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Review Article

Genetic Engineering Strategies for Improving Saccharification Efficiency of Lignocellulosic Biomass in Bioenergy Plants

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Abstract

The first-generation biofuels have limited capability to meet the growing transportation fuel demands of the burgeoning world population because food availability and food security are the prime concerns. Therefore, lignocellulosic biomass from plant cell walls is being explored as an alternate yet significant resource to produce second-generation biofuels. Current biofuel production technologies are largely dependent on using sugarcane and corn due to the ease in substrate handling and processing. However, commercial production of bioethanol from lignocellulosic biomass requires substantial improvements in plant feedstocks, pretreatment conditions, saccharification or sugar release processes and fermentation of released simple sugars. Plant biotechnology provides an effective means of developing targeted structural alterations in the lignocellulosic secondary cell walls of bioenergy plants for improved saccharification. This review provides a comprehensive and critical assessment of the published reports about genetic modifications of bioenergy plants toward reducing the secondary cell wall recalcitrance to enhance bioethanol production.

Keywords: *Cellulosic bioethanol, Chemical pretreatments, Secondary cell walls, Genetic modification, Saccharification.*

Introduction

Plants fix atmospheric carbon via photosynthesis. Plant cell walls act as important sinks for irreversible sequestering of fixed carbon in the form of a variety of biopolymers such as cellulose, hemicellulose and lignin. About 70% carbon fixed by the plants is stored in the cell walls (Pauly. *et al*., 2008). The growing demands on transportation fuels and establishing a variety of renewable fuel sources led researchers to genetically modify the cell wall polymers for improving biofuel production. Although the first-generation biofuels have made significant contributions to the global energy needs, additional energy requirements can only be fulfilled by using alternative resources such as lignocellulosic biomass that have about 60% energy conversion efficiency (Chen and Peng. 2013; Pauly and Keegstra. 2008). Moreover, using biomass does not directly conflict with the food supply. Secondary cell walls in plants may provide abundant biomass but the release of energy from this type of biomass includes various steps: chemical pretreatments to provide ease in cell wall deconstruction, enzymatic digestion to release simple sugars i.e saccharification and yeast fermentation to produce cellulosic bioethanol (Xu. *et al*., 2011). Plant cell walls have evolved into a strong complex structure to cope up with biotic and abiotic stresses. Therefore, there is significant recalcitrance during the harvesting of energy at each of this processing step. Genetic modification provides an answer to some of these complexities associated with energy conversion (Chen and Dixon. 2007; Demura and Ye. 2010).

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Plant feedstocks provide the raw materials for saccharification (release of sugars from complex carbohydrates), therefore it has always been one of the research interests for bioenergy researchers. Plant cell wall is an intricate network of cellulose copolymerized with non-cellulosic biopolymers like hemicellulose and lignin. Therefore, such complexities of cell walls make the biomass more recalcitrant to the sugar release. Moreover, crystallinity of cellulose is another factor limiting saccharification (Nookaraju. *et al*., 2013). Chemical pretreatments of biomass could solve this issue to some extent but it is an additional cost to the final product. Genetic engineering provides a way to understand the causes of complexity of biomass. Hence, large number of model crops have been examined on to meet the second-generation biofuel production goals. First generation biofuels derived from food crops initially gained enormous popularity to produce bioethanol. Corn and sugarcane are also the prime food sources being utilized for bioethanol production. If all the ethanol production in US is from corn crop, then producing 10 billion gallons of ethanol would almost consume 30% of its harvest which is quite unwelcoming for the food security in the US (Wescott. 2007). Therefore, the global demands have made the researchers to focus on lignocellulosic biomass from non-food crops such switchgrass, poplar and eucalyptus for the bioenergy production to avoid such food scarcity and security problems. The high energy output and low carbon emissions enable lignocellulosic biofuels as a better choice for bioethanol production (Farell & Turner. 2006). Any other biomass processing steps like chemical and enzymatic pretreatments which helps the release of sugar (saccharification) will also increase the overall biofuel processing costs. Third generation biofuels are obtained from algal sources for producing biodiesel. Fourth generation biofuel crops are genetically engineered bioenergy plants for the improved biofuel production. The woody biomass of poplar consists of 45-50% of cellulose, 25-30% of hemicellulose and lignin contributing about 20-25% (Anderson. *et al*., 2006). The major hydrophobic hindrance in the cell wall is conferred by lignin which decreases the cellulolytic activity on polysaccharides. Therefore, pretreatments are required to solubilize the biomass. The improved biomass should require no or lesser pretreatments. Pretreatments with chemicals like hot acid or alkaline treatment increase the final processing cost of biomass into bioethanol. It breaks down the macroscopic structure of cell walls and bonds between the biopolymers and solubilizes the biomass making it more accessible to the fermenting enzymes. Pretreatments always add further costs in the final processing of biomass and sometimes, the byproducts of pretreatments hinder the process of fermentation. Therefore, researchers are now working on genetically modifying the plant feedstocks where they can alter the complexity of cell wall structure enabling efficient conversion of biomass into bioethanol.

