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INVITED REVIEW

Genetic modification of lignin biosynthesis for improved biofuel production

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Abstract The energy in cellulosic biomass largely resides in plant cell walls. Cellulosic biomass is more difficult than starch to break down into sugars because of the presence of lignin and the complex structure of cell walls. Transgenic down-regulation of major lignin genes led to reduced lignin content, increased dry matter degradability, and improved accessibility of cellulases for cellulose degradation. This review provides background information on lignin biosynthesis and focuses on genetic manipulation of lignin genes in important monocot species as well as the dicot potential biofuel crop alfalfa. Reduction of lignin in biofuel crops by genetic engineering is likely one of the most effective ways of reducing costs associated with pretreatment and hydrolysis of cellulosic feedstocks, although some potential fitness issues should also be addressed.

Keywords Biomass · Biofuel crops · Genetic engineering · Lignin modification

Introduction

Transgenic technology has greatly contributed to breakthroughs in plant improvement and is expected to play a crucial role in coming years in genetic modification of crops for biofuel production by modifying quantity or quality of biomass (Sánchez and Cardona 2007; Gressel 2008). Global industrialization, the increase in world population, and faster economic growth, especially in developing countries, call for continuous and steady increases in the demand for energy (Li et al. 2008; Yuan et al. 2008). High fluctuation in the global oil market, decrease in oil reserves, global warming due to the emission of greenhouse effect gases, and other problems associated with the use of fossil fuels make the development of alternative sources of energy highly imperative to meet worldwide rising energy demands (Gray et al. 2006; Koonin 2006; Yuan et al. 2008).

In recent years, the exploitation of renewable and sustainable energy sources is taking center stage in science, research, media, and politics (Schubert 2006). Bioethanol, biodiesel, biomethanol, and other biofuels that can replace or can be mixed with fossil fuels are renewable energy sources (Gray et al. 2006; Ragauskas et al. 2006). To date, bioethanol production in the USA has been mainly based on the use of maize and other crops. However, high biomass producing non-grain crops like switchgrass or *Miscanthus* are being considered as a primary source of feedstocks to produce biofuels. Biofuels produced from these sources are called lignocellulosic biofuels, which represent an alternative fuel for future use (Yuan et al. 2008). These energy-rich cellulosic non-food or non-grain crops are mostly perennial grasses that can be grown in marginal lands with minimal nutrition inputs. Although cellulosic biofuel can overcome the limitations associated with starch-based ethanol production, the main obstacle is the high production cost incurred during the conversion process from the lignified plant cell walls which limits large-scale adoption of cellulosic ethanol production (Li et

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al. 2008). Other obstacles include the lack of infrastructure associated with harvest, transportation and storage of cellulosic biomass.

Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin; these are major components of the secondary cell walls of all vascular plants. Cellulose, consisting of glucose (6-carbon sugar) units linked by glycosidic bonds, is the most abundant substance on earth. Hemicellulose consists of 5-carbon sugars such as xylose or arabinose along with glucose. Hemicellulose forms complex cell wall network by cross-linking cellulose microfibrils with lignin (Rubin 2008). This complex network should be broken down for efficient biofuel production. The process of cellulosic biofuel production involves three major steps: (1) pretreatment of biomass feedstock; (2) hydrolysis and saccharification; and (3) fermentation of sugars into ethanol. After collection and processing of feedstocks, a pretreatment with acid or steam releases the polysaccharides. In the second step, the released complex polysaccharides are enzymatically converted into simple sugars by cellulase and hemicellulase enzymes. The final step converts the simple sugars into ethanol via microbial fermentation, as in the case of starch-based biofuel. However, the association of lignin with cellulose and hemicellulose has a negative impact in cellulosic ethanol production as it inhibits the release of polysaccharides during the pretreatment process and also absorbs the enzymes used for saccharification or reduces the accessibility of enzymes during the conversion process. The use of increased acidity or steam also reduces the efficiency of the saccharification and fermentation process at a later stage (Keating et al. 2006). The high cost incurred during processing is the major limiting factor in cellulosic biofuel production and makes the price of the cellulosic ethanol two- to threefold higher than starch-based ethanol (Sticklen 2006, 2008).

