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REVIEW & INTERPRETATION

Feedstock Crop Genetic Engineering for Alcohol Fuels

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ABSTRACT

One of the goals of the U.S. government is to have "cellulosic ethanol" produced from a variety of sources, including feedstock crop biomass (a mass of raw material used in alcohol fuels processing), because these biomass sources contain polysaccharides that can be converted into fermentable sugars. Furthermore, the feedstock biomass sources are renewable and could become available at a billion tonnes per year in the United States. There are three major steps associated with the conversion of feedstock biomass into cellulosic ethanol. The first is the production of hydrolysis enzymes such as microbial cellulases, which convert the cellulose of feedstock biomass into fermentable sugars. The second step is the pretreatment processes used to break down the recalcitrant lignocellulose complex of feedstock into more reactive intermediates and to remove the lignin residues so the cellulase enzymes can have access to cellulose. The third step is fermentation of sugars into ethanol. The first two steps are the subject of this review. Plant genetic engineering has been used to directly express heterologous versions of cellulase and hemicellulase enzymes in situ. Plants have also been genetically modified for less lignin content or for more digestible lignin. An increase in feedstock polysaccharides and an increase in overall crop biomass via crop genetic engineering have also been reported. This article reviews the advancements made in feedstock crop genetic engineering in the above areas and discusses possible near-future perspectives.

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Abbreviations: AFEX, ammonia fiber explosion; PAL, phenyl ammonia lyase.

Feedstock crops are crops that can be used in industrial processes such as fermentation into alcohol fuels. The idea of producing biofuel ethanol from feedstock crops goes back to 1925. In an interview published in the *New York Times* on 24 Sept. 1925, Henry Ford predicted: "The fuel of the future . . . is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust—almost anything. There is fuel in every bit of vegetable matter that can be fermented" (Proquest Historical News, 1925).

After the United States' gasoline shortages of the 1970s, research in conversion of crop feedstock biomass into alcohol fuels began earnest. Today, as a result of early research efforts, many microorganisms containing biomass conversion enzymes have been discovered, and several pretreatment processes have been examined to recover from energy shortages (Greene et al., 2004; Lynd et al., 2005) and reduce the accumulation of atmospheric greenhouse gases (Farrell et al., 2006; Ragauskas et al., 2006).

Presently, most ethanol produced in the United States is corn (*Zea mays*) ethanol, which is from the conversion of corn grain starch (a polysaccharide) into glucose via enzymatic hydrolysis and subsequent fermentation of glucose into ethanol. The ethanol produced from starch is more costly than ethanol produced directly from fermentation of sugarcane (*Saccharum* sp.) sugar (Dias de Oliveira et al., 2005).

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Using ethanol as transportation liquid fuel is not a new idea. Henry Ford's first car, the 1896 Quadracycle, ran on pure ethanol (http://www.ideafinder.com/history/inventors/ford.htm#STORY). And the first gas station to offer 10% corn ethanol blend opened in 1933 (Fig. 1).

Figure 1. Photo, taken in April 1933, shows a Lincoln Nebraska gas station of the Earl Coryell Co. selling "Corn Alcohol Gasoline." By permission of the Nebraska Historical Society.

To date, more than 100 corn ethanol plants are in operation in the United States, with a production capacity of more than 18.9 billion L (5 billion gal) per year. More than 100 new corn ethanol plants are currently under construction or near completion, which will add about 30 billion L (8 billion gal) to the U.S. annual capacity.

In the United States, there are plans for the construction of six commercial cellulosic ethanol plants (ethanol produced from plant lignocellulosic matter), with a total capacity of approximately 530 million L (140 million gal) per year (USDOE, 2007). These include Abengoa Bioenergy in Kansas, Alico, Inc. in Florida, Bluefire Ethanol in southern California, POET in Iowa, Iogen Biorefinery Partners in southeastern Idaho, and Range Fuels in Georgia. Michigan has also planned on constructing a cellulosic ethanol plant to convert wood chips and other forest residues into ethanol. In Canada, there is currently a small commercial biomass ethanol plant, with a few more planned to be constructed soon.

Because of recent increases in corn yields and advances in farm operations, corn ethanol technology now has a positive net energy balance (MacDonald et al., 2003; Demain et al., 2005). According to Farrell et al. (2006), corn ethanol provides about 25% more energy than it consumes during its production. The problem with production of corn ethanol is that an increase in corn consumption results in more demand and higher price of corn in general. While this results in more prosperity for farmers, it also means higher prices for all corn-dependant products such as meat and dairy products. Regardless, if all corn seeds presently produced in the United States were converted to ethanol, only 15% of the country's transportation fuels consumption would be covered (Houghton et al., 2006). Therefore, corn ethanol is not considered to offer a long-term solution to the U.S. transportation fuel needs; rather, it represents a great transitional technology (Somerville, 2006).