Figure1 depicts how the lignocellulosic biomass is converted into bioethanol after a series of chemical and enzymatic treatments. Complex sugar is first broken down into simple sugar which is then further fermented into bioethanol by a series of enzymatic reactions. Chemical pretreatments are employed to break down the complex structural bonds of non-cellulosic polysaccharides to make them accessible to degrading enzymes. These enzymes break complex carbohydrates into simple sugars. Some released pentose sugars hinder the fermentation process for the production of bioethanol.

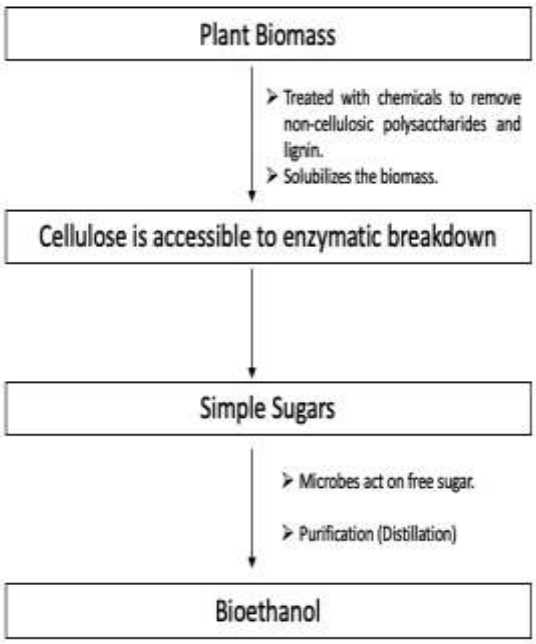

Figure 1: Flow diagram showing processing of plants feedstock and production of liquid biofuels

Genetic Modification of the Lignocellulosic Plant Biomass Results in Increased Saccharification Efficiency

Although lignin is present in relatively low quantities (20-25%) in secondary cell walls than the polysaccharides (40-50%), it is a major recalcitrance factor in the processing of lignocellulosic biomass to bioethanol. Lignification occurs during the cell differentiation stages in specific cell types as well as during biotic and abiotic stresses. Dicots have the ability to use phenylalanine as precursor which may produce three different types of monolignols via phenylproponoid pathway. These monolignols undergo dehydrogenation in the cell wall by certain cell wall specific enzymes called laccases and peroxidases resulting in lignin polymerization. Three different kinds of lignin are produced namely, H, G and S-lignin. The proportion of each lignin type is cell- and species-specific. For example, tracheary-elements are mostly G-rich and xylary- fibers have mostly G and S-lignin. S-lignin is easier to remove from the cell walls than H- and G-lignin during the process of delignification. Therefore, it is researchers have attempted to produce transgenic plants producing higher amounts of S-lignin for easier cell wall deconstruction for biofuels. Most of the lignin pathway genes (Figure 2) have been well characterized from many different plant model species for their functions in lignification (Review: Nookaraju. *et al*., 2013).

During the last decade, several research groups have genetically modified production of lignin pathway enzymes for producing improved biomass with increasing saccharification efficiency. The first such report was published by Chen and Dixon in 2007 who demonstrated increased saccharification efficiencies in the six antisense suppression lines of alfalfa plants that showed reduced expression of C4H, HCT, C3H, CCoAOMT, F5H and COMT genes. Overall, 15-18% decrease in lignin content and 67-79% increase in the saccharification efficiency from HCT and C3H lines than control was observed indicating a negative correlation between lignin content and saccharification. Moreover, the saccharification efficiency in the untreated antisense lines of HCT and C3H was greater than the pretreated biomass from control plants indicating that high-cost chemical pretreatments could be avoided to increase the release of simple sugars. In another report by using alfalfa plants, two more lignin biosynthesis pathway genes, CAD and CCR downregulated transgenic lines depicted 50-60% increase in saccharification efficiency than control plants suggesting them as potential candidates for increasing saccharification in the biomass (Jackson. *et al*., 2008). However, this genetic modification resulted in negative effect on plant growth compromising the final biomass yield. Since, Alfalfa is one of the world's most important forage crop with an annual production of $8 billion in the US, significant improvements in agronomic performance of alfalfa are needed. Another lignin pathway enzyme, COMT was downregulated by RNAi in transgenic sugarcane (Jung. *et al*., 2012) where a range of lignin reductions in downregulated lines was observed from 3.9% to 13.7% that also decreased Syringyl/Guaiacyl (S/G) ratios