Although breeding plant biomass feedstock for reduced lignin content or increased biomass production will solve this problem (Bouton 2007), it will take a long time to achieve the goal. In this circumstance, modern biotechnological approaches offer great alternative opportunities to conventional plant breeding techniques to reduce the cost of cellulosic ethanol production (Gressel 2008). The genetic engineering approaches include up-regulation of cellulose and hemicellulose pathway enzymes or other enzymes involved in increasing plant biomass characteristics or production of recombinant cellulases or hemicellulases in plants (Ziegelhoffer et al. 1999; Ericksson et al. 2000; Biswas et al. 2006; Oraby et al. 2007; Ransom et al. 2007). These approaches will possibly compensate the reduced saccharification efficiency due to the presence of lignin or minimize the use of enzymes during saccharification (Sticklen 2008). A direct and effective approach is to down-regulate the enzymes involved in lignin biosynthesis to reduce lignin content or to modify its composition (Ralph et al. 2006; Chapple et al. 2007; Chen and Dixon 2007)

Lignin Biosynthesis

Lignin is a phenolic biopolymer of complex structure, synthesized by all plants. The deposition of lignin in the cell wall is considered critical for plant growth and development (Dixon et al. 2001; Rogers and Campbell 2004). The biosynthesis of lignin begins with the synthesis of cinnamic acid from the amino acid phenylalanine by phenylalanine ammonia lyase (PAL) in the cytosol. Lignin is made up of three main *p*-hydroxycinammyl alcohol precursors or monolignols, namely *p*-coumaryl, coniferyl, and sinapyl alcohols, which later undergo dehydrogenative polymerizations by peroxidase (PER) and laccase (LAC) to form *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin, respectively (Weng et al. 2008). The relative proportion of each lignin unit varies with species, plant parts, and maturity. The biosynthetic pathway to lignin has been under constant revision during the past decade, mainly as a result of genetic and transgenic studies. These studies question the *in vivo* specificities of the monolignol pathway enzymes as initially extrapolated from *in vitro* studies (Chen et al. 2006). The current view of the general pathway of lignin biosynthesis in higher plants is shown in Fig. 1 (Chen et al. 2006; Li et al. 2008). Many studies have proposed that the following enzymes are required for monolignol biosynthesis through phenylpropanoid pathway: phenylalanine ammonia lyase; cinnamate 4-hydroxylase (C4H); 4-coumarate-CoA ligase (4CL); cinnamoyl CoA reductase (CCR); hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT); coumarate 3-hydroxylase (C3H); caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H); caffeic acid 3-*O*-methyltransferase (COMT); and cinnamyl alcohol dehydrogenase (CAD). In addition, transcription factors like MYB, LIM, and NAC genes are thought to be coordinately regulating the expression of these genes for lignin biosynthesis (Rogers and Campbell 2004). The functions of many lignin genes have been well-studied in several plant species, especially in dicot plants using either mutants or transgenic plants. With the availability of information of genes involved in lignin biosynthesis and by taking advantage of developments in plant transformation technology, it is now possible to modify or reduce lignin content in biofuel crops by overexpression, down-regulation, or suppression of genes involved in either lignin synthesis, regulation, or polymerization (Li et al. 2003; Ralph et al. 2006; Chen and Dixon 2007). The extent of lignin reduction or modification depends on the kind of gene which is down-

