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# Genetic modification of wood quality for second-generation biofuel production

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How the abundant tree biomass resources can be efficiently used for future biofuel production has attracted a great deal of interest and discussion in the past few years. Capable technologies are expected to be developed to realize the production of biofuel from wood biomass. A significant effort is put into the field of modifying wood properties of trees and simplifying the process of biomass-to-ethanol conversion, which includes mainly genetic engineering of lignin, cellulose and hemicellulose of woods. Current research in this field has achieved some promising results and opened up new opportunities to utilize wood biomass efficiently. This review will discuss the main developments in genetic modification of lignin, cellulose and hemicellulose biosynthesis in trees as well as other potential genetic technology of biofuel production from wood biomass.



## Introduction

With the increasing demand for energy and growing concerns of accelerated greenhouse gas emission, great efforts have been made worldwide to develop nonfossil-based renewable energy from biological materials.[1-3] Raw materials that have been used for this purpose include mainly starch or sugar produced by food crops such as corn and sugarcane. However, using food crops as raw materials has caused problems because of agricultural land limitation. Thus, food crop-derived biofuel is considered only the first-generation biofuel. A second-generation biofuel will be made from more sustainable biological materials. Trees, which produce large amounts of lignocellulosic biomass and are able to grow on previously uncultivated land, are accounted a great potential source of biomass for biofuel production.

Tree biomass (wood) consists mainly of cellulose, hemicellulose and lignin. Cellulose is embedded in the matrix of hemicellulose and lignin. The proportion of cellulose, hemicellulose and lignin varies among plant species and cell types within a species.[4]

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Angiosperm wood, such as poplar wood, contains ~45% cellulose, ~30% hemicellulose and ~20% lignin, respectively; while the proportions in gymnosperm wood are roughly 42, 27 and 28%, respectively.[5] Lignin in gymnosperm wood consists of two phenylpropane units, *p*-hydroxyphenyl (H) unit and guaiacyl (G) unit; however, there is an additional syringyl (S) unit in angiosperm wood, which increases the extractability of lignin during conversion of angiosperm wood to ethanol.

The conversion of wood to ethanol can be divided into three main steps: pretreatment, hydrolysis of cellulose into sugars and the fermentation of sugars into ethanol.[6] The pretreatment step, which disrupts the lignocellulosic matrix to increase the accessibility of chemicals or enzymes to cellulose, is extremely important in improving the configuration and efficiency of the following hydrolysis step.[7-11] Wood with low lignin content and a high S/G ratio is easier to pretreat. In a few cases, the pretreatment step can be bypassed if low lignin biomass is used.[12] After pretreatment, cellulose is hydrolyzed chemically or by enzymes. The crystalline and noncrystalline forms of cellulose further affect the efficiency of hydrolysis.[13] The noncrystalline form of cellulose is more accessible to chemicals and enzymes and is more efficiently converted into sugars.

Research so far has shown that the production of ethanol may be significantly improved when woody feedstocks with more cellulose, less hemicellulose, less lignin or lignin with a high S/G ratio and cellulose with reduced crystallinity are used as starting materials. Great efforts are being made to produce such materials through genetic modification. This review will summarize and discuss recent progress of genetically modifying trees for second-generation biofuel production.

## Lignin Biosynthesis and Genetic Modification

Lignin is a complex biopolymer composed of H, G and S units, which are derived from three monolignols, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively.[14,15] Lignin disrupts the access of chemicals or enzymes to cellulose for hydrolysis and is one of the major barriers in biomass-to-ethanol conversion. Reducing the lignin content or changing the monolignol ratio may help overcome this barrier.

The pathway of monolignol biosynthesis in trees involves at least ten gene families which encode phenylalanine ammonia-lyase (PAL), cinamate 4-hydroxylase (C4H), 4-coumarate:CoA

ligase (4CL), *p*-hydroxycinnamoyl-CoA:D-quinate/shikimate *p*-hydroxycinnamoyltransferase (HCT), *p*-coumaroyl shikimate/quinate 3'-hydroxylase/coumarate 3-hydroxylase (C3H), caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), coniferaldehyde 5-hydroxylase/ferulate 5-hydroxylase (F5H), cinnamyl/sinapyl alcohol dehydrogenase (CAD/SAD) and caffeic acid/5-hydroxyconiferaldehyde 3-*O*-methyltransferase (COMT), respectively.[16,17] Up or downregulation of these genes for alternation of lignin content or composition has been achieved in various tree species through genetic modification using sense, antisense or RNAi approaches.