from 0.79 to 1.27 relative to 1.47 in the control plants. One of the enzymes that chemically modify the phenolic moiety of lignin precursors, 4-O methyl transferase was overexpressed in aspen without affecting any plant growth and biomass yield (Cai. *et al*., 2016). The study demonstrated that MOMT4 etherifies the phenolic moieties of monolignols disrupting S-lignin and increasing the intensity of G-lignin. Therefore, it was expected to increase the lignin condensation in the cell walls and hence the recalcitrance. However, MOMT4 transgenic lines showed 62% increase in saccharification efficiency and 49% increase in bioethanol production. The increase in saccharification could be attributed to the 19-20% decrease in lignin content and the structural alterations in the cell wall. High S/G ratio of lignin in wood pertains to higher saccharification (Yoo. *et al*., 2017). Silencing of 4CL, CSE and CAD1 genes reduced lignification and improved saccharification in poplar (Voelkar. *et al*., 2010: Saleme. *et al*., 2017: Acker. *et al*., 2017). 25% lignin reductions in case of CSE downregulation resulted in 86% increase in saccharification efficiency without affecting the plant growth whereas poplar downregulated with 4CL significantly affected plant growth with 10% decrease in lignin content. Reduction in lignin was most likely responsible for 35-92% increase in glucose release than controls. In case of CAD deficient poplar plants, 10-13% decrease in lignin was reported to result in 81% increase in glucose release. All these results demonstrate that there is a negative correlation between lignin content and glucose release. In another study on poplar by (Mansfield. *et al*., 2012) transgenic plants modified with C4H:F5H (fusion) did not affect the biomass growth in the fusion construct but RNAi:C3H lines were impacted the biomass and growth. Silencing of 4CL, CAD and COMT in switchgrass resulted in increased saccharification with decrease in lignin content (Xu. *et al*., 2011; Saathoff. *et al*., 2011; Fu. *et al*., 2011). Lignin was reduced to 12.2% in the case of COMT lines, saccharification efficiencies was increased by 14%. 4CL1 is an important enzyme catalyzing one of the key steps of monolignol pathway and its downregulation in switchgrass reduced the lignin to 17-32% however no significant change in biomass was reported from T0-T1 generations. Most of the genetic modification in switch-grass appeared to have no negative effect on agronomic performance which is also the desirable trait for the crop yield. It is possible that downregulating one of the key enzymes in lignin biosynthetic pathway can decrease the proportion of G (one of a lignin monomer) units which makes the lignin fragile and less intensive in the biomass and therefore release of sugars becomes easier. Apart from lignin modification, overexpression of sucrose synthase (SUS3) and ARAF genes increased the saccharification efficiency in rice proving them as one of the potential genes for increasing saccharification (Fan. *et al*., 2017; Sumiyoshi. *et al*., 2013). SUS3 transgenic lines showed normal plant growth during the two-year field study with 6-8% increase in biomass yields and 23-33% increase in saccharification than control. It seems like a powerful strategy for genetic modification in biofuel crops. Similarly, rice plants overexpressed with ARAF genes showed no visible change in plant growth in any of the phenotypic characteristics. Although lignin was increased, transgenic lines still showed an increase in saccharification from 46-69%. It is believed that the decrease of 75% arabinose was compensated by increase in cellulosic glucose. Arabinoxylans bind to cellulosic chains with strong H-bonding and to lignin through ester linkages. Overall, in the current literature, it has been observed that any genetic change in the genes responsible for cell wall biosynthesis creates modification in plant cell wall structure which is accompanied by the increase in fermentable sugar production. Therefore, modulating lignin biosynthesis or other cell wall component in the lignocellulosic feedstock could help in improving the biomass quality for bioethanol production while sustaining the normal growth of healthy plant.