Cellulosic ethanol is produced from fibrous lignocellulosic biomass matter. Cellulose is a polysaccharide with linkages that impart a microcrystalline structure that is more difficult to saccharify than starch. Compared with corn ethanol, lignocellulosic biomass is much more available in the United States, about 1 billion Mg per year (Perlack et al., 2005; Somerville, 2006), and much greater amounts at the global level (Y-H.P. Zhang et al., 2006; Ragauskas et al., 2006). Rice (*Oryza sativa* L.) straw, which produces about half of the agronomic biomass worldwide (Kim and Dale, 2004), is routinely burned around the globe. This creates pollution, which causes asthma and other health problems (Sticklen, 2006; McCurdy et al., 1996; Kayaba et al., 2004; Golshan et al., 2002). In addition, unlike corn ethanol, lignocellulosic biomass is not used for human consumption. Therefore, there is no negative impact on the global food supply (Golshan et al., 2002).

Crop lignocellulosic biomass structure and composition vary depending on plant taxa, plant age, plant parts (Ding and Himmel, 2006), cell types, and individual cell wall layers. Plant lignocellulosic biomass is essentially composed of plant cell wall materials that consist mostly of crystalline cellulose embedded in a matrix of hemicellulose and pectin, which are surrounded by lignin (Bothast and Schlicher, 2005; Ding and Himmel, 2006).

Significant knowledge exists concerning the structure of cellulose. Discoveries have been reported on genes associated with cellulose biosynthesis and over all understanding of the cellulose biosynthesis pathways (Somerville, 2006; Hayashi et al., 2005; Saxena and Brown, 2005; Robert et al., 2004; Taylor et al., 2004; Doblin et al., 2003, 2002).

Plant cellulose is found in both primary and secondary cell walls in the form of microfibrils. These microfibrils are about 30 nm in diameter and composed of about 36 polysaccharide subunits. The most likely location of

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assembly of microfibrils is the Golgi. After assembly, the microfibrils are moved to the plasma membrane, where they become activated (Gibeaut and Carpita, 1993). Cellulose microfibrils are composed of linear chains of up to 15,000 unbranched glucose units. These chains stack together via extensive interchain hydrogen bonding to form microfibriles (Somerville, 2006).

Along with cellulose, hemicellulose is found in lignocellulosic matter. Hemicellulose polysaccharides are composed of xylan backbone (a polymer of β-1,4-linked xylose) found in all plant cell walls. However, unlike cellulose, hemicellulose has a random amorphous structure, and it is hydrolyzed by dilute acid as well as numerous hemicellulase enzymes. Plant hemicellulose consists of about 200 branched sugar residues. These residues include xyloglucans with a heavily substituted β-1,4-glucan, glucomanans with β-1,4-linked mannose, glucoronoarabonoxylans with β-1,4-linked xylan, and mixed-linkage glucans with glucosyl residues containing both β-1,3- and β-1,4-glycocyl linkage backbones (Carpita and McCann, 2000).

Basic research is in progress to better understand the functions of some of the genes and proteins associated with hemicellulose biosynthesis (Liepman et al., 2007; Cavalier and Keegstra, 2006). Bauer et al. (2006) and Persson et al. (2007), for example, revealed certain xylan biosynthesis pathway genes in *Arabidopsis* mutants.

The process of biosynthesis of plant cell wall polysaccharides has been elegantly illustrated, and certain proteins have been identified that play important roles in cell wall polysaccharide biosynthesis (Lerouxel et al., 2006). Certain phenolics compounds such as ferulate dehydrotrimers that are known to cross-link plant-derived polysaccharides have also been identified (Ralph et al., 2004a). These cross-linking compounds provide the plant cell wall strength but decrease the degradability of lignin by pretreatment processing and decrease the plant digestibility by livestock (Bunzel et al., 2004). In grasses, for example, ferulate dimmers and trimmers cross-link between individual polysaccharides and between lignin and polysaccharides (Schatz et al., 2006). Certain monomers substitute for monolignols in some wild-type and transgenic plants. These monomers display the same function as monolignols in their chemical radical coupling and cross-coupling. These substitutes could improve the feedstock biomass conversion processing of fermentable sugars for alcohol fuels (Ralph, 2006).

Willats et al. (2001), Ridley et al. (2001) and O'Neill et al. (2004) have reviewed the structure and function of pectins. Pectins are complex polysaccharides in the middle lamella (i.e., the layer between adjacent plant cells) in form of a mixture of homogalacturonan, rhamnoglacturonan I, and a minor amount of rhamnoglacturonan II polymers (Voragen et al., 1995). However, no research has been reported on conversion of pectins into fermentable sugars for alcohol fuels.

