ELSEVIER

Plant genetic engineering to improve biomass characteristics for biofuels

Mariam Sticklen

Currently, most ethanol produced in the United States is derived from maize kernel, at levels in excess of four billion gallons per year. Plant lignocellulosic biomass is renewable, cheap and globally available at 10–50 billion tons per year. At present, plant biomass is converted to fermentable sugars for the production of biofuels using pretreatment processes that disrupt the lignocellulose and remove the lignin, thus allowing the access of microbial enzymes for cellulose deconstruction. Both the pretreatments and the production of enzymes in microbial tanks are expensive. Recent advances in plant genetic engineering could reduce biomass conversion costs by developing crop varieties with less lignin, crops that self-produce cellulase enzymes for cellulose degradation and ligninase enzymes for lignin degradation, or plants that have increased cellulose or an overall biomass yield.

Addresses
Department of Crop and Soil Sciences, Michigan State University, East Lansing, MI 48824, USA

Corresponding author: Sticklen, Mariam (stickle1@msu.edu)

Current Opinion in Biotechnology 2006, **17**:315–319

This review comes from a themed issue on
Energy biotechnology
Edited by Jonathan R Mielenz

Available online 15th May 2006

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2006.05.003

Introduction

Lignocellulosic biomass is renewable, cheap and readily available with over 180 million tons produced per year in the United States [1] and 10–50 billion tons produced per year at global level [2]. In fact, half of the agronomic biomass produced worldwide is rice straw, which is burned to waste causing environmental and health problems [3]. Currently, most ethanol produced in the US is derived from the starch of maize kernels with a net energy balance [4]. However, starch by itself is a valuable high energy food and feed commodity.

The idea that fermentable sugars for use in the production of alcohol fuels could be derived from crop biomass has been well received by the US Federal government; however, major economical downsides of biomass refineries include the pretreatment processing of the lignocellulosic matter and the cost of production of the microbial cellulases needed to convert the cellulose of biomass into fermentable sugars [5]. Recent plant genetic engineering studies have aimed to decrease both of these costs and to further increase the cellulose and/or overall crop biomass yield [6••]. Several approaches have been considered.

Firstly, efforts have been made to reduce the lignin content of plants. Lignocellulosic biomass is composed of crystalline cellulose embedded in a hemicellulose and lignin matrix. Pretreatment methods are currently used to disrupt the lignocellulosic matter and to remove most of the lignin, thus allowing the cellulases to access the cellulose. Plant genetic engineering can decrease the lignin content and/or change the composition of lignin, thereby reducing the need for expensive and harsh pretreatments. Genetic engineering can also be employed to produce microbial ligninases within the biomass crops, so the lignin content of the biomass could be deconstructed during or before bioprocessing.

A second approach has considered the overexpression and engineering of enzymes for cellulose degradation in plants. Three different groups of cellulases work in concert to convert cellulose into glucose: namely, endoglucanases, exoglucanases and β-glucosidases. Plant genetic engineering has been successfully used to produce these enzymes in plants.

Lastly, there might be ways to increase biomass through plant genetic engineering. These can include the genetic manipulation of plant growth regulators or photosynthetic pathways. Delay in flowering can also increase plant biomass.

This review addresses the recent improvement of biomass characteristics that have been obtained through plant genetic engineering, along with strategies to produce new genetic materials for biofuel exploitation.

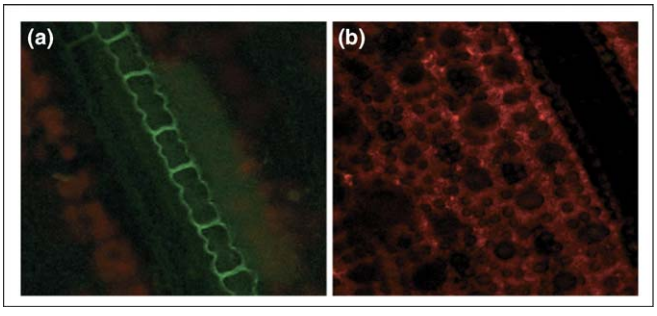
The production of cellulase enzymes within the crop biomass

