Review

Biofpr Biofuels Bioproducts & Biorefining

Expediting the biofuels agenda via genetic manipulations of cellulosic bioenergy crops

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Received January 30, 2009; revised version received March 16, 2009; accepted April 8, 2009
Published online May 22, 2009 in Wiley InterScience (www.interscience.wiley.com); DOI: 10.1002/bbb.157;
***Biofuels, Bioprod. Bioref.* 3:448–455 (2009)**

Abstract: Cellulosic bioenergy crops are crops whose cellulosic matter could be converted into biofuels. The petroleum industry makes its profit not only from petro fuels, but also from petroleum-derivative coproducts, such as lubricants and aromatic petrochemicals, that are used for production of other hydrocarbon compounds. This review explains how using the petroleum industry model in cellulosic biofuels industry could sharply increase profitability via production of high-value, low-volume recombinant coproducts, such as biopharmaceuticals, and high-value industrial chemicals in cellulosic bioenergy crops. The two major expenses associated with the production of cellulosic biofuels include the costs of pre-treatment processes and the costs of microbial cellulases. This review summarizes the role of biomass crop genetic manipulations to reduce these costs. It also describes the challenges that farmers will soon face because of the monoculture of new bioenergy crops, such as perennial grasses, and how genetic manipulations of these crops could overcome such challenges. Finally, this review addresses concerns about the effect of cellulosic biomass removal from soil on the soil carbon reserve and the role that crop transgenic technology will play to assure sufficient carbon sequestration in soil. It also addresses the role that modern crop genetic engineering technology plays in avoiding the presence of herterologous coproducts in the food chain to overcome consumer concerns.

Keywords: bioenergy crop; plant genetic engineering; biofuel; lignin down regulation; cellulase; industrial enzymes; biopharmaceuticals

Coproduction of high-value, low-volume products in bioenergy crops

The petroleum industry makes its profits not only from petro fuels (gasoline or petrol, petrodiesel, ethane, kerosene, liquefied petroleum gas and natural gas), but also from petroleum-derivative coproducts such as alkenes (olefins), lubricants, wax, sulfuric acid, bulk tar, asphalt, the solid fuel called petroleum coke, paraffin wax, and aromatic petrochemicals that are used for production of other hydrocarbon chemicals (http://en.wikipedia.org/wiki/Petroleum). The United States is in the process of building six feedstock crop cellulosic biomass conversion plants for biofuels. An easy way to make the cellulosic biofuels industry profitable is

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to first genetically modify biomass crops that use the freely available energy from the sun for the production of high-value, low-volume products, such as cellulases, along with major biopharmaceuticals and industrial products. Then, extract such high-value matter before converting the cellulosic biomass into fermentable sugars for alcohol fuels.[1]

It was accepted over a decade ago that transgenic plants could be used as hosts for efficient expression systems for large-scale production of biologically active recombinant proteins,[2] and plants could be used as biofactories for the production of molecules that are presently expensively produced in microbes.[1,3,4] Indeed, plants have already been used successfully for molecular farming and biopharmaceuticals,[5,6] industrial enzymes,[7,8] and specific carbohydrates.[9]

In the twenty-first century, when many aspects of gene expression are understood and the phenomena of protein folding and the need for molecular chaperons including folding catalysts and quality control molecules are mostly known, with the exception of a few 'difficult to express proteins', most proteins can be successfully produced with their own natural biological activities in plant cells or in whole plants. For example, transgenic plants have been developed that produce high-value proteins[10] including antibodies,[11] biodegradable plastic[12,13] and industrial enzymes, including cellulases.[1,3,4]

Among high-value, low-volume proteins is the human saliva leukocyte protease inhibitor (SLPI). The human saliva SLPI is known as the reason that HIV is not transmitted via saliva.[14] Due to its antimicrobial activities and the difficulties associated with its protein folding, this high-value, low-volume protein cannot be efficiently and sufficiently produced in *E. coli* for preclinical testing. As such, R&D Systems produces human SLPI in *E. coli* and sells it at $245/100 micrograms (http://www.rndsystems.com). Despite challenges which might be associated with its production in cellulosic bioenergy crops, this extremely valuable protein could be produced in plants in large quantities to meet its preclinical testing requirement of 20 grams (communication with Dr Andrew Badley, Director, HIV Research Center, Mayo Clinic, Rochester, MN) and for curing HIV as the most important infectious disease. Studies of the production of human saliva SLPI in plants is in progress in the author's laboratory (Park *et al.,* unpublished). One can only imagine the profitability that coproduction of SLPI in cellulosic bioenergy crops can bring to the cellulosic biofuels industry.