The polysaccharides of feedstock biomass, such as cellulose and hemicellulose, could be converted into fermentable sugars using hydrolysis enzymes. However, the lignin residues physically block the exposure cellulosic matter to hydrolysis enzymes (Zhang and Lynd, 2006; Ragauskas et al., 2006).

Three major steps are associated with biological conversion of lignocellulosic biomass into ethanol fuels. First is the production of hydrolysis enzymes (Kabel et al., 2005) such as cellulases including microbial endoglucanase (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4), exoglucanase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91) and β-glucosidase (cellobiase or β-D-glucoside glucohydrolase; EC 3.2.1.21). While β-glucosidase completes the hydrolysis by converting the cellabiose and cellu-oligosaccharides into monomeric molecules of glucose (Sternberg, 1976), the endo- and exo-glucanases act synergistically and promote the solubilization of crystalline cellulose into cellubiose (Wood et al., 1989; Bhat and Bhat, 1997). β-glucosidase also relieves the cellobiose-induced inhibition of endo- and exo-glucanases (Wood and McCrae 1982; Bhat et al., 1993). To hydrolyze the hemicellulose of the feedstock lignocellulosic matter into fermentable sugars, different groups of hemicellulases such as endo-xylanases and exo-xylanases (Collins et al., 2005) would also be needed for such conversion.

The second step is the lignocellulose pretreatment processes, which is still expensive (Eggeman and Elander, 2005). This step chemically and physically breaks down the recalcitrant feedstock lignocellulose complex into more reactive intermediates and disrupts the lignin structure so the cellulase enzymes can have access to cellulose. Presently, lignocellulosic pretreatment technologies include dilute acid, hot water flow-through, ammonia fiber explosion (AFEX), ammonia recycle percolation, steam water explosion, lime, and organosolv (Eggeman and Elander, 2005; Mosier et al., 2005; Wyman et al., 2005a,b; Pan et al., 2005). An ideal pretreatment technology must have low initial capital investment costs, low sugar degradation (McMillan, 1994) during its processes, and reasonable operating costs.

The third step, which is beyond the scope of this review, is the fermentation of sugars into cellulosic ethanol. The fermentation can also produce biobased materials such as lactic acid and succinic acid (L. Zhang et al., 2006; Ragauskas et al., 2006; Wyman et al., 2005b).

Because the first two steps are expensive for commercial cellulosic ethanol (Ragauskas et al., 2006), this article reviews the use of feedstock crop genetic engineering as a more economical alternative technology. I also review other feedstock genetic engineering approaches that might prove useful in increasing the crop biomass by prolonging the vegetative growth stage. At the end, I discuss possible future perspectives.

Production of Hydrolysis Enzymes Within Crop Biomass

Over the last few decades, technologies associated with the production of recombinant cellulase enzymes in microbes and the efficiency of producing biologically active enzymes within microbes have improved. More recently, the cost of cellulase production in microbes has been dramatically reduced (Knauf and Moniruzzaman, 2004; Ragauskas et al., 2006). Despite these advances, this technology remains economically unfeasible at commercial level.

During the last few years, biologically active heterologous thermostable endo-1,4-β-endoglucanase (E1) enzyme of *Acidothermus cellulolyticus* (Tucker et al., 1989; Baker et al., 1994) expressed in *Arabidopsis* (Ziegler et al., 2000), potato (*Solanum tuberosum* L.) (Dai et al., 2000) and tobacco (*Nicotiana* sp.) (Ziegelhoffer et al., 2001) plants. This enzyme was produced in plants to use the free energy of sun via photosynthesis. At the time, however, there was concern that the harsh pretreatment conditions might damage the biological activity of plant-produced heterologous E1 enzyme. In other words, it was not known whether transgenic feedstock could be directly put into pretreatment processes while performing enzymatic hydrolysis. To investigate this question, the biological activity of heterologous E1 was assayed after AFEX, which is a relatively mild pretreatment. The results of this investigation demonstrated that about two-thirds of the activity of the heterologous E1 were lost due to the AFEX pretreatment (Teymouri et al., 2004). Therefore, in follow-up studies, the E1 enzyme was genetically expressed in corn (Biswas et al., 2006; Fig. 2) and rice (Oraby et al., 2007), which are both emblematic biomass crops. Plant total soluble proteins including the E1 were then extracted from the dry transgenic biomass and added to AFEX-pretreated lignocellulosic matter for enzymatic hydrolysis. The E1 expressed in corn and rice successfully converted the AFEX-pretreated corn stover and rice straw into glucose (Ransom et al., 2007; Oraby et al., 2007). In the transgenic corn and rice, E1, with addition of β-glucosidase (Novozyme 188, St. Louis, MO), successfully converted 30% of corn stover and rice straw into glucose, whereas the commercially available mix enzymes (Genencor commercial Spezyme CP microbial cellulase) and β-glucosidase converted about 90% of the crop biomass into glucose (unpublished results; see Fig. 3 and 4). Β-glucosidase was added to complete the hydrolysis and to relieve the cellabiose-induced inhibition of endo- and exo-glucanases (Bhat et al., 1993). These experiments demonstrate that E1 produced in feedstock biomass crops is a viable alternative to commercially available enzymes. The results shown in Fig. 3 and 4 are comparable to those recently published by the author's team (Oraby et al., 2007; Ransom et al., 2007).