The path from lignocellulosic matter to alcohol fuels has recently been illustrated, and the reasons why ethanol production from biomass is not yet economically feasible have been explained [6••]. Nevertheless, thanks to engineers and scientists in the field, the cost of production of cellulases for cellulosic ethanol at $5 per gallon has recently been reduced to $0.30–$0.50 per gallon, and the goal is to further reduce these costs to $0.10 per gallon

of ethanol. One possible route to further reduce costs is to produce cellulases within the crop biomass itself [7], rather than producing cellulases in microbial tanks. Along these lines, the gene coding for the catalytic domain of the thermostable *Acidothermus cellulolyticus* 1,4-β-endoglucanase E1 enzyme was successfully expressed in *Arabidopsis,* tobacco and potato plants and the translation product targeted to the apoplast (i.e. the extracellular pathway provided by the continuous matrix of cell walls). This study demonstrated the possibility of producing this enzyme within the biomass, in the case of *Arabidopsis* at levels up to 25% of the plant total soluble protein. Recently, work was carried out in our laboratory to constitutively express the catalytic domain of the *A. cellulolyticus* 1,4-β-endoglucanase E1 in rice (H Oraby, V Balan *et al.*, unpublished) and maize (Figure 1), again targeting the enzyme into the apoplast. The amount of endoglucanase E1 enzyme produced in rice and maize leaves accounted for up to 4.9% and 2% of the plant total soluble proteins, respectively, and the enzyme accumulation had no apparent deleterious effects on plant growth and development. Furthermore, when the crude extract of rice total soluble proteins was added to ammonia fiber explosion (AFEX) pretreated rice straw or maize stover, ~30% and 22% of the cellulose of these plants, respectively, was converted into glucose (H Oraby, V Balan *et al.*, unpublished; C Ransom *et al.*, personal communication).

Initially, there were three concerns associated with production and use of cellulase enzymes within the crop biomass. The first concern was whether the harsh conditions (acid, alkaline and/or heat) of pretreatment would destroy the biological activity of these enzymes. The second issue was whether sufficient enzymes could be expressed within the biomass to convert polysaccharides into fermentable sugars without the need to add further commercial enzymes. Lastly, it was necessary to consider whether increasing the level of production of these heterologous enzymes within the plant cells would cause harm to plant growth and development.

Figure 1



The production of the *Acidothermus cellulolyticus* endoglucanase (E1) gene product in corn leaf apoplast. **(a)** E1 transgenic maize leaf tissue shows apparent storage of E1 in the plant apoplast (green areas around each of the cells). Image produced with immunofluorescent confocal laser microscopy using the E1 primary monoclonal antibody and the fluorescein isothiocyanate (FITC) anti-mouse secondary antibody. **(b)** Leaf tissue from an untransformed control maize leaf, showing the characteristics of no expression of E1 enzyme.

To address the first concern, the mildest method of pretreatment (i.e. AFEX) was used on the thermostable *A. cellulolyticus* endoglucanase E1-producing tobacco biomass. In this experiment, about two-thirds of the activity of this heterologous enzyme were lost [8•]. It was concluded, therefore, that a better approach would be to extract the heterologous enzyme in either a crude or a pure form, and then to add this to the pretreated matter for the production of fermentable sugars. In a follow up study, up to 30% of rice and 22% of maize cellulose were converted into glucose when the rice-produced endoglucanase E1 was extracted in crude form, frozen for three months and then added to the AFEX pretreated matter (H Oraby, V Balan *et al.*, unpublished).

To address the second issue — whether or not it is possible to express sufficient enzyme levels — it is possible to increase the level of gene expression by regulating transcriptional, post-transcriptional and post-translational factors. However, the best way to increase the production of heterologous proteins is to target them away from the cytosol for accumulation into non-cytosolic cellular compartments [9]. This approach would also address the third concern, because enzyme accumulation inside these compartments will not interfere with the plant cytosolic metabolic activities. Another advantage of this approach is that there are distinct molecular chaperone systems in targeted compartments to translocate or fold certain proteins. However, it is necessary to choose the correct compartment for accumulation, because factors that influence transcription and translation efficiency, recombinant protein accumulation, and protein stability strongly depend on the compartment itself.