Suppression of *4CL* expression through the antisense approach has repeatedly shown significant reduction of lignin content in both angiosperm and gymnosperm trees.[18-20] The reduction can be up to 55% by a decrease in both G and S units in aspen (*Populus tremuloides* Michx) or up to 50% by the depletion in G unit in pine (*Pinus radiate*). Lignin reduction in antisense *4CL* transgenic aspen trees was accompanied with a normal or enhanced growth rate and an increased cellulose content;[18,19] while the growth of *4CL*-downregulated pine trees was dwarfed and the content of galactose, a sugar monomer in hemicellulose, was found to be doubled in these transgenic pine trees,[20] suggesting differential responses to downregulation of *4CL* expression in angiosperm and gymnosperm trees.

Downregulation of *C3H* gene using the RNAi approach in hybrid poplar trees, *P. grandidentata* x *P. alba* resulted in up to a 60% reduction of the total lignin content by dry weight of wood, accompanied with an up to a 13% increase of cell wall carbohydrates.[21] The lignin reduction is mainly due to a decrease in G unit, since the content of S unit remains constant and the content of H unit is increased in *C3H* transgenics compared with those in wild type trees. The increased carbohydrates included glucose, xylose and arabinose, suggesting both hemicellulose and cellulose contents were increased.[21] Moreover, downregulation of *C3H* led to the production of phenylglucosides which increased poplar resistance against biotic pests.[21]

Poplar (*P. tremula* x *P. alba*) harboring a sense or an antisense *CCoAOMT* transgene showed 12 or 40% lignin reduction by decreases in both G and S units.[22,23] Suppression of *CCoAOMT* also led to an increase in S/G ratio.[23,24] However, the alternation of lignin had no significant effect on plant growth and morphology.[22]

Expression of an antisense *CCR* gene in Norway spruce resulted in up to an 8% reduction in lignin content.[25] The transgenics exhibited a normal phenotype but smaller stem widths compared to control plants.[25] Downregulation of *CCR* by sense or antisense approaches in 8 year-old, field-grown transgenic poplar (*P. tremula* x *P. alba*) caused up to a 50% reduction in lignin content and a low S/G ratio resulting from a greater reduction in S than G units.[26] In addition, a reduced hemicellulose biosynthesis and increased remodeling of hemicellulose was observed in *CCR*-downregulated poplar. The reduced levels of lignin and hemicellulose were associated with an increased proportion of cellulose.[26]

F5H catalyzes hydroxylation at the 5-position on the aromatic ring of cinnamic intermediates and is a key enzyme controlling S lignin biosynthesis.[27,28] Overexpression of this enzyme is a promising strategy for improving trees for ethanol production and has been tested in various transgenics.[19,29] Overexpression of an Arabidopsis *F5H* gene under the control of cinnamate 4-hydroxylase (C4H) promoter in hybrid poplar trees (*Populus tremula* x *P. alba*) resulted in a significant increase in S lignin content.[29,30] Expressing a *F5H* transgene in quaking aspen resulted in a three-fold S/G ratio increase.[19] In quaking aspen expressing an antisense *4CL* and a sense *F5H* transgene, up to 52% lignin reduction, 64% higher S/G ratio and 30% more cellulose was found.[19]

Downregulation of *COMT* by antisense approach in a *P. tremula* x *P. alba* clone caused up to a 6-fold S/G ratio reduction due to a decrease in S lignin and an increase in G lignin, with the level of lignin content similar to that in controls.[31] Similar results were also obtained by Lapierre et al. (1999).[32] Whereas, downregulation of COMT activity by introduction of a sense homologous transgene in the same hybrid poplar species caused a 17% reduction in total lignin content with an almost complete lack of S units and the incorporation of 5-hydroxyguaiacyl (5-OH-G) units.[33]

*CAD* is involved in the last step of monolignol biosynthesis. Downregulation of *CAD* by an antisense transgene resulted in a slight lignin reduction and an increased proportion of syringaldehyde and diarylpropane structures in 2 year-old or older transgenic poplar trees (*P. tremula* x *P. alba*).[32,34] The growth indicators and interactions with insects were normal compared to that of control plants, confirming the possibility of modifying wood for biofuel production without interfering with tree growth or fitness.[32,34]



Thus, up or downregulation of genes involved in monolignol biosynthesis has been successfully employed to reduce lignin content and alter monomeric composition in trees. Though the extent of lignin content reduction and monomeric composition alteration depends on the gene manipulated, the approach used and the tree species targeted, the results certainly confirmed the promising utilization of transgenic woods as raw materials for ethanol production.