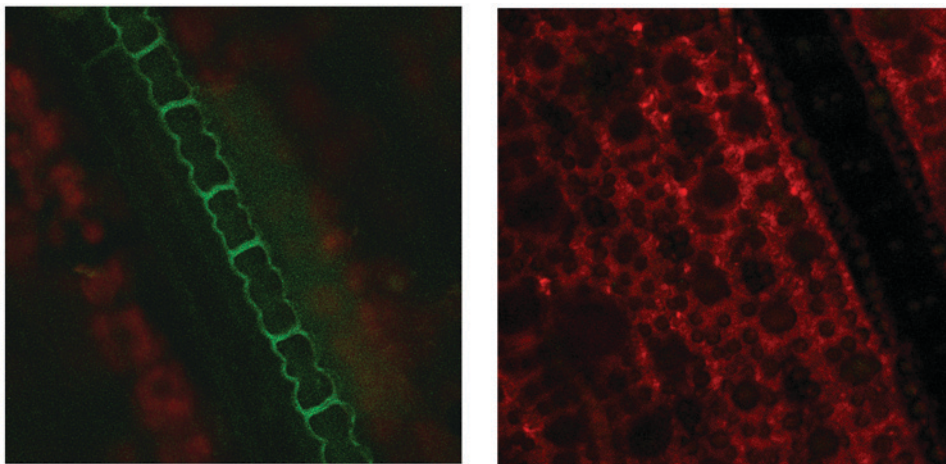

Figure 2. Immunofluoresence confocal microscopy to confirm the localization of the hetrologous *A. cellulolyticus* endo-1,4-β-endoglucanase E1 enzyme in transgenic maize leaf (left) compared with that of untransformed maize leaf (right). Both transgenic and the control leaves were treated with the E1 monoclonal primary antibody and the fluorescein isothiocyanate (FITC) anti-mouse secondary antibody. Green areas around cells of transgenic maize sample (left) indicate the apparent accumulation of E1 in apoplast. By permission of Current Opinion in Biotechnology (Sticklen, 2006).

Production of the single thermostable E1 in rice and corn (Oraby et al., 2007; Biswas et al., 2006) had no apparent harm to the plants' normal growth and development. This is probably because this specific thermophilic E1 enzyme is not active under plant temperature in vivo, plant cellulose is mostly in crystalline form, and the plant cell wall cellulose is covered by a matrix of hemicellulose and lignin, which protects against cell wall damage.

It is not clear, however, that heterologous E1 did not damage the plant cell wall. A comprehensive study reported by the Biotechnology Group from the Danish Institute of Agricultural Sciences (Sorensen et al., 2000) demonstrated that the tuber pectin organization in transgenic potato (*S. tuberosum* L. cv. Posmo) was disturbed by the expression of a fungal endo-galactanase gene regulated by a tuber-specific promoter. Similar to the above study of transgenic rice, transgenic potato plants had no apparent abnormalities in growth and development. The disturbance of transgenic plant cell wall pectin was evidenced by Fourier transform infrared microspectroscopy, immune-gold labeling, sugar analysis, and the isolation of rhmnogalacturonan I fragments compared with the wild-type nontransgenic potato tubers. This group also reported that the expression of endo-a-1,5-arabinanase protein targeted into the potato Golgi compartment interfered with the rhamnogalacturonan in Golgi vesicles. In these transgenic plants, arabinose content of the cell wall was reduced by 70% (Skjot et al., 2002). Therefore, the results for transgenic potato tuber suggest that the E1

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produced in rice and corn may have damage that was not readily apparent, and further investigations are necessary.

Production of biologically active β-glucosidases in tobacco have been reported (Reggi et al., 2005; Kiran et al., 2006). However, these studies did not investigate cell wall deconstruction. Further research is needed to see whether β-glucosidases expressed in plants can convert cellubiose into glucose.