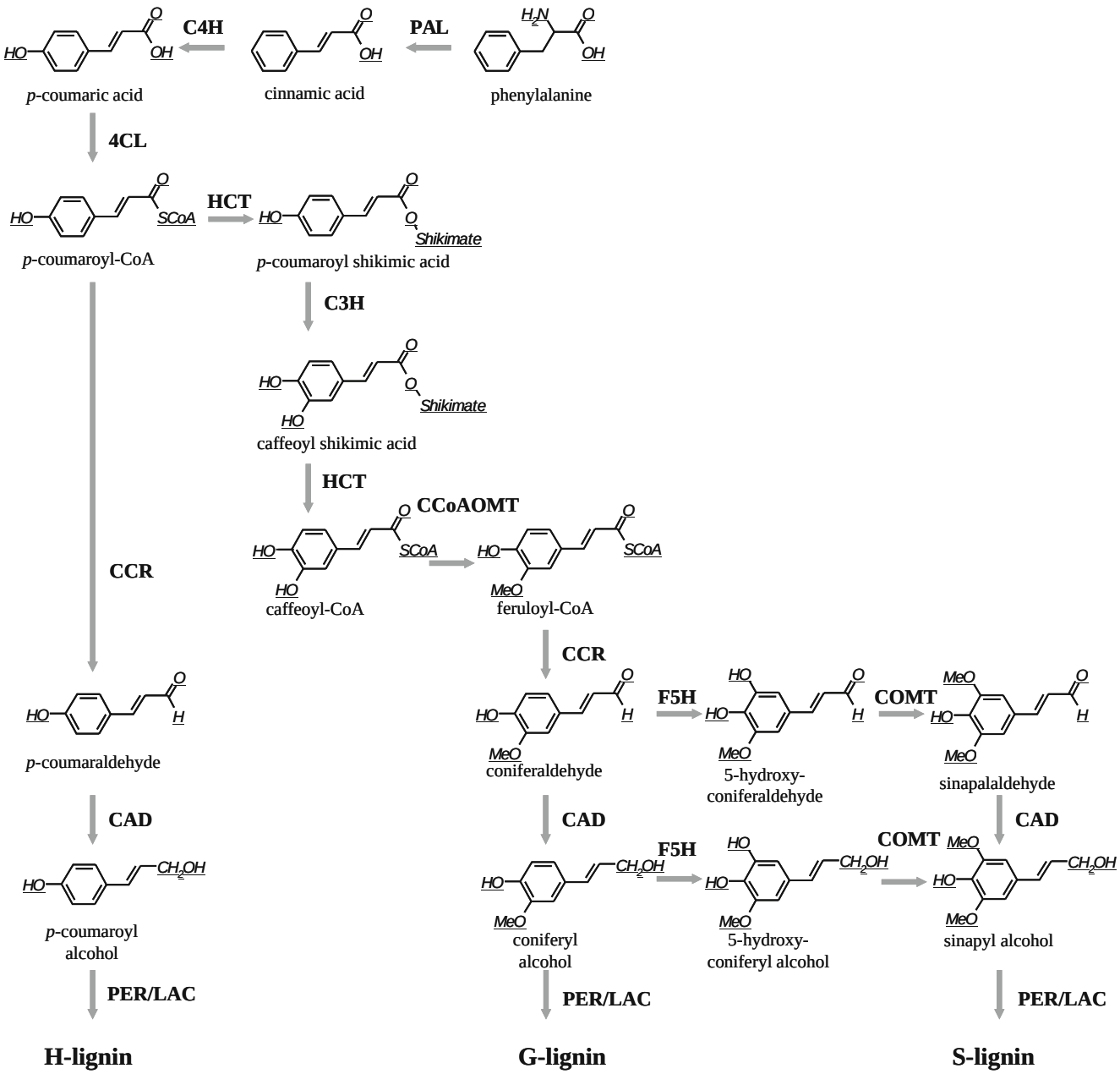

Figure 1. One of the current views of lignin biosynthetic pathway. The enzymes involved in the pathway are: phenylalanine ammonia lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate-CoA ligase (*4CL*), cinnamoyl-CoA reductase (*CCR*), hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (*HCT*), coumarate 3-hydroxylase (*C3H*), caffeoyl CoA 3-*O*-methyltransferase (*CCoAOMT*), ferulate 5-hydroxylase (*F5H*), caffeic acid 3-*O*-methyltransferase (*COMT*), cinnamyl alcohol dehydrogenase (*CAD*), peroxidase (*PER*), and laccase (*LAC*).

regulated. For example, the down-regulation of the upstream genes like C3H, HCT, or 4CL leads to reduction in lignin content, while the down-regulation of F5H and COMT resulted in changes of S/G ratio (Weng et al. 2008). Although many studies and findings were first reported in non-feedstock model plants such as tobacco and *Arabidopsis* (Zhou et al. 2009), it is assumed that similar approaches can be applied to cellulosic feedstock crops as the lignin pathway is conserved among plant species.