A set of less valuable, but very useful enzymes that could be easily produced in bioenergy crops is a combination of cellulases and hemicellulases. A recent review[3] summarizes the research performed in this area. Microbial cellulases are already produced in biomass of maize[15,16,17] and rice straw,[18] and such cellulase could convert maize stover and rice straw into fermentable sugars for biofuels.[16,18] However, recombinant cellulases lose most of their biological activities during pre-treatment processes presently available.[19] Therefore, the crude or purified recombinant cellulases produced within cellulosic bioenergy crops must be isolated, lignocellulosic biomass pre-treated, and then such plant-produced recombinant cellulases added to the pre-treated matter for production of fermentable sugars for biofuels in the cellulosic biorefinery. An ideal scenario would also be the development and use of specific pre-treatment processes that would not destroy the biological activities of recombinant enzymes and other recombinant coproducts in plants.

Higher transgene copy numbers usually cause the silencing of transgene expression. Because the gene gun device is mostly used to genetically transform cereals and perennial grasses and such method causes the integration of a high number of transgenes in plants, many independent transgenic lines must be developed and those with the least transgene copy numbers and high expressions should be selected for ideal cellulase production in crops. Alternatively, the recombinant gene expression cassettes could be flanked with DNA containing the matrix attachment region (MAR) sequence to lower the transgene copy number and to increase the transgene expression.[20]

When the use of transgenic cellulosic bioenergy crops that produce high-value enzymes and polymers becomes common practice, obviously the more recombinant molecules produced in these crops, the higher the profitability. To increase the level of recombinant enzyme and other proteins in plants, over-expression of certain molecular chaperons along with genetic engineering of high-value proteins in plants could become useful. For example, certain proteins, including cellulases, contain disulfide bonds. Disulfide bonds form, but also break constantly to recycle the amino acids. The Disulfide Isomerase (PDI) molecular chaperon

catalyzes the disulfide bond formation of disulfide bond proteins.[21] It is believed that the presence of PDI in protein folding is so important that it constitutes about 2% of the total ER protein.[22,23] PDI is involved in the correct disulfide bonds-protein folding,[24] and therefore co-transfer of PDI along with genes that encode for disulfide bond proteins can increase such herterologous protein production and activity.

Another important molecular chaperon is the Luminal Binding Protein (BiP). BiP plays an important role in protein quality control[25] by blocking the entry of misfolded proteins and also by helping with the degradation of misfolded proteins. Therefore, the over-expression of BiP in crops can increase the production of recombinant proteins. BiP chaperons are also known to play the role of the quality controllers of newly synthesized recombinant proteins.[26,27] For example, studies have shown that the over-expression of BiP has improved the transcription of recombinant immunoglobulin (IgG) antibody in transgenic tobacco.[28] In fact, the over-expression of both BiP and PDI can increase protein production and protein folding.[29] Therefore, production of high-value recombinant molecules in cellulosic bioenergy crops might need over-expression of chaperons, such as PDI and/or BiP proteins.

Decreasing the costs of pre-treatment processes via lignin genetic manipulation

Plant cell walls are mostly composed of cellulose, hemicellulose and lignin polymers. Cellulose and hemicellulose are sugar-containing molecules and therefore valuable and worth improving in cellulosic bioenergy crops. Lignin is the vascular plant cell wall phenolic polymer mostly within plant sclerenchyma and vascular elements, providing pleiotropic positive effect on structural integrity and physical strength to higher plants to avoid lodging. As the component of vascular elements, lignin also assists in the transport of water and solutes via lowering of cell wall permeability[30] causing positive impact on water conductivity with hydrophobicity required for the transport of water and solutes,[31] and for providing chemical defense against plant pathogens and insects.[3] At the same time, lignin is well known for its 'negative impact' on the manufacturing of paper as this component of the plant cell wall must be removed in paper manufacturing to allow quality paper production. It is also well known for its 'negative impact' on the quality of forage crops because cellulosic matter of silage is imbedded with cross-linking bonds[32] in a matrix of lignin[4] and therefore rumen cellulases can not easily accessed and digest the cell wall cellulose.[33] Lignin also adsorbs cellulase enzymes, and certain degraded products of lignin blocks fermentation of the fermentable sugars to alcohol fuels.[34]