Microbial xylanases have been produced, in their biologically active forms, specifically in the endosperm of barley (*Hordeum vulgare*) grain (Patel et al., 2000) and constitutively in tobacco (Herbers et al., 1995; Kimura et al., 2003a), rice (Kimura et al., 2003b), and potato (Yang et al., 2007). However, no reports are available on the use of these heterologous xylanases for the hydrolysis of feedstock hemicellulose.

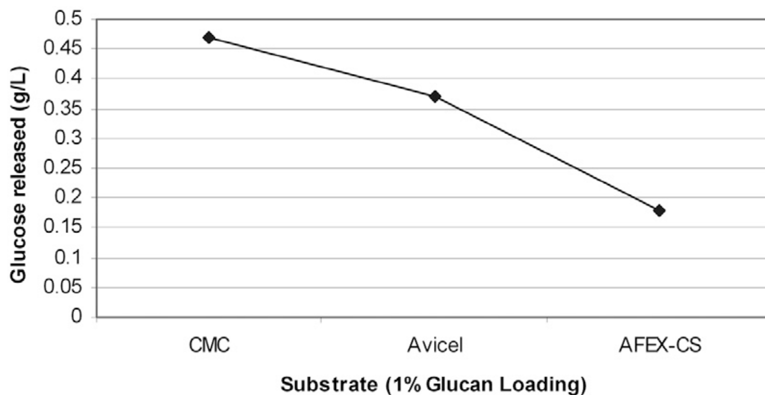

Figure 3. Production of glucose from conversion of substrates including carboxymethyl cellulose (CMC), Avicel ,and ammonia fiber explosion–treated corn stover (AFEX-CS) using the transgenic corn-produced heterologous *A. cellulolyticus* endo-1,4-β-endoglucanase E1 enzyme. The enzymatic hydrolysis was conducted for a period of 72 h, at 50°C with 90 rpm shaking (unpublished data). These results are similar to those published in Ransom et al. (2007).

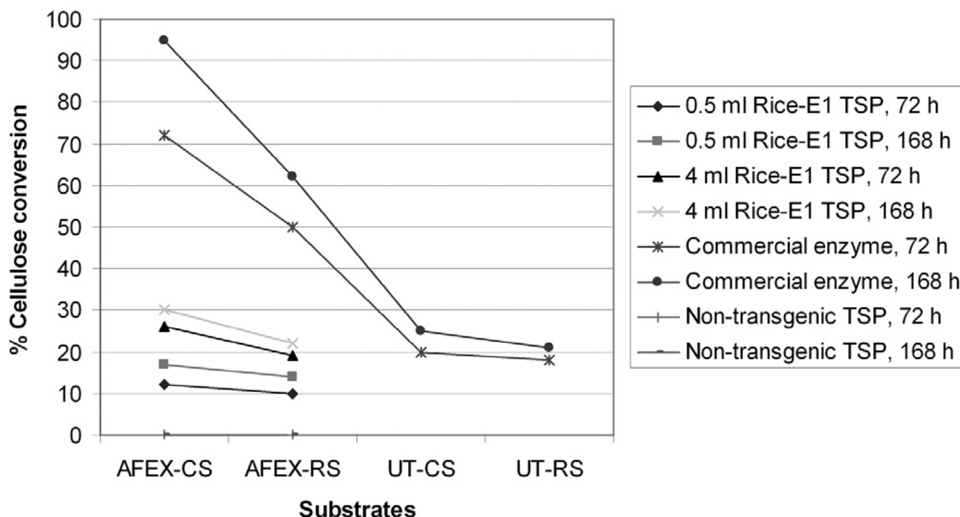

Figure 4. Comparison of percentage of feedstock crop cellulose converted into glucose through conversion of ammonia fiber explosion (AFEX)-treated and untreated (UT) corn stover (CS) and rice straw (RS) cellulose hydrolyzed using 4 mL of transgenic rice soluble proteins containing 4.9% rice E1 heterologous enzyme compared with 15 filter paper units (FPU) of Genencor commercial Spezyme CP microbial endoglucanase and exoglucanase mix (unpublished data). Novozymes commercial microbial β-glucosidase (6.5 mg 15 mL^{-1}) was added to both the rice E1 heterologous enzyme and to the commercial mix enzymes to inhibit cellubiose inhibition. These results are similar to those published in Oraby et al. (2007).