Plant Transformation and Gene Regulation Methods for Lignin Modification

Because plant genetic engineering plays a major role in lignin modification, availability or establishment of a well-defined, highly efficient transformation system for feedstock crops is an important prerequisite for the successful manipulation of lignin pathway genes to modify the quality or quantity of biomass (Gressel 2008). Since most of the

cellulosic feedstock crops are perennial grasses, which are considered recalcitrant for transformation procedures, the choice of transformation method will also have a great impact. To date, genetic transformation of plants has been performed by two main methods: *Agrobacterium*-mediated transformation and particle bombardment. The *Agrobacterium* method was originally used for dicotyledonous (dicot) plants such as tobacco, alfalfa, and poplar because these plants are natural hosts for *Agrobacterium*. After extensive studies, the *Agrobacterium* method has been extended to various monocotyledonous (monocot) plants including some feedstock species such as maize and switchgrass (Ishida et al. 1996; Somleva et al. 2002). The early reports on grass transformation were mainly based on particle bombardment (Conger et al. 1993; Denchev et al. 1997; Wang and Ge 2006). Considering the advantages of *Agrobacterium*-mediated transformation (lower copy number, fewer rearrangements of the transgene), it is the method of choice for transforming biofuel crops (Somleva et al. 2002).

As far as the modification of lignin genes is concerned, constant or specific "knockdown" and "overexpression" techniques are being used (Capell and Christou 2004). Initially, reduced lignin plants were identified from natural or chemically induced (e.g., ethylmethane sulfonate treatment) mutants. With the development of genetic transformation techniques, antisense, RNA interference (RNAi), and virus-induced gene silencing (VIGS) have been used to knock down or silence the target gene(s) (Lu et al. 2003; Chen et al. 2006; Chen and Dixon 2007). Antisense method is to introduce and express RNAs equivalent to an antisense strand of the mRNA of target genes. RNAi usually involves stable transformation with a gene construct that, when expressed, produces a small double-stranded RNA homologous to a portion of the target gene sequence. This is usually generated via an inverted repeat of the short target sequence interrupted by a plant intron sequence (Wesley et al. 2001). This approach has been effectively used for modifying a number of plant traits through targeted down-regulation of a specific gene or genes (Miki et al. 2005; Dixon et al. 2007). Overexpression of target genes sometimes causes cosuppression, in which the endogenous gene is silenced. Since RNAi or antisense may not totally abolish expression of the gene, the technique is sometimes referred to as a "knockdown" to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated. RNAi technology has emerged as an attractive tool to study the gene functions in plants through genetic engineering. VIGS takes advantage of an endogenous defense mechanism against viral infection and is used for high throughput tests of gene functions in plants (Lu et al. 2003). However, VIGS is a transient system which does not lead to stable integration of the transgenes.

Lignin Modification in Monocots

Because of the negative correlation between lignin and forage digestibility, lignin modification has been one of the breeding goals in grasses to improve feed quality. In recent years, several monocot species have been recognized as major candidates of biomass materials for cellulosic ethanol production; lignin modification in these species has received much attention (Stewart 2007).