## Cellulose Biosynthesis and Genetic Modification

It is estimated that approximately 1.5 x $10^{15}$ kilograms of cellulose is produced annually, which makes it an abundant renewable biomaterial.[35,36] Cellulose is a simple and linear polymer typically having a DP of 500–14,000 glucosyl residues.[37] The glucoses are linked together through β-1→4 glycosidic bonds by condensation. In plant cell walls, 36 glucan chains are held firmly together through hydrogen bonds that are formed among hydroxyl groups of the glucose residues.[37] The aggregated chains form cable-like structural microfibrils, which are cable-like structures with high strength. In microfibrils, the glucan chains are oriented in parallel and form highly ordered, crystalline regions that are interspersed with more disordered, amorphous regions.[38] The crystalline regions are highly resistant to chemical or enzymatic hydrolysis; while the amorphous regions are more susceptible. Strategies of genetic modification of cellulose for ethanol production include enhancing cellulose biosynthesis and reducing

cellulose crystallinity. The later allow easier access of chemicals and enzymes to the glucan chains for hydrolysis.

The crystalline state and degree of crystallinity depend on the source of cellulose.[39] There are two distinct types of native crystalline cellulose: Iα and Iβ.[40,41] They differ from each other in the manner of chain packing. Type Iα has a one-chain triclinic unit cell, whereas type Iβ has a two-chain monoclinic unit cell.[42] Type Iβ is more stable and more difficult to hydrolyze than cellulose Iα.[43,44] Generally, cellulose from natural sources contains both types in varying proportions.[45,46] Higher plants such as trees and corn contain a higher proportion of type Iβ and the Iβ/Iα ratio is usually greater than four.[47] The regulatory mechanisms of cellulose crystallization in plant cell walls are poorly understood.

Cellulose is synthesized in the plasma membrane by coordination of multiple proteins.[37,48-50] These proteins are assembled into terminal complexes (TCs), which subsequently use uridine diphospho-glucose (UDP-Glc) as a substrate for glucan chain elongation.[49,51] TCs can be observed as particle arrays by freeze-fracture electron microscopy.[52] In bacteria and some algae, TCs are arranged in single or multiple rows; however, in higher plants, they are hexagonal rosette structures with six-fold symmetry.[49,50] The rosettes are assembled in the Golgi and then transported to the plasma membrane.[53] Organisms with TCs in single or multiple rows tend to synthesize cellulose with lower Iβ/Iα ratio, whereas organisms containing rosettes synthesize cellulose with higher Iβ/Iα ratio.[49]

The only identified components of rosette are cellulose synthases (CesAs), which include multiple isoforms in plants.[54,55] Generally, to form a functional rosette, three different isoforms are required.[50] These enzymes have eight transmembrane domains, two at the amino terminus and six at the carboxy terminus, which form a channel in the plasma membrane for secretion of the glucan chain. Between the two regions of transmembrane domains, there is a large central, cytoplasmic domain containing a highly conserved D,D,D,Q/RXXRW motif, which are thought to participate in the enzyme's catalytic activity.[37,49,56] In the amino terminal region of CesA, there are eight highly conserved cysteine residues in four pairs of CX2C, forming the putative LIM-like zinc-binding domain/RING finger domain.[57] This domain may mediate the interaction of CesA proteins to form homo or heterodimers under oxidative conditions.[49,50,58] The half-life of CesAs is very short in vivo (less than 30 min for GhCesA1 protein).[49,59,60]

Mutations in CesAs affect cellulose crystallization. The genetic evidence first came from the analysis of the Arabidopsis *rsw1* mutant.[57] This mutant has a single base pair change in *AtCesA1* gene, which results in substituting Val for Ala[549] in the translated protein. *rsw1* grows normally at 21°C, but exhibits swelling of roots and stunted growth at 31°C. Shoots of *rsw1* seedlings grown at the restrictive temperature (31°C) have less crystalline cellulose but more non-crystalline cellulose than wild-type seedlings. Rosettes are rare and sometimes irregular in the plasma membrane of root cells cultured at 31°C.[57] Analysis of the Arabidopsis mutant *ixr1-2*, which contains a mutation in a highly conserved consensus sequence among the C-terminal transmembrane region within *CESA3*, revealed a cellulose crystallization reduction and a saccharification efficiency improvement compared to wild type, demonstrating cellulose crystallinity can be altered by mutations in the transmembrane region of a cellulose synthase.[61]