Lignin Manipulations Via Feedstock Crop Genetic Engineering

It is believed that reduction in feedstock lignin or modification of lignin structure may reduce the needs for pretreatment processes (Ragauskas et al., 2006). Lignin, the second most abundant polymer (cellulose being the first) on earth has a biosynthesis pathway (Fig. 5) that can be readily manipulated. Cell wall structure has been studied (Carpita and McCann, 2000), and an excellent recent review article (Boerjan et al., 2003) and a book have discussed lignin content, structure variations, functions, and lignification (Hayashi, 2006; Ralph, 2006). Strategies have also been considered on how to manipulate the lignin biosynthesis pathway for different purposes such as an increase in feedstock digestibility and bleaching (Boudet, 2000; Dean, 2005; Ralph, 2006).

By definition, lignin is a complex mixture of phenylpropanoid polymers that are attached together by radical coupling (Ralph et al., 2004b) derived from three hydroxycinnamyl alcohol monolignols, including para-coumaryl, coniferyl, and sinapyl alcohols (Fig. 5). Each of these residues results from separate but interconnected biosynthesis pathways. Manipulation of each of the lignin biosynthesis pathways is expected to modify plant lignin. Lignin biosynthesis pathways are also linked to other functional and defense responsibilities such as those associated with protecting plants from pathogens and insects (Ragauskas et al., 2006).

Jung and Ni (1998) studied the downregulation of lignin in alfalfa (*Medicago sativa*) to improve digestibility of this crop by rumen. Other examples of lignin downregulation were modification of the transgenic tobacco cell wall lignin structure via the use of homologous antisense technology (Blaschke et al., 2004) and the effect of downregulation of 4-hydroxycinnamate 3-hydroxylase or C3H (Fig. 5) on lignin structure. Downregulation of C3H predictably increased the proportion of para-hydroxyphenyl units relative to the normally dominant guaiacyl/syringyl ratio (Ralph et al., 2006). Furthermore, the downregulation of hydroxycinnamoyl-CoA:NADPH oxidoreductase or CCR (Fig. 5) in poplar (*Populus*)

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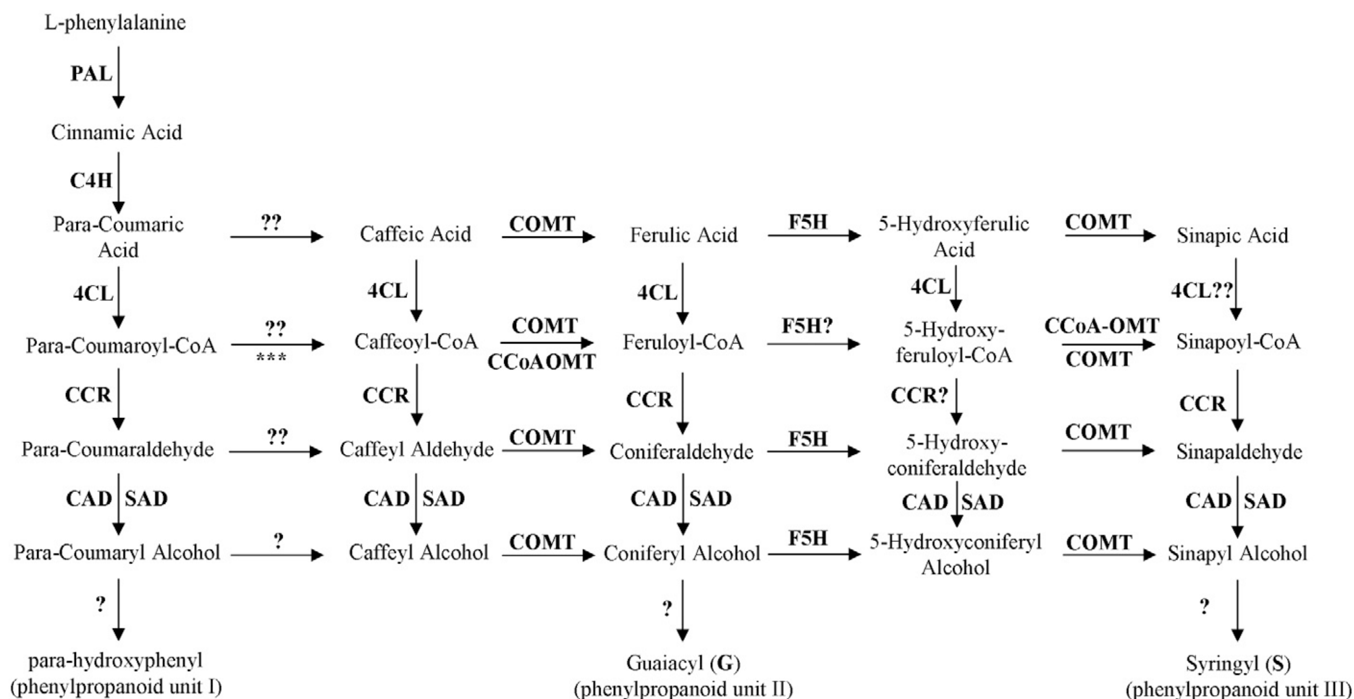