Table 3 shows all the bibliography on different genetic improvements for increasing

saccharification efficiency literature reported from 2007 until 2018. The effect of genetic modification on the model plant in terms of lignin content, plant growth and saccharification is explained. It shows a list of genes responsible for SCW biosynthesis and how their genetic modification using specific promoters affects plant growth and other wood properties:

Plant	**Gene Modified**	**Overexpression or Downregulation**	**Change in Saccharification**	**Decrease in Lignin Content**	Reference
Panicum virgatum	MYB4 transcription factor	Overexpression	9.3-16.2%	no change	Liu. *et al.*, 2018
Panicum virgatum	microRNA156	Overexpression	12-18%	8%	Baxter. *et al.*, 2018
Arabidopsis thaliana	MYB46:4cl1	Overexpression	127%	10-15	Ko. *et al.*, 2018
Populus deltoides	GAUT4	Downregulation	64-97%	not reported	Biswal. *et al* l., 2018
Oryza sativa	Sucrose synthase3	Overexpression	23-49%	decreased	Fan. *et al.*, 2017
P.tremula x *P. alba*	acetyl xylan esterase	Overexpression	23-30%	not reported	Pawar. *et al.*,2017
P.tremula x *P. alba*	Cinnamyl alcohol dehydrogenase1	Downregulation	81%	10%	Acker. *et al.*, 2017
Populus trichocarpa	caffeoyl shikimate esterase	Downregulation	62-91%	19-25%	Saleme. *et al.*, 2017
Nicotiana benthamiana	DUF579 and KNAT7	Downregulation	74.5%, 40%	no change	Pandey. *et al.*, 2016
Brachypodium distachyon	COMT	Downregulation	20%	10%	Kuang. *et al.*, 2016
Populus deltoidus	LAC2	Downregulation	100%	No change	Bryan. *et al.*, 2016
Zea mays	GA-20OXIDASE1	Overexpression	11%	6%	Voorend. *et al.*, 2016
P.tremula x *P. alba*	monolignol 4-O methyl tranferase	Overexpression	62%	19-20%	Cai. *et al.*, 2016
Saccharum officinarum	CCoAMOT, F5H, COMT	Downregulation	53.5-57.9%	no change	Bewg. *et al.*, 2016
Eucalyptus urophylla x *E. grandis*	C3H and C4H	Downregulation	90-95%	18.9-21.7%	Sykes. *et al.*, 2015
Arabidopsis thaliana	3-dehydroshikimate dehydratase	Overexpression	79-116%	45-54%	Eudes. *et al.*, 2015
Panicum virgatum	GA2-oxidase	Overexpression	15%	not reported	Wud-dineh. *et al.*, 2014

P.tremula x *P. alba*	CCR	Downregulation	27-29%	12%	Acker. *et al*., 2014
Oryza sativa	ARAF	Overexpression	28-34%	Not reported	Sumiyoshi. *et al*., 2013
Arabidopsis thaliana	C4H	Downregulation	200%	9-12%	Yang. *et al*., 2013
Arabidopsis thaliana	CCR1	Downregulation	88%	21-59	Acker. *et al*., 2013
Panicum virgatum	MYB4	Overexpression	300%	1.9-7.4	Shen. *et al*., 2012
Saccharum officinarum	COMT	Downregulation	29%, 34%	29-32	Jung.*et al*., 2012
Panicum virgatum	miR156	Overexpression	19-30%	8% up	Fu. *et al*., 2012
Populus tremuloides	4CL1	Downregulation	50-60%	15-19	Min. *et al*., 2012
Brachypodium distachyon	CAD1	Downregulation	44-46%	20-26	Yvoire. *et al*., 2012
Panicum virgatum	COMT	Downregulation	16.5-21.5%	14-22	Fu. *et al*., 2011
Panicum virgatum	CAD1	Downregulation	2-11%	No change	Saathoff. *et al*., 2011
Panicum virgatum	4CL1	Downregulation	57%	17-32	Xu. *et al*., 2011
Arabidopsis thaliana	Laccase4 and 17	Downregulation	9-35%	20-40	Berthet. *et al*., 2011
Nicotiana benthamiana	Peroxidase TP60	Downregulation	45-50%	20	Kavousi *et al*., 2010
P.tremula x *P. alba*	4CL1	Downregulation	35-92%	50	Voelkar. *et al*., 2010
Arabidopsis thaliana	xylan mutation-gux1 gux2	Downregulation	200%	not reported	Mortimer. *et al*., 2010
P.tremula x *P. alba*	Glucosyltransferase , GT47	Downregulation	24-48%	not reported	Lee. *et al*., 2009
Sorghum bicolor	bmr6 and bmr12 (double mutant)	Downregulation	88%	18.7-24.1	Dien. *et al*., 2009
Arabidopsis thaliana	CesA3	Downregulation	15%	not reported	Harris. *et al*., 2009
Medicago truncatula	CCR	Downregulation	50-60%	20-30	Jackson. *et al*., 2008
Medicago truncatula	C4H, C3H, COMT, F5H, HCT, CCoAOMT	Downregulation	166%	15-18	Chen & Dixon. 2007