Monocot mutants with reduced lignin. Maize is not only used for food; it has also been widely used as silage for animal production. Moreover, maize stover and cobs are considered important sources of biomass for cellulosic ethanol production. The brown-midrib (*bm*) mutants of maize, which differ in quality and quantity of lignin from normal genotypes, were known as pioneering models to study lignifications and digestibility (Kuc and Nelson 1964, Rook et al. 1977, Barriere et al. 2004, Guillaumie et al. 2007). It is a simple recessive trait that phenotypically produces a reddish-brown pigmentation associated with lignified tissues (Cherney et al. 1991). Lignin contents in *bm* genotypes are consistently lower than their normal counterparts. *In vitro* digestibility of *bm* genotypes has been consistently higher than normal (Cherney et al. 1991). Activities of several enzymes involved in lignification differed between *bm* and normal genotypes (Grand et al. 1985; Cherney et al. 1991; Pillonel et al. 1991). However, differences in enzyme activities were not consistent across species and genotypes, indicating that different modifications of the lignification pathway may result in a similar *bm* phenotype.

Maize *bm3* mutant is severely deficient in OMT activity, with only 10% of the activity found in normal plants (Grand et al. 1985). Two independent maize *bm3* mutations were analyzed concerning their effects on expression of *COMT* gene (Vignols et al. 1995). By sequencing the *COMT* clones obtained from the *bm3-1* and *bm3-2* maize, the *bm3-1* allele was found to arise from an insertional event producing a *COMT* mRNA altered in both size and amount, and the *bm3-2* was resulted from a deletion of part of the *COMT* gene (Vignols et al. 1995). These results demonstrated that mutations at the *COMT* gene lead to the *bm3* phenotype.

In maize *bm1* genotypes, CAD activity was significantly reduced by 60–70% in stem tissue (Halpin et al. 1998). A *CAD* cDNA was isolated and used as a probe to map the location of the *CAD* gene. The *CAD* gene is located very closely to the known location of *bm1* and co-segregates with the *bm1* locus in two independent recombinant inbred populations. The results strongly suggest that maize *bm1* directly affects expression of the *CAD* gene (Halpin et al. 1998).

Functions of *bm2* and *bm4* mutants are still unknown, although both mutants showed reduced lignin content of

15–25%, especially G lignin unit (Marita et al. 2003, Barriere et al. 2004). Guillaumie et al. (2007) showed that *bm2* and *bm4* mutations could affect regulatory genes involved in the regulation, polymerization, or transportation of coniferaldehyde in maize tissues. In addition to maize, brown-midrib mutants have been induced in other monocots such as sorghum and pearl millet that also showed a red-brown color of midribs with modified lignin composition and improved digestibility (Cherney et al. 1991, Barriere and Argiller 1993). The *bm* mutants are good candidates for studying the relationship between lignin reduction and biofuel production.

Transgenic maize with modified lignin. There were two reports on generating transgenic maize through the particle bombardment method for lignin modification. Piquemal *et al*. (2002) produced COMT down-regulated transgenic maize by the antisense approach and showed decreased lignin contents in the transgenic plants. In this case, *COMT* antisense sequence was driven by maize alcohol dehydrogenase 1 (*Adh1*) promoter which showed good expression in vascular tissues and lignifying sclerenchyma. One transgenic line carrying antisense *COMT* had only 15–30% residual COMT activity and showed similar phenotype as the maize *bm3* mutant. Further analyses revealed that several transcription factor genes, cell signaling genes, transport and detoxification genes, genes involved in cell wall carbohydrate metabolism, and genes encoding cell wall proteins were differentially expressed and mostly over-expressed in COMT-deficient plants (Guillaumie et al. 2008). A separate study obtained similar results by introducing a sorghum *O*-methyltransferase (*OMT*) antisense construct into maize (He et al. 2003). The transgenic plants showed red-brown midrib phenotype with reduced OMT activity by 60% and reduced lignin contents by an average of 17%. Digestibility was significantly improved in transgenic plants by 2% in leaves and 7% in stems (He et al. 2003). The studies demonstrated the feasibility of using transformation technology to modify lignin biosynthetic pathway and to alter the lignin profile in maize. Further research is needed to evaluate bioethanol production from maize stover with reduced lignin.