Lignin pathway is not yet well understood.[3] A recent book describes the most advances made in lignin research[35] and a few recent review articles explain lignin genetic manipulations performed to date in order to improve production of fermentable sugars for biofuels.[3,31,33]

Amongst other values, maize is important as a feed crop. The importance of four naturally existing mutated maize brown midrib (bm) on their increased digestibility by rumen have been known for decades.[36,37,38,39] The mutated genes of these bm maize are known to receive and follow the normal Mendelian inheritance.[39,40]

Maize bm breeding, microscopy and molecular analyses have contributed toward more understanding of the phenomenon of down-regulation mutations of certain lignin biosynthesis enzymes in this major crop.[41,42] The mutated genes in bm 2 and bm 4 are not yet known. However, the mutated gene in bm 1 maize is known to be cinnamyl alcohol dehydrogenase or CAD;[43] and the mutated gene in bm 3 is known to be caffeic acid O-methyltransferase or COMT.[44,45] The naturally existing bm 3 maize is similar to the developed COMT down-regulated transgenic maize plant[46]. To further understand the midrib mutations, sorghum and pearl millet bm mutants (bmr) have been generated via chemical mutagenesis[47] where the bmr mutants also showed better digestibility by rumen.[47,48]

Two cinnamoyl-CoA reductase (CCR) cDNAs have been isolated from maize and characterized.[49] The author's team recently down regulated the transcription of CCR in maize via the RNAi transgenic technology, and self-bred transgenic plants creating a new homozygous recombinant bm maize crop (Fig. 1) that has low CCR transcription along with normal growth and development.[50] This novel homozygous transgenic bm maize shows a decrease in lignin content with a concomitant increase in cellulose similar to the results shown in CCR down regulated quaking aspen (*Populus sp*) trees.[51]

Figure 1. Presence of reddish-brown midrib, internode area on the stem, and husk covering the seeds in CCR down-regulated maize plant. Wildtype: Control non transgenic; CCR_RNAi: CCR down-regulated transgenic maize.

CCR converts CoA-ester to aldehyde in monolignols biosynthesis pathways. In wheat, CCR is mostly expressed in stem, where it is progressively increased as the plant matures correlating with higher Klason lignin contents, and improving the structural strength of the stem.[30]

Most of the lignin down-regulation studies have been performed on dicot herbaceous crops, such as forage crops, due to their use as animal feed. Although initially performed for digestibility research, studies of different lignin biosynthesis enzymes down regulation in alfalfa and their use in acid pre-treatment research was a classical study showing possible reduction of needs for pre-treatment processes of lignin down-regulated crops.[52] Similar studies need to be applied to major cellulosic energy crops (i.e., corn, wheat, switchgrass, miscanthus, etc.) because these crops have a different lignifications mechanism than dicot crops[33] such as alfalfa.

Most lignin down-regulation studies have manipulated genes associated with the biosynthesis of one or more of the classical monolignols (p-coumaryl, coniferyl and sinapyl alcohol). It has been suggested rather to genetically manipulate bioenergy crops in ways that they can synthesize more easily breakable lignin polymers, such as transgenic plants with more hydrolysable inter-unit lignin linkages. In this case, transgenic plants will sustain their structural strength while their lignin content will degrade easier after crop harvest and before their conversions into biofuels.[31]

It is also suggested to manipulate the expression level of lignin biosynthesis-related transcription factors because certain transcription factors are known to bind to monolignols biosynthetic promoters and cause gene regulations in plants,[31] and/or use specific sclerenchyma-specific promoters in a non-vascular tissue-specific manner so vascular elements are not affected by lignin down-regulation phenomena.[33] This suggestion might become valuable because a major role of lignin is the establishment and strength of the vascular systems for the transfer of water and solutes in plants, and the lowering of lignin in the vascular tissues, easily causing dysfunctioning of vascular system causing problems with water conductivity in plants.[31] The use of Schleremchyma-specific promoters in down-regulation of lignin in cellulosic bioenergy crops might not be needed for all crops, all lignin biosynthesis pathway enzymes and/or might be needed depending on the level of enzyme down-regulation; the author's laboratory recently developed a CCR down-regulated homozygous transgenic maize genotype (Fig. 1) showing no apparent harm to the plant growth and development including the strength of the stem at the greenhouse level. Such normality is assumed to be due to the intact nature of the vascular system of transgenic CCR down-regulated plants as compared to their wild-type non-transgenic control plants (Fig. 2).