Engineering the Feedstock Affects the Agronomic Performance

The energy stored in the plant cell walls is an attractive target for bioenergy researchers. But extractions of this energy from the cell walls come with adding cost and complexities. Therefore, to reduce the cell wall recalcitrance, genetic modification in biomass is one of the most desirable traits for biofuel production. But modifying the genes responsible for SCW biosynthesis sometimes result in unexpected consequences on the plant growth and biomass. The defects in the plant growth due to genetic changes in biomass affects certain agronomic traits like plant lodging resistance, biomass yield and stress tolerance (Li. *et al*., 2015; Casler. *et al*., 2002). As most of the published research is based on laboratory or green house studies, therefore, to evaluate the actual effect of these genetic modifications on the agronomic performance (Macaya. *et al*., 2017), it is imperative to replicate the same effects in field condition under repeated trials for a realistic understanding of these changes in biomass. It is often observed to have different results in green house and field conditions for any genetic change in biomass (Poorter. *et al*., 2016). For example, genetic modification of 4CL gene in Populus *tremuloides* showed dramatic effects in poplar when its silencing produced different effects on the plant growth in green house and field conditions. Under green-house conditions, plant growth was increased with reduction in lignin (Bonawitz and Chapple. 2010) whereas growth was significantly reduced in the fields in subsequent years (Hu. *et al*., 1999).

Concluding Remarks and Future Directions

The conventional energy sources are limited in nature because of the growing demands of population and rapid industrialization. Increasing global warming issues and environmental concerns associated with present day fossil fuel problems have made researchers to search for the alternative sources of energy. Second generation biofuels such as bioethanol from lignocellulosic biomass, have proven to be a great alternative to the current fossil fuels. A number of gaps have been recently filled in providing deep insights of plant cell wall structure, formation and breakdown, which helps researchers find ways of enhancing saccharification. The pathway of lignin biosynthesis is well characterized and engineered for the improved saccharification without affecting plant growth and physiology. Incorporation of system biology-based approaches can improve our understanding about the plant responses to altered lignin synthesis and modification of secondary cell walls for enhancing bioethanol production. Cell wall deconstruction is critically important aspect for the economical production of biofuels. Structural complexity of lignocellulosic biomass and cellulosic crystallinity are some of the factors that increase the recalcitrance of the cell wall (Harris. *et al*., 2012). Incorporation of genetic modifications that enhance the amorphous nature of the cellulosic biomass and decrease in the cellulose crystallinity could provide solutions to reduce the complexity and hindrance during the release of sugars. Once the complexity of lignocellulose biomass is unraveled, it is feasible to produce such substrates that require lesser or no chemical treatments for the biomass solubilization and hence, it might cost less for downstream processing. Proper engineering of secondary cell walls will reduce the manual labor, downstream processing of the crude extract and therefore the economic cost incurred as a result of lignocellulosic biomass. Although breeding of bioenergy crops might be an inexpensive option, it is a time-consuming process. Instead we should select some of the genes responsible for SCW biosynthesis by creating their overexpression and mutant lines to decrease recalcitrance in the biomass, increase the biomass content and enhance ease of cell wall digestibility by enzymes without much additional cost. Simultaneously, some cost-effective mild pretreatments could also be applied to help release of sugars. There are always some challenges associated with plant feedstock such as feedstock transportation, pretreatment, saccharification and fermentation etc. (Balan. *et al*., 2014).

Overcoming challenges during these processes may result in reduced economic cost of bioethanol production. Altering the lignin structure and lignin content increases the saccharification efficiency and reduces the downstream cost of processing to some extent. Recent advancements in plant biotechnology have helped us introduce manipulations in monolignol synthesis pathway and learn how interactions between lignin and cellulose affect the cell wall digestibility and saccharification. Producing transgenic plants with reduced crystallinity will help cellulases to digest more cellulose efficiently, although some negative effects on plant growth have also been observed. There has been a substantial progress in the area of biofuels in terms of funding and research, yet a lot has to be unraveled to improvise promising ways for improving saccharification.

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