Genes encoding plant cellulose synthases were first identified in cDNA libraries made from developing cotton fibers by sequencing random clones.[54] Characterizing Arabidopsis cellulose-deficient mutants has identified 10 *CesA* genes.[55] Among them, *AtCesA1*, *AtCesA2*, *AtCesA3*, *AtCesA5* and *AtCesA6* are required in primary wall synthesis;[50,57,62-66] while *AtCesA4*, *AtCesA7* and *AtCesA8* are believed to coordinate cellulose biosynthesis in secondary walls.[67-70] Gene expression profiling in quaking aspen, black cottonwood, eucalyptus and loblolly pine trees has identified CesAs that are preferentially expressed in developing secondary xylem.[71-82]

Although CesAs are the only identified components of the rosettes, several other proteins are also associated with cellulose biosynthesis. For example, sucrose synthase (SuSy), an enzyme catalyzing the formation of UDP-Glc from sucrose, is postulated to play a role in providing UDP-Glc to CesA for cellulose chain elongation.[83] In cotton, suppression of *SuSy* gene expression represses the initiation and elongation of cotton fiber cells and the development of seed coat fibers;[84] while overexpression of SuSy in hybrid poplar (*P. alba* x *P. grandidentata*) enhances the content of secondary cell wall cellulose without influencing plant growth.[85] Another example is the KOR protein that is a membrane-localized β-1,4-glucanase.[59,86-92] Transcripts of *KOR* gene accumulate highly in the developing secondary wall of Arabidopsis cells and cotton fibers.[93,94] Similar to *rsw1*, Arabidopsis *kor* mutants show deficiency of crystalline cellulose and accumulation of soluble linear β-1,4-glucan when seedlings are grown at restrictive temperatures.[86] Since KOR has cellulose-hydrolyzing activity,[88,95,96] it is thought that KOR may play roles in removing non-crystalline glucan chains during the assembly of glucan chains into a microfibril.[37,89] Recently, *KOR* gene has been tested as a target for genetic modification of cellulose. Overexpression of *KOR* resulted in increases of non-crystalline cellulose biosynthesis in transgenic Arabidopsis and poplar.[97,98]



## Hemicellulose Biosynthesis and Genetic Modification

Hemicellulose is the second most abundant polysaccharide in nature.[99,100] It is usually a heterogeneous branched polysaccharide and consists of a variety of sugar monomers. The degree and type of branch and monomer proportions vary among plant species and cell wall types.[16,99] In hardwood, the dominant hemicellulose is glucuronoxylan that is composed of linear chains of β-1,4-linked xylosyl residues, α-1,2-linked glucuronic acid residues and 4-*O*-methylglucuronic acid residues. The minor hemicellulose is glucomannan, which has a backbone of β-1,4-linked mannosyl residues. Softwood hemicellulose is composed mainly of galactoglucomannan and a small amount of arabinoglucuronoxylan and arabinogalactan.[100,101] Galactosyl residues are linked to glucomannan by α-1,6-bonds in galactoglucomannan; while arabinosyl residues are linked to glucuronoxylan by α-1, 3-bonds in arabinoglucuronoxylan. Arabinogalactan has a backbone of β-1, 3-linked galactoyl residues.[100,101]

The biosynthesis of hemicelluloses is a complicated bioprocess that includes glycan backbone assembly and side chain addition. A large number of genes are involved in hemicellulose biosynthesis. However, few have been identified and characterized. Known genes include some of the cellulose synthase-likes (*CSLs*) that seem to be responsible for hemicellulose backbone elongation and glycosyltransferases (GTs), such as galactosyltransferase, fucosyltransferase, α-xylosyltransferase and others, which are associated with side-chain additions.[55,102-108] Analysis of mannan formation in guar (*Cyamopsis tetragonoloba*) seeds led to the identification of β-mannan synthase (CtManS) that catalyzes the biosynthesis of β-1, 4-linked-D-mannan backbone of galactomannan.[106] This gene belongs to the *CslA* subfamily of *Csl* superfamily. Three Arabidopsis *CslA* genes, *AtCslA2*, *AtCslA7* and *AtCslA9* and two poplar genes, *PtCslA1* and *PtCslA3*, were consequently identified by homology to be associated with mannan formation.[79,108] PtCslA1 is a glucomannan synthase catalyzing the biosynthesis of β-1, 4-linked-D-glucomannan.[79] Further functional analysis of the members of CslA gene family from diverse plant species supports the hypothesis that the function of the CslA genes is conserved in all plants.[109] Additionally, a member of the rice CslF subfamily was found to be involved in the biosynthesis of β-1, 3-1,4-D-glucans, which are usually absent in dicots.[110]