The question has been asked as to why the heterologous endoglucanase E1 targeted and accumulated in the apoplast of rice, maize and other plants did not harm the plant cell wall cellulose. There are three possible reasons for this. First, the heterologous E1 enzyme does not have direct access to the plant cellulose, because cellulose is present as a compact mixture together with lignin and hemicellulose. Second, the plant cellulose is in crystalline form, which is less amenable to hydrolysis by cellulase. Third, the heterologous endoglucanase E1 from thermophilic *A. cellulolyticus* might have limited activity at *in vivo* temperatures in plants.

Enzyme compartmentalization

Cell compartments are discrete structures within plant cells, each having specific biological functions. Among the plant cell compartments, the nucleus, chloroplast

and mitochondria contain distinct genetic materials. However, although the nucleus has no translation capability, chloroplast and mitochondrial DNA are transcribed and translated within their own compartments. In addition to their own proteins, the chloroplast and mitochondria receive proteins from the cytoplasm through the action of specific nuclear transit peptide DNA sequences that are designed to target certain proteins into these compartments. Certain other specific nuclear transit peptides target proteins into compartments that do not contain genetic material and therefore are not capable of producing their own proteins. These compartments include the apoplast, vacuole, endoplasmic reticulum, golgi apparatus, and microbodies such as liposomes and peroxisomes. Using these specific transit peptide sequences, scientists can transfer heterologous proteins into these compartments for accumulation.

As mentioned above, the choice of compartment for targeting heterologous enzymes is important, but how do we know which compartment to choose? The apoplast can provide more space than other compartments for higher levels of accumulation; for example, ProdiGene targeted the heterologous laccase to the maize seed endosperm cell walls or apoplast for high accumulation. Targeting the chloroplast requires specific targeting signal peptide sequences; for example, in a maize study [10], the first 24 amino acids of the coding sequence of the mature rubisco small subunit (rbcS) protein were used together with the pea rubisco transit peptide to direct three polyhydroxybutyrate pathway enzymes into the maize chloroplast. Targeting of heterologous peptides to the vacuole has also been performed in several cases.

The targeting of heterologous proteins for high accumulation has successfully been achieved without causing harm to plant growth and development. It might now be desirable to test a battery of different polysaccharide- and lignin-degrading enzymes within the same crop biomass by targeting each enzyme to the same or different compartments. Also, one might wish to target the same enzyme to different cellular compartments of the same plant to maximize production of a single enzyme. For example, in *Arabidopsis* when a heterologous fungal xylanase was targeted to either the chloroplast, the peroxisome or both of these compartments, the dual compartment targeted xylanase accumulated 160% of that targeted to the chloroplast alone and 240% of that targeted to the peroxisome alone [11].

Regulation of lignin synthesis

After cellulose, lignin is the second most abundant polymer on earth. In the lignocellulosic biomass, crystalline cellulose is embedded in a hemicellulose and lignin matrix. In order for cellulases to access the cellulose for degradation, costly acid and/or heat pretreatment of the biomass is required to remove lignin and hemicellulose and to disrupt the lignocellulosic matter. Tremendous efforts have been made to improve pretreatment methods and to reduce costs [12,13].

