83 in Arabidopsis. These master switches together turn on downstream transcription factors and directly regulate the biosynthetic genes of the secondary cell wall [9,33]. Feed-forward loops between different transcription factors make the network robust in regulating secondary cell wall synthesis. To describe this network, Taylor-Teeple *et al.* utilized DNA-protein binding assays to study the complex regulatory feed-forward cascades controlling Arabidopsis lignocellulosic biosynthesis [34]. In terms of feedstock genetic engineering, one advantage of transcription factor manipulation is that multiple genes can be simultaneously regulated to restrict or enhance flux towards lignocellulose. Such an approach avoids the necessity of stacking of biosynthetic genes (or their mutants) to achieve desired phenotypes. For example, overexpression of *MYB4* in switchgrass, a transcriptional repressor of lignification, reduces the transcription of 10 lignin biosynthetic genes, decreases lignin content and increases cellulosic ethanol yield up to 2.6 fold [35,36].

It was recently demonstrated that transcriptional regulation of phenylpropanoid metabolism in plants is also dependent on the Mediator complex. Subunits of Mediator, MED5a and MED5b, suppress the steady state level of phenylpropanoid biosynthetic mRNAs in Arabidopsis [37]. Knocking-out *MED5a* and *MED5b* enhances phenylpropanoid gene expression and end product accumulation. Surprisingly, it also substantially alleviates the mis-regulation of transcription in the stunted and lignin-deficient Arabidopsis *c3'h* mutant, in which one-third of the transcriptome is differentially expressed, rescues its arrested growth and restores lignin deposition to wild-type levels (Figure 4) [38,39]. Most importantly, due to the defect in *C3'H*, the mutant's lignin is almost exclusively derived from H subunits, demonstrating that these monomers can be polymerized into a functional lignin that supports normal growth. The deposition of this novel lignin doubles the saccharification efficiency of the mutant's biomass compared to the wild-type [38].

In addition to regulation at the transcriptional level, posttranslational regulation also controls lignin biosynthesis [40]. It has recently been shown that ubiquitination and degradation of phenylalanine ammonia lyase (PAL), the first enzyme in the phenylpropanoid pathway, is mediated by Kelch-repeat-containing F box (KFB) proteins, components of the SCF type E3 ligase [41,42]. Overexpression of the KFBs facilitates the proteolysis of PAL and results in a reduction in lignin content and soluble phenylpropanoids in Arabidopsis [41,42] and may provide a novel approach to biomass modification in the future.

Engineering of energy crops for lignocellulosic utilization

An increasing number of studies conducted directly on energy crops have identified promising strains for biofuel production, including spontaneous or chemically-induced mutants. Mutant screens are particularly facile when visible markers are available. Brown midrib mutations, first found in natural maize mutants, are clearly visible to the unaided eye, and are associated with

reduced lignin content or altered lignin composition [43]. Random mutagenesis screens of sorghum were conducted to identify *brown midrib* (*bmr*) mutants and so far three independent loci have been isolated and found to encode 4CL (*bmr2*), CAD (*bmr6*), COMT (*bmr12*), respectively [43-45]. Characterization of these mutants showed a 7-20% increase in saccharification efficiency and a further 20% increase when *bmr6* and *bmr12* mutations are stacked [45,46].

Translation of genetic engineering strategies established in model plants into energy crops requires a comprehensive knowledge of lignification in these target species. Advanced sequencing technologies have enabled the systematic identification of lignin biosynthetic candidate genes in poplar (*Populus trichocarpa*), *Eucalyptus grandis*, switchgrass (*Panicum virgatum*), and sugarcane (*Saccharum spp.*) thus facilitating reverse genetic analysis in these crops [47-50]. For example, RNAi mediated down-regulation of *C3'H* in poplar resulted in lower lignin content and higher glucose release by enzymatic hydrolysis (Figure 2) [51,52] and the CRISPR/Cas9 system was successfully employed to mutate *4CL1* and *4CL2* in hybrid poplar [53].

To be economically viable for biofuel production, modified crops will need to thrive in the field and yield high amounts of biomass. A five-year field trial study showed a significant increase in enzymatic hydrolysis of cellulose in transgenic poplar deficient in CCoAOMT but no impacts on its growth or biomass [54]. Similarly, field-grown *COMT*-downregulated or *MYB4* overexpressing switchgrass yielded increased amounts of fermentable sugars and comparable amounts of dry biomass [55,56]. Importantly, in the field these transgenic plants displayed no differences in disease susceptibility compared to wild-type controls. Elevated saccharification of lignocellulose was also observed in *COMT*-suppressed sugarcane and *CCR* downregulated poplar in field trials, but the improvements were accompanied by a reduction in biomass [57,58]. Although the higher cellulose conversion in the transgenic sugarcane and poplar compensates for the reduced biomass, the overall yield penalty in these lines remains an obstacle to be overcome before industrial utilization.

Conclusions

The production of cost-effective biofuels from abundant and renewable lignocellulosic feedstocks is impeded by the inherent recalcitrance of the biomass. Various genetic manipulations of lignification have demonstrated the power to reduce this stumbling block and increase the yield of fermentable sugars from engineered biomass. Alteration of lignin content and composition can be achieved by modification of endogenous plant genes or expression of heterologous genes. The introduction of novel monomers and increased levels of non-conventional subunits result in lignin with new functional groups and ease the removal of lignin for saccharification. Further, regulation of lignification at both transcriptional and post-translational levels is shown to be effective targets to engineer lignin. Extension of the established strategies from model systems into energy crops and assessment of the mutants in the field are necessary for successful industrial application. Future studies must focus on overcoming the yield loss that often accompanies lignin modification.