More studies are needed to see why CCR down-regulation in maize caused no apparent harm to the vascular system (Fig. 2), and what the broader effect of a decrease in CCR transcription on other chemical components of the transgenic cell walls has been. As such, studies of biology systems may become important for discovering interactions between biosynthesis of lignin and the whole plant metabolism, such the role of lignin down-regulation on starch metabolism and photorespiration, and/or on regulatory genes.[31] Furthermore, these CCR down-regulated maize plants (Fig. 1) must be field tested, preferably in a windward field location to assure lack of plant lodging, and be tested against invasions by pathogens and insects as one major role of lignin is defense against these invaders.

With all the studies performed on lignin metabolic pathway enzymes, our knowledge of non-lignin-related biochemical and physiological aspects of plant growth and development, and other unexpected roles that changes in the level of one pathway enzyme might cause are still so limited; it is very important to remember that any non-lignin-related genetic change in a plant might cause a change in lignin, and any change in lignin content might have an effect on the crop agronomic values. For

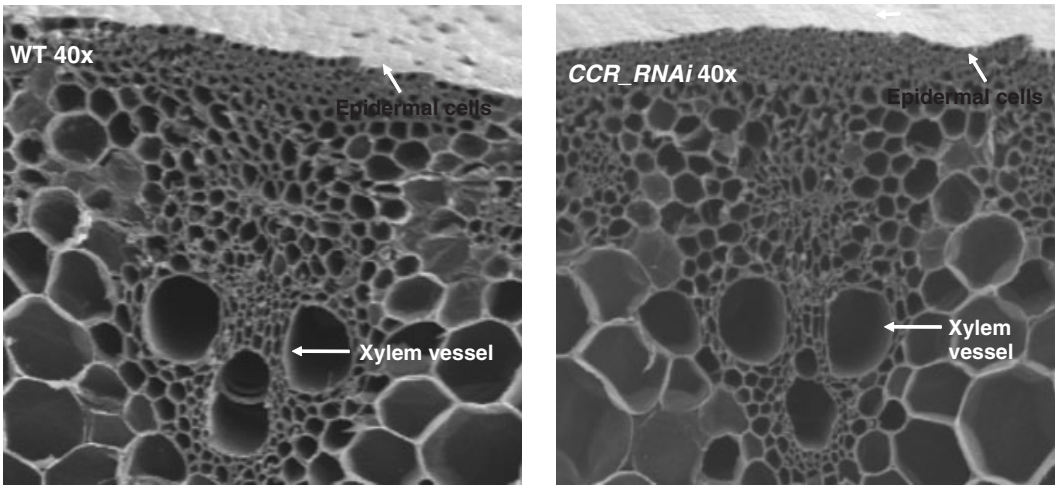

Figure 2. Scanning electron microscopy of CCR down-regulated transgenic maize leaf midrib as compared to that of wild-type non-transgenic control plant.

example, it has been reported that the BT corn (transgenic corn expressing the *Bacillus thuringienses* Cry 1Ab insect resistance gene) showed significantly higher (33 to 97%) lignin content than non-BT corn,[53] and Arabidopsis mutants with low COMT content showed higher susceptibility to different pathogens. Furthermore, such changes in lignin content unexpectedly resulted in an increase in sexual reproduction of downy mildew pathogen[54] showing the importance of lignin metabolic pathway enzymes on plant-pathogen interactions.

Improving the agronomic performance of cellulosic bioenergy crops via transgenic technology

When the new lignocellulosic bioenergy crops such as miscanthus, switchgrass and other perennial grasses are cultivated as monoculture crops, like all domesticated crops they would be exposed to invasions by weeds, insects and pathogens. Transgenic technology could be applied to these crops for control of these biological agents, as well as for their resistance to abiotic stresses such as drought, cold, salt, etc. For example, several herbicide-resistance DNA sequences could be successfully used as selectable marker genes for genetic transformation of bioenergy crops. These herbicide resistance transgenic crops could be sprayed with the correlating herbicide for weed control. Examples of herbicide resistance genes that could be included in the production of genetically modified bioenergy crops include Streptomyces hygroscopicus that encodes Phosphinothricin acetyl transferase (PAT) for resistance to the glufosinate herbicide[55] and the acetohydroxyacid synthase (AHAS) mutant which encodes for resistance to sulfonylurea;[56] the 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) that encodes for resistance to glycophosphate;[57] and the acetyl-CoA carboxylase (ACCase) inhibitor that encodes for resistance to phenoxy proprionic acids and cycloshexones herbicides.[58] Some of these genes could be transferred to the cellulosic bioenergy crops for an efficient weed control.