Decreases in lignin content through the manipulation of different lignin biosynthetic pathway genes have been reported [14•,15•]. For example, downregulation of one of the major enzymes involved in lignin biosynthesis, 4-coumarate:coenzyme A ligase (*Pt*4CL1) in transgenic aspen (*Populus tremuloides*), resulted in a 45% decrease in lignin with a compensation of 15% increase in cellulose, doubling the plant cellulose:lignin ratio without any change in lignin composition and without any apparent harm to plant growth, development or structural integrity [15•]. The *Pt*4CL1 is a structurally and functionally distinct protein in the lignin biosynthetic pathway of aspen, associated with catalysis of the CoA ligation of hydroxycinnamic acids (i.e. intermediates in the biosynthetic pathway of lignin), resulting in the production of phenolic precursors for lignin biosynthesis in developing xylem. Therefore, downregulation of this important protein in aspen has resulted in reduced lignin biosynthesis. It is believed that a decrease in lignin content could be further amplified by manipulation of multiple genes associated with the lignin biosynthetic pathway [6••].

Although modification of the lignin biosynthetic pathway enzymes has been proven to decrease lignin content, one must ensure that this modification will not interfere with the plant defense against invading pathogens and insects. In addition, because lignin deposition of specialized plant cells is known to occur through a sophisticated spatially and temporally coordinated response to the internal and external needs, more basic research is needed to understand the genetic basis of lignin pathway regulation [16]. Basic research is also currently in progress to obtain a better understanding of the lignin biosynthetic pathway [14•]. It is hoped that, in light of this new knowledge, it will be possible to reduce the lignin content without causing long-term harm to the plant.

Increasing the number of polysaccharides or the overall plant biomass

Basic research is also in progress to understand the cellulose biosynthetic pathway (e.g. [17,18]), with the ultimate aim of increasing quantities of this polysaccharide. The regulation of certain growth regulators, such as brassinosteroids, has been reported to increase plant biomass without the need for increased fertilizer applications [19]. In this study, a brassinosteroid-deficient mutant exhibited an erect leaf phenotype associated with enhanced grain yields. In a different approach, tobacco biomass was significantly increased through the nuclear insertion of a single *Arabidopsis thaliana* Flowering Locus C (*flc*) gene known to delay flowering [20]. In this example, the transfer of a single *flc* gene was able to

significantly increase plant biomass, because the energy needed for reproduction to occur at the correct time is shifted into biomass growth.

Attempts have also been made to increase biomass through increasing the availability of key nutrients. Phosphorus is one of the least available nutrients in soil, yet has an important role in photosynthesis, respiration and the regulation of many enzymes. The expression of the *Medicago truncatula* gene for purple acid phosphatase (*Mt*PAP1) in transgenic *Arabidopsis* resulted in a twofold increase in biomass production when 2 mM phytate was supplied as the sole source of phosphorus in soil [21]. As phytate is a plant phosphate storage compound that is not readily available as a phosphorous source to plants, the increase in biomass is probably due to the auxiliary role of *Mt*PAP1 in the utilization of the exogenous phytate which increases the availability of phosphorous to transgenic plants.

Plants have the capacity to fix more carbon than they do under standard growth conditions. For example, following an increase in atmospheric CO_2 concentration, maize produced 20% more biomass [22]. However, neither seed yield [22] nor plant biomass yields are directly related to maximizing the photosynthetic rate. It is not surprising to see that an increase in photosynthesis does not increase plant biomass, because several other factors such as plant nutrients, oxygen, water and plant respiration also need to be regulated. An increase in photosynthesis also relates to the correct matching of the plant circadian clock [23] with that of the external light-dark cycle [22]. The fact that maize produced 20% more biomass under high CO_2 concentration could be because C4 maize has a greater capacity to synthesize sucrose, starch and overall biomass under elevated conditions of CO_2 [22]. This observation needs to be tested in C3 plants.

Lastly, one study aimed to increase biomass through the manipulation of key metabolic enzymes. The chloroplastic fructose-1,6-bisphosphatase (FBPase) is known to have a key role in CO_2 assimilation and in coordinating carbon and nitrogen metabolism to increase sucrose production. When the pea FBPase was downregulated in transgenic *Arabidopsis*, the lower levels of FBPase production resulted in increased sucrose production [24].