Transgenic tall fescue with modified lignin. Tall fescue, a predominant cool-season grass in the USA, has been used as a model species to study lignin deposition at defined developmental stages (Chen et al. 2002). Lignification of cell walls of tall fescue increased drastically from elongation stage to reproductive stage. The relative S lignin content and S/G ratio increased when plants matured, while relative G and H lignin content decreased during the same period. It seemed that G lignin was deposited at the early stage of plant growth, and S lignin was preferentially deposited at the later developmental stage (Chen et al. 2002). It has been noted that the aromatic composition of lignin of monocot plants is characterized by the presence of H unit (Iiyama and Lam 2001). However, H unit only comprises a small portion of total lignin when compared with S and G lignin in most monocot species (Baucher et al. 1998).

Transgenic tall fescue plants with down-regulated CAD and COMT were obtained by particle bombardment of embryogenic cell cultures (Chen et al. 2003, 2004). Transgenic *CAD* plants showed reduced lignin content and altered S/G ratio. There were no significant changes in levels of celluloses, hemicelluloses, neutral sugar composition, *p*-coumaric acid, or ferulic acid in the transgenics. The *CAD*-transgenic plants showed a significant increase in dry matter digestibility of 7.2–9.5% (Chen et al. 2004). Similarly, transgenic tall fescue down-regulated with *COMT* gene showed substantially reduced levels of *COMT* transcripts, significantly reduced COMT activity, reduced lignin content, and increased dry matter digestibility (Chen et al. 2004). These results indicated that down-regulation of lignin genes could lead to development of grass germplasm with improved forage quality and improved characteristics for bioethanol production.

Transgenic Alfalfa with Modified Lignin

Alfalfa is a perennial forage crop that has also been proposed as a feedstock for biofuel purposes. Lignin genes have been systematically down-regulated in alfalfa (Guo et al. 2001a; Reddy et al. 2005; Shadle et al. 2007). The first lignin gene down-regulated in alfalfa was *CAD* (Baucher et al. 1999). Reduction of the CAD enzyme was associated with a red coloration of the stem. Although lignin quantity remained unchanged, lignin composition was altered, and the rate of disappearance of dry matter *in situ* was increased (Baucher et al. 1999). Later, Guo et al. (2001a) produced transgenic alfalfa plants down-regulated with COMT and CCoAOMT. The cDNA sequences of these genes in sense and antisense orientations were controlled by the vascular-specific bean PAL promoter. Strong down-regulation of COMT resulted in decreased lignin content, a reduction in total guaiacyl (G) lignin units, a near total loss of syringyl (S) units in monomeric and dimeric lignin degradation products, and appearance of low levels of 5-hydroxy guaiacyl units and a novel dimer. In contrast, strong down-regulation of CCoAOMT led to reduced lignin levels, a reduction in G units without reduction in S units, and increases in β-5 linked dimers of G units (Guo et al. 2001a). Analysis of rumen digestibility of alfalfa forage revealed improved digestibility of forage from COMT down-regulated plants but a greater improvement in digestibility following down-regulation of CCoAOMT (Guo et al. 2001b).

Several cytochrome P450 enzymes, C3H, C4H, and F5H, were subsequently down-regulated in transgenic alfalfa (Reddy et al. 2005). Down-regulation of C4H, C3H, or F5H produced plants with greatly reduced lignin without significant impact on composition, lignin-rich in *p*-hydroxyphenyl (H) units, or lignin rich in G-units with reduced S content, respectively. There was a strong negative relationship between lignin content and forage digestibility, but no relationship between lignin composition and digestibility was detected in the transgenic lines (Reddy et al. 2005). Down-regulation of a recently discovered enzyme, HCT, resulted in strongly reduced lignin content and striking changes in lignin monomer composition, with predominant deposition of 4-hydroxyphenyl units in the lignin (Shadle et al. 2007). Vascular structure was impaired in the strongly down-regulated lines, and forage digestibility was increased by up to 20% (Shadle et al. 2007).