Figure 5. Lignin biosynthesis pathway. PAL, phenyl ammonia lyase; C4H, cinnamate 4-hydroxylase; C3H, para-coumarate 3-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; 4CL, 4-coumarate:CoA ligase; 4CL??, certain species have 4CL activity toward sinapic acid; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase; HCT, para-hydroxycinnamoyl-CoA:quinate shikimate para-hydroxycinnamoyltransferase; CCR? And F5H?, enzymes whose substrates have not been tested; F5H: ferulate 5-hydroxylase; ?, conversion has been demonstrated; ??, direct conversion not convincingly been demonstrated. ***, enzymatic assays in *Arabidopsis* have shown that the shikimate and quinate esters of para-coumaric acid are the ideal substrates for para-coumarate 3-hydroxylase (C3H). This means that in *Arabidopsis*, 4CL first converts the para-coumarate to para-coumaroyl-CoA, and then the C3H converts the para-coumaroyl-shikimate and para-coumaryoyl-quinate. This figure is a less-comprehensive summary of a 2003 report (Boerjan et al., 2003) and the 2002 redrawing of lignin biosynthesis pathway (Humphreys and Chapple, 2002).

resulted in more digestible cellulose by *Clostridium cellulolyticum* and twice the fermentable sugar production (Dean, 2005, p. 4–26).

Plant lignin concentration has also been reduced via genetic engineering. Downregulation of hydroxycinnamate-CoA/5-hydroxyferuloyl-Co-A- ligase or 4CL (Fig. 5) in transgenic quaking aspen (*Populus tremuloides*), for example, resulted in a 45% decrease in lignin with a concomitant 15% increase in cellulose (Hu et al., 1999)· It is believed that such compensation occurred because the quantitative or qualitative changes of one cell wall component often results in alteration of other cell wall components (Boudet et al., 2003).

The downregulation of cinnamyl alcohol dehydrogenase in poplar has caused an increase in less-conventional syringyl units and β-*O*-4-bonds, and more free phenolics groups (Lapierre et al., 2004). The downregulation of phenyl ammonia lyase or PAL (Fig. 5), which is the master enzyme responsible for the downstream regulation of the whole lignin biosynthesis flux, could depend on the level of PAL suppression (Elkind et al., 1990; Bate et al., 1994). It is also believed that lignin downregulation could be further amplified by multiple gene cotransformations (Ragauskas et al., 2006).

Success in research on reducing lignin content and/or modifications of lignin configuration has recently shown very fast progress. This is a result of the recent advancements in technologies associated with multidimensional nuclear magnetic resonance, pyrolysis–mass spectrometry, and UV microspectrometry, which have allowed the observation of the subcellular lignin structural changes at an extremely high resolution (Rogers and Campbell, 2004; Ralph and Bunzel, 2005; L. Zhang et al., 2006).

Despite all the research on lignin biosynthesis pathway enzymes, several questions associated with the pathway remain. As lignin deposition is both complex and highly variable even within a single plant cell, more basic research is needed to further understand the genetic basis for lignin biosynthesis, regulation of genes associated with the pathway, lignin deposition, and overall coordination (Dean, 2005).

Other Feedstock Crop Genetic Engineering Approaches to Alcohol Fuels

Understanding plant cellulose biosynthesis has long been considered important, and basic research in this area is underway in different laboratories (e.g., Kawagoe and Delmer, 1997; Arioli et al., 1998; Balwell, 2000; Persson et al., 2005; Haigler, 2006; Andersson-Gunneras et al., 2006).

A promising area of research for possible increase in crop biomass is to delay the feedstock crop flowering time. Several reports indicate that the switch from vegetative to reproductive growth (flowering) is a key developmental change in the plant life cycle. This switch is controlled by both environmental and developmental signals (Reeves and Coupland, 2000; Simpson and Dean, 2002; Jang et al., 2003; Henderson and Dean, 2004). The regulation of this switch and genes associated with the mechanism of the switch have been studied (Sheldon et al., 1999; Araki, 2001). A single floral repressor gene, *FLOWERING LOCUS C* (*FLC*), was identified in *Arabidopsis* (Michaels and Amasino, 2000). Several genes act to promote the expression of the *FLC* gene, which is known to delay flowering by suppressing a group of floral promotion genes called *floral pathway integrators* (Scortecci et al., 2001). Plants overexpressing the *FLC* gene prolong their vegetative growth phase unless they are exposed to vernalization (Michaels and Amasino, 2000; Sheldon et al., 1999).