Conclusions

The production of ethanol from plant biomass is receiving increased attention and developments in plant genetic engineering are going some way to reducing the costs of biomass conversion. Several avenues have been investigated, including the engineering of plants that self-produce cellulase and/or ligninase enzymes, the development of plants with reduced lignin content, and the production of crops with increased cellulase or overall biomass. In roads have been made in all of these areas, as discussed above. For example, the future for the large-scale production of cellulases within the crop biomass is bright, and the prospect of replacing microbial tank reactors with plants as biofactories for the commercial production of these and other industrial enzymes seems a realistic possibility.

Plant genetic engineering to improve biomass characterization for a better biofuel economy is a new technology. By definition, a new technology is economically feasible if the social benefits from adopting the technology are greater than its social costs. Here, the social costs include the cost of resources used without government subsidies, while the social benefits are the low production costs.

Following on from the above discussion, it sounds as though it will be easy to produce low cost transgenic biofuel related resources and to bring them to market for a better biofuel economy; however, we have all observed the closure of a successful industry owing to a single incident causing consumer suspicions. Thus, if plant genetic engineering is able to reduce the cost of resources needed in biomass refineries, the use of methods for the 'bioconfinement of genetically modified' plants [25••] must also be considered.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Kim S, Dale BE: **Global potential bioethanol production from wasted crops and crop residues**. *Biomass and Bioenerg* 2004, **26**:361-375.
2. Greene N: (principal author): **Growing energy: how biofuels can help end America's oil dependence**. *Natural Resources Defense Council*; 2004.
3. Kayaba H, Meguro H, Muto H, Kamada Y, Adachi T, Yamada Y, Kanda A, Yamaguchi K, Hamada K *et al.*: **Activation of *Eosinophils* by rice-husk dust exposure: a possible mechanism for the aggravation of asthma during rice harvest**. *Tohoku J Exp Med* 2004, **204**:27-36.
4. Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM: **Ethanol can contribute to energy and environmental goals**. *Science* 2006, **311**:506-508.
5. Kabel MA, Van der Maarel MJEC, Klip G, Voragen AGJ, Schols HA: **Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials**. *Biotechnol Bioeng* 2005, **93**:56-63.
6. •• Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL *et al.*: **The path forward for biofuels and biomaterials**. *Science* 2006, **311**:484-489.

This review article describes new manufacturing concepts for biomass conversion, economic downsides of biomass refineries, and the possible integration of plant genetic engineering to reduce costs.

7. Sticklen M, Teymouri F, Maqbool S, Salehi H, Ransom C, Biswas G, Ahmad R, Dale B: **Production of microbial hydrolysis enzymes in biomass crops via genetic engineering**. *2nd International Ukrainian Conference on Biomass for Energy* 2004. pp. *133, 20–22.*

8. Teymouri F, Alizadeh H, Laureano-Perez L, Dale BE, Sticklen M:
• **Effects of ammonia fiber explosion treatment on activity of endoglucanase from *Acidothermus cellulolyticus* in transgenic plants**. *Appl Biochem Biotechnol* 2004, **116**:1183-1192.
Demonstrates the effect of a mild method of pretreatment on the biological activity of a plant self-produced cellulose-degrading enzyme, and the conversion of plant biomass into fermentable sugars using transgenic plants.

9. Hood EE: **Bioindustrial and biopharmaceutical products from plants.** In *New Directions for a Diverse Planet*. *Proceedings of the 4th International Crop Science Congress, Brisbane, Australia, Sept 26–Oct 1*, 2004; ISBN 1 920842 20 9.

10. Zhong H, Teymouri F, Chapman B, Maqbool S, Sabzikar R, El-Maghraby Y, Dale B, Sticklen MB: **The dicot pea (*Pisum sativum* L) rbcS transit peptide directs the Alcaligenes eutrophus polyhydroxybutyrate enzymes into the monocot maize (*Zea mays* L.) chloroplasts**. *Plant Sci* 2003, **165**:455-462.

11. Hyunjong B, Lee BS, Hwang I: **Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein in plant cells**. *J Ex Bot* 2006, **57**:161-169.