Lignin Modification and Cellulosic Ethanol Production

The relationships between lignin content/composition and chemical/enzymatic saccharification were first convincingly documented by Chen and Dixon (2007). They analyzed transgenic alfalfa down-regulated with different antisense gene constructs. Lignin content of mature stems decreased in the order: F5H and control (most lignin) > COMT and CCoAOMT > C4H, C3H, and HCT (lowest lignin level). Down-regulation of genes early in the pathway (C4H, C3H, and HCT) was most effective at reducing lignin content, in some cases leading to plants that contain less than half the lignin present in wild type. Plants with the least lignin had the highest total carbohydrate levels in untreated biomass, reflecting compensation for the reduction in lignin level on a mass balance basis. The amount of carbohydrate released by acid pretreatment increased in proportion to the reduction in lignin levels. A strong negative correlation between lignin content and sugar released by enzymatic hydrolysis was observed. Some lines showed two- to threefold greater yield of monosaccharides (the substrates for ethanol production) compared with wild-type materials (Chen and Dixon 2007).

The results from transgenic alfalfa demonstrated that genetic reduction of lignin content effectively overcame cell wall recalcitrance to bioconversion. For ethanol production, the current paradigm is that biomass must first be subjected to a costly pretreatment to make cell walls accessible to enzymes. However, several transgenic HCT and C3H alfalfa lines produced greater amounts of sugar from untreated biomass than that obtainable from pretreated biomass of control plants. Thus, it may be possible to reduce or eliminate the pretreatment step by using biomass from low-lignin transgenic plants, thereby greatly reducing the cost of biofuel production. Moreover, the simplified process without harsh chemical pretreatment allows for taking advantage of other traits, such as *in planta* expression of enzymes to increase enzymatic processing efficiency (Chen and Dixon 2007).

Conclusions

The energy in plant biomass largely resides in plant cell walls. The major obstacle for ethanol production from cellulosic feedstocks is the high cost of obtaining sugars from cell walls (Boudet et al. 2003). Because of the presence of lignin and the complex structure of cell walls, cellulosic biomass is more difficult than starch to break down into sugars. Transgenic down-regulation of major lignin genes led to reduced lignin content, increased dry matter degradability, and improved accessibility of cellulases for cellulose degradation. Wall polysaccharides of lignin-down-regulated plants were more easily hydrolyzed, and the proportion of released sugars in the transgenic material was much greater than that of the control. Thus, improvements of downstream procedures can be achieved by genetically redesigning the properties of the feedstocks. Furthermore, since lignin degradation products are known inhibitors of ethanol fermentation, reduction of lignin may help to reduce the degradation products that inhibit the fermentation process.

Although there have been many reports on down-regulation of lignin biosynthesis in dicot species (e.g. *Arabidopsis*, tobacco, alfalfa, poplar), only limited information is available in monocots (corn and tall fescue). To date, no public information on lignin modification is available in the major biofuel crops, switchgrass and *Miscanthus*. The lack of reports on successful modification of lignin in these monocot species is mainly due to the difficulties in obtaining transgenics and identifying transgenic plants having changes in lignin. The biosynthetic pathways to lignin monomers are conserved across species, and knowledge gained from dicots should be applicable to monocots. There is an urgent need to systematically characterize lignin genes in monocot species and to develop strategies to improve ethanol production for the major biofuel crops.

Reduction of lignin in the biofuel crops by genetic methods is likely one of the most effective/economic ways of reducing costs associated with pretreatment and hydrolysis of lignocellulosic feedstocks. However, some potential negative issues should also be addressed. For example, some lignin-down-regulated alfalfa lines had reduced biomass production. Although increases in fermentable sugar production could compensate for the decreases in biomass, high productivity of biofuel crops is a basic

requirement for the industry. Future studies are needed to break up the negative relationship between lignin reduction and biomass productivity. The problem seems solvable by combining lignin modification with other approaches, such as manipulation of cellulose and hemicellulose biosynthesis and deposition. It is also important to evaluate the impact of cell wall manipulation on plant structure and tolerance to biotic and abiotic stresses. The development of new cultivars with optimized biomass yield and quality will greatly benefit the biofuel industry.

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