Because delay in flowering time results in prolonged vegetative growth, it was conceptually predicted that *FLC*-transgenic plants would produce higher vegetative biomass yields (Sheldon et al., 1999). This hypothesis was recently proven in the author's laboratory in a late-flowering tobacco, confirming that expression of the single *Arabidopsis FLC* gene that delayed flowering by three week significantly increased transgenic plant biomass at the greenhouse level (Salehi et al., 2005).

An increase in overall crop biomass may occur via the regulation of plant growth regulators. Increased gibberellin biosynthesis in transgenic hybrid poplar, for example, promoted plant growth and biomass (Eriksson et al., 2000).

As new biomass crops such as switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus* × *giganteus*), and other perennial grasses are considered for use in production of cellulosic ethanol. New lines of studies in these crops will become important in the near future. For example, the correlation between the photosynthetic rate (Richards, 2000) and an increase in atmospheric CO_2 concentration increased the overall plant biomass (Maroco et al., 1999). Other factors such as plant nutrients, oxygen, water, respiration, circadian clock (Dodd et al., 2005), and the capacity of C_4 plants to store more carbon (Maroco et al., 1999) must also be taken into research considerations for these crops. In addition, how these plant genotypes influence carbon sinks and the ability to acquire sufficient nitrogen and other resources (Sinclair et al., 2004) are all important physiological studies to be considered. At present, there are no reports on genetic modification of plants in any of these areas.

CONCLUSIONS AND FUTURE PERSPECTIVES

Successful production of biologically active *A. cellulolyticus* E1 endo-1,4-β-glucanase in different crop species and the capability of this plant-produced enzyme to help the conversion of feedstock cellulose into glucose (Fig. 2 and 3) are most encouraging. This could be an excellent start for production of a battery of all the different hydrolysis enzymes targeted for storage in different subcellular compartments (e.g., apoplast, chloroplast, mitochondria, endoplasmic reticulum) of the same feedstock biomass. Multitargeting enzymes in cell compartments could potentially generate high levels of enzymes yield.

Theoretically, plant-produced hydrolysis enzymes must be cheaper than the same produced in microbes. The ideal scenario would be to produce designer biomass crops that express their own cell wall hydrolysis enzymes and have less lignin or more easily deconstructable lignin residues (Sticklen, 2006). This may be as realistic as producing single designer microbes that secrete all of the necessary hydrolysis enzymes and also utilize all sugars in an "integrated bioprocessing" for fermentation (Lynd et al., 2005).

Plants are known to be used as "green bioreactors" for the production of large amounts of biomolecules such as essential enzymes, carbohydrates, lipids (Horn et al., 2004; Breithaupt, 2004; Bailey et al., 2004; Cai et al., 2006; Fischer et al., 2004; Qi et al., 2004; Liu et al., 2005; Chiang et al., 2005), polymers such as polyhydroxybutyrate (Bohmert et al., 2002; Saruul et al., 2002; Zhong et al., 2003), and especially higher-value compounds such as pharmaceuticals (Howard and Hood, 2005). The level of production of such compounds could be drastically increased using approaches such as boosting of transcription level, direct transcription in tissue suited for protein accumulation, transcript stabilization, translation optimization (Streatfield, 2007), and subcellular targeting (Sticklen, 2006).

To date, reports on lignin pathway enzymes have concentrated on improving the pulping industry or livestock feed digestibility. Decrease in feedstock lignin content and especially genetic alteration of lignin for a less-expensive lignin deconstruction could well decrease the costs of biomass pretreatment processes and reduce the needs for environmentally undesired chemicals presently used in pretreatment processes.

Basic research such as advancement in plant lignin transcript profiling (Ehlting et al., 2005) would certainly enhance the lignin modifications to improve cellulosic fuel technology in the near future.

Understanding the plant cell walls may require a system-based approach to integrating biophysical, developmental, and genetic information into a useful and functional model (Somerville et al., 2004). One aspect of future research may concentrate on how to modify the lignin content and lignin

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chemistry without interfering with defense against invading pathogens and insects.

Studies of carbon sequestration must also be considered as we move toward the long-term use of crop feedstock for alcohol fuels. In addition, looking at the overall alcohol biofuels picture, problems associated with ethanol fuel technology include distillation costs, since ethanol is highly hydrophilic; transportation costs, because it cannot be transported through pipelines; and ethanol toxicity to fermentation microbes. Some would argue that butanol fuel may be a better option because, despite its few drawbacks, it is much less hydrophilic and can partition out of the aqueous phase (Somerville, 2006). Further research comparing the economic feasibility of ethanol versus butanol will determine the best course for the biofuel industry.

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