12. Lynd LR, van Zyl WH, McBride JE, Laser M: **Consolidated bioprocessing of cellulosic biomass: an update**. *Curr Opin Biotechnol* 2005, **16**:577-583.

13. Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladish MR, Lee YY: **Coordinated development of leading biomass pretreatment technologies**. *Bioresour Technol* 2005, **96**:1959-1966.

14. Ralph J, Akiyama T, Kim H, Lu F, Schatz PF, Marita JM, Ralph SA,
• Srinivasa Reddy MS, Chen F, Dixon RA: **Effects of coumarate 3-hydroxylase down-regulation on lignin structure**. *J Biol Chem 2006,* 10.1074/jbc.M511598200 [Epub ahead of print].
This research article demonstrates the downregulation of the lignin biosynthesis pathway enzymes in alfalfa. Downregulation of the gene encoding 4-coumarate 3-hydroxylase massively, but predictably, increased the proportion of *p*-hydroxyphenyl (P) units relative to the normally dominant guaiacyl (G) and syringyl (S) units. This, in turn, led to an increase in crop digestibility, which might also translate into increased bio-energy.

15. Dean JFD: **Synthesis of lignin in transgenic and mutant plants**.
• In *Biotechnology of Biopolymers, from Synthesis to Patents.* Edited by Steinbüchel A, Doi Y. Weinheim, DE: Wiley-VCH Verlag; 2005:4-26.
This book chapter reviews articles on lignin mutants of brown midrib monocots, loblolly pine and *Arabidopsis*. Lignin-modified transgenic plants are also considered, along with the author's future perspectives in the area.

16. Rogers LA, Campbell MM: **The genetic control of lignin deposition during plant growth and development**. *New Physiologist* 2004, **164**:17-21.

17. Persson S, Wei H, Milne J, Page GP, Somerville CR: **Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets**. *Proc Natl Acad Sci USA* 2005, **102**:8633-8638.

18. Anderson-Gunneras S, Mellerrowicz EJ, Love J, Segerman B, Ohmiya Y, Coutinho PM, Nilsson P, Henrissat B, Moritz T: **Sundberg: Biosynthesis of celluloseriched tension wood in populus: global analysis of transcripts and metabolites identifies biochemical and developmental regulations in secondary wall biosynthesis**. *Plant J* 2006, **45**:144-145.

19. Sakamoto T, Morinaka Y, Ohnishi T, Sunohara H, Fujioka S, Ueguchi-Tanaka M, Mizutani M, Sakata K, Takatsuto S, Yoshida S, Tanaka K, Kitano H, Matsuoka M: **Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice**. *Nat Biotechnol* 2006, **24**:105-109.

20. Salehi H, Ransom C, Oraby H, Sticklen M: **Delay in flowering and increase in biomass of plants expressing the *Arabidopsis* floral repressor gene *FLC (FLOWERING LOCUS C)***. *Plant Physiol* 2005, **162**:711-717.

21. Xiao K, Harrison M, Wang Z: **Cloning and characterization of a novel purple acid phosphatase gene (MtPAP1) from *Medicago truncatula* barrel medic**. *J. Integrative Plant Biol* 2006, **48**:208-212.

22. Sinclair TR, Purcell LC, Sneller CH: **Crop transformation and the challenge to increase yield potential**. *Trends Plant Sci* 2004, **9**:70-75.

23. Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibert JM, Miller AJ, Webb AAR: **Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage**. *Science* 2006, **309**:630-633.

24. Sahrawy M, Avila C, Chueca A, Canovas FM, Lopez-Gorge J: **Increased sucrose level and altered nitrogen metabolism in *Arabidopsis thaliana* transgenic plants expressing antisense chloroplastic fructose-1,6-bisphosphatase**. *J Exp Bot* 2004, **55**:2495-2503.

25. Report NRC: *Bioconfinement of Genetically Engineered*
•• *Organisms*. The US National Academy of Sciences. *Natl Acad Sci Press* 2004.
This book contains a compilation of different methods that could be employed in transgenic crops productions to reduce the risks associated with growing genetically modified organisms.