Among biotic stress-resistance genes with great potential for transfer to bioenergy crops are those encoding insect resistance such as lectin genes;[59] *Bacillus thuringiensis* (BT) endotoxin genes that encode proteins used to control certain insects;[60,61,62] avidin that has larvicidesal activity against insect larva; and genes that encode protease inhibitors such as rice cysteine protease inhibitor.[63] When the new cellulosic bioenergy crops become monocultured, transfer of some of these genes can increase their resistance to the damaging insects.

Among genes to be transferred to bioenergy crops for their antifungal activities are chitinase genes,[64] viral coat protein genes,[65] and genes that encode specific antibodies.[66]

Among several other genes, the drought and/or salt tolerance of the new cellulosic bioenergy crops could be enhanced via transfer of, for example, the Hva1 gene which encodes for resistance to drought and/or salt.[67]

Soil carbon sequestration via feedstock crop genetic manipulations

A major concern with the long-term removal of cellulosic biomass from soil is the reduction of soil carbon reserve. Therefore when crop biomass, such as corn stover, is used for production of biofuels, methods of carbon sequestration need to be developed and practiced to assure the sustainability and health of microbial populations in soil. Among these methods are increasing the amount of cellulosic biomass per plant via delay in flowing,[68] increasing polysaccharides in plants via genetic manipulation of these crops[69,70] and possibly by concomitant increase in polysaccharides while down-regulating certain lignin biosynthesis enzymes such as when 45% down-regulation of CCR caused a 15% increase in plant cellulose.[51] Also keeping in mind to allow at least one-third of the crop residues in soil for carbon sequestration.[71]

Conclusions

Considering the coproduction of highly valuable recombinant coproducts such as biotech drugs and major industrial enzymes in transgenic bioenergy crops, the biofuels industry would most probably gain most of its profits from recombinant coproducts rather than from biofuels. In this model, Western countries, especially the USA, would become most capable of economically producing biofuels as one of the means is to reduce the import of petroleum to have less dependence on foreign oil. If so, the world might benefit from such a change as foreign oil has always been at least one of the reasons for political instability and weak international relations between the developed nations (especially the USA) and the rest of the world.

Recombinant high-value coproducts produced within bioenergy crops would fit quite well into an ethanol biorefinery and the high-value coproducts could be recovered during the biorefining processes. Per unit mass, biopharmaceuticals and industrial enzymes are worth many times more than ethanol product. Such high-value coproducts can undergo a high degree of processing and separation. It is important to note that transgenesis technology can also be largely applicable to the production of other industrial products such as lactic acid, 1, 3 propanediol, etc., that one might wish to produce in cellulosic bioenergy crops.

Genetic modifications of lignin biosynthesis pathway in bioenergy crops in ways in which such modifications would not interfere with plant health and its defense against insects and pathogens will soon play a very major role in reducing the costs of expensive pre-treatment as reported.[52,72]

Much more research is needed in this area. For example, further studies of the CCR down-regulated transgenic maize (Fig. 1) might become important in understanding the enzyme kinetics of CCR protein and the physiological and biochemical aspects of lignin biosynthesis in maize especially because monocot lignin contains 'ester- and ester-linked hydroxycinnamic acid' causing more complexity in the understanding of lignin biosynthesis in these crops.[30]

Overcoming the removal of carbon from soil via modern methods of carbon sequestration to soil is an important issue which more allocations of Federal funds, innovations and scientific efforts will resolve within the next decade. The rational concerns regarding genetically modified organisms[73] in the food chain should mostly not exist due to the non-food nature of most bioenergy crops and by avoiding production of recombinant products in pollens and seeds of bioenergy crops.[17]

Acknowledgement

The author acknowledges financial support from the Consortium for Plant Biotechnology Research (CPBR), the United States Department of Energy (USDA) via the Iowa State University, Edenspace Systems Corp., the National Institute of Health, Corn Marketing Program of Michigan (CMPM) and the National Corn Growers Association (NCGA) for her team's research reported in this review.

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