Several Arabidopsis GTs have been characterized for their biochemical function in side chain additions in primary cell walls. It includes a galactosyltransferase,[111,112] two fucosyltransferases (AtFT1 and AtFUT1),[113,114] and an α-xylosyltransferase.[115] Moreover, several GTs were found to be associated with hemicellulose synthesis in secondary wall formation, though their biochemical function has not been completely elucidated. For instance, Arabidopsis FRA8/F8H, IRX8, IRX9 and PARVUS/GATL1 appear to be involved in glucuronoxylan biosynthesis;[116-123] however, due to lack of enzymatic functions, it is not clear how they are involved in the biosynthesis of glucuronoxylan. Peña and coworkers' results further suggest that IRX9 is required for normal elongation of the glucuronoxylan backbone, while IRX8 and IRX7/FRA8 play roles in the addition of residues at the reducing end.[123] Overexpression of poplar homologous in *fra8*, *irx8*, *irx9* and *parvus* plants respectively rescues the defects in secondary cell wall thickness and glucuronoxylan synthesis, suggesting that poplar GT47C, GT8D, GT43A/B and PdGATL1.1/1.2/GT8E/F are functional orthologs of FRA8/IRX7, IRX8, IRX9 and PARVUS, respectively.[117-121,124]

Hemicellulose binds to cellulose via hydrogen bonds and can be cross-linked to lignin, so the structure and content of hemicellulose are critical factors affecting biomass-to-ethanol conversion. Modifying sugar composition or changing structure of hemicellulose by genetic engineering may improve the efficiency of ethanol production. For example, suppression of poplar *PoGT47C*, an ortholog of the Arabidopsis GT47 family member involved in glucuronoxylan biosynthesis, resulted in a drastic reduction in the thickness of secondary cell walls, a deformation of vessels and a decreased amount of glucuronoxylan without causing a significant alteration in plant growth.[120,122] *PoGT47C*-suppressed transgenic wood is more easily digested by cellulases, demonstrating that the quality of wood used for ethanol production can be improved by modification of glucuronoxylan.[120] On the other hand, suppression of *AtBXL1*, a putative Arabidopsis β-xylosidase gene involved in hemicellulose biosynthesis in secondary cell walls, resulted in the alteration of hemicellulose in secondary cell walls, indicating another possible approach to modify hemicellulose.[125] Additionally, hemicellulose content can be modified by downregulation of UDP-glucuronate decarboxylase that catalyzes the production of UDP-xylose, a precursor for xylan biosynthesis.[126] The xylem cell walls of transgenic tobacco plants with downregulated UDP-glucuronate decarboxylase exhibit a reduced hemicellulose content and show an increase of cellulose extractability.[126]

## Concluding Remarks

Significant advances have been made in genetic modification of trees for improved wood quality for second-generation biofuel production. Reducing lignin content or altering monomeric composition by regulating monolignol biosynthesis genes has been successfully achieved. Modification of cellulose and hemicellulose content and composition in woods has also shown promising results, such as the increase of non-crystalline cellulose and the change of sugar composition in cell walls. These approaches have shed light on wood quality improvement for ethanol production. Moreover, recent studies on transcription factors and small RNAs indicate that they are able to regulate total biomass production or specific biomass component during plant growth and development;[127-138] however, transcription factors and small RNAs have not been used to modify wood quality and quantity for biofuel production.

The current main challenge in wood-based biofuel production is how the novel knowledge and experimental discoveries can be transferred into capable technology for large scale utilization. More has to be done. For example, various genetically modified woods have been produced; however, few studies were performed to evaluate their ethanol conversion efficiency. It is also unclear how wood compostion affects ethanol coversion quantitatively. Fitness of transgenic trees grown in the field and the influence of genetically modified trees on environments also need to be examined in more detail. Above all, transgenic woods have shown a good potential in replacing food crops for biofuel production; while more studies are expected in order to develop capable technologies of cost-competitive ethanol production from wood on a large scale.



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