Figures

Figure 1. A simplified pathway illustrating the enzymes and metabolites involved in lignin biosynthesis. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; HCT, hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase; C3'H, *p*-coumaroyl shikimate 3'-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl CoA O-methyltransferase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid O-methyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; LAC, laccase; PRX, peroxidase. Structures of the five hydroxycinnamyl alcohols are included. Reactions in grey indicate that these steps are of only minor importance in wild-type plants.

Figure 2. A simplified model illustrating that manipulations of lignin biosynthetic genes change pathway flux. In flowering plants, the pathway generates H, G, and S lignin. When *C3'H* is perturbed (blue X), the flux towards G and S is blocked and mutants deposit H lignin (blue line). When *F5H* is silenced (red X), the S branch is blocked and mutants contain almost exclusively G lignin (red line). When *F5H* is overexpressed (green check), the G flux is redirected towards S monomers so transgenics accumulate predominantly S lignin (green line).

Figure 3. A model of lignin polymer illustrates the native and modified monomers and structural units. The major monomers are coniferyl alcohols (R=OMe, R'=H) and sinapyl alcohols (R=R'=OMe), and the major structural units are β -ether units (black) in wild-type plants. Wild-type lignin also includes phenylcoumaran units (green), resinol units (purple), and biphenyl ether units (blue). Cinnamyl alcohols (R=OMe, R'=OMe or H) (pink) and hydroxybenzaldehydes (R=OMe, R'=OMe or H) (cyan) are often seen as end groups. Incorporation of caffeyl alcohol (R=H) or 5-hydroxyconiferyl alcohol (R=OMe) results in benzodioxane units (brown) in the lignin polymers. Incorporation of monolignol ferulate (red) introduces the ester bond into the lignin backbone, forming ZipLigninTM. Oxidation of hydroxyl group on the α -carbon results in α -keto β -O-4 linkages (orange).

Figure 4. Knocking-out *MED5a* and *MED5b* substantially rescues the arrested growth of *Arabidopsis ref8-2* mutant that contains a T-DNA insertion in the gene encoding C3'H. Wild-type, *med5a/5b*, *ref8-2*, and *med5a/5b ref8-2* plants were photographed four weeks after planting. The *ref8-2* mutant is dwarf and sterile, but the triple mutant *med5a/5b ref8-2* has restored growth and is fertile. Photograph provided by Nicholas D. Bonawitz.

Acknowledgements

The preparation of this manuscript was supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomic Science program, under Award Number DE-SC0008628.

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Figure 1. A simplified pathway illustrating the enzymes and metabolites involved in lignin biosynthesis. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; HCT, hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase; C3'H, *p*-coumaroyl shikimate 3'-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl CoA O-methyltransferase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid O-methyltransferase; CCR, cinnamoyl CoA reductase; CAD, cinnamyl alcohol dehydrogenase; LAC, laccase; PRX, peroxidase. Structures of the five hydroxycinnamyl alcohols are included. Reactions in grey indicate that these steps are of only minor importance in wild-type plants.

Figure 2. A simplified model illustrating that manipulations of lignin biosynthetic genes change pathway flux. In flowering plants, the pathway generates H, G, and S lignin. When *C3'H* is perturbed (blue X), the flux towards G and S is blocked and mutants deposit H lignin (blue line). When *F5H* is silenced (red X), the S branch is blocked and mutants contain almost exclusively G lignin (red line). When *F5H* is overexpressed (green check), the G flux is redirected towards S monomers so transgenics accumulate predominantly S lignin (green line).

Figure 3. A model of lignin polymer illustrates the native and modified monomers and structural units. The major monomers are coniferyl alcohols ($R=\text{OMe}$, $R'=\text{H}$) and sinapyl alcohols ($R=R'=\text{OMe}$), and the major structural units are β -ether units (black) in wild-type plants. Wild-type lignin also includes phenylcoumaran units (green), resinol units (purple), and biphenyl ether units (blue). Cinnamyl alcohols ($R=\text{OMe}$, $R'=\text{OMe}$ or H) (pink) and hydroxybenzaldehydes ($R=\text{OMe}$, $R'=\text{OMe}$ or H) (cyan) are often seen as end groups. Incorporation of caffeyl alcohol ($R=\text{H}$) or 5-hydroxyconiferyl alcohol ($R=\text{OMe}$) results in benzodioxane units (brown) in the lignin polymers. Incorporation of monolignol ferulate (red) introduces the ester bond into the lignin backbone, forming ZipLigninTM. Oxidation of hydroxyl group on the α -carbon results in α -keto β -O-4 linkages (orange).

Figure 4. Knocking-out *MED5a* and *MED5b* substantially rescues the arrested growth of *Arabidopsis ref8-2* mutant that contains a T-DNA insertion in the gene encoding C3'H. Wild-type, *med5a/5b*, *ref8-2*, and *med5a/5b ref8-2* plants were photographed four weeks after planting. The *ref8-2* mutant is dwarf and sterile, but the triple mutant *med5a/5b ref8-2* has restored growth and is fertile. Photograph provided by Nicholas D. Bonawitz.

lignocellulose with native lignin

pretreatment and saccharification

free sugars

genetic manipulations affect lignin content, composition, structural units, and/or polymerization

lignocellulose with modified lignin

pretreatment and saccharification

increased saccharification efficiency

enhanced yield of free sugars