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The quest for alternatives to microbial cellulase mix production: corn stover-produced heterologous multi-cellulases readily deconstruct lignocellulosic biomass into fermentable sugars

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Abstract

BACKGROUND: Production of cellulosic ethanol is still expensive compared with corn (maize) grain ethanol due to the high costs of bulk production of microbial cellulases. At least three cellulases including endo-cellulase, exo-cellulase and cellobiase are needed to convert cellulosic biomass into fermentable sugars. All these cellulases could be self-produced within cells of transgenic bio-energy crops. The production of heterologous *Acidothermus cellulolyticus* (E1) endo-cellulase in endoplasmic reticulum and mitochondria of green tissues of transgenic corn plants was recently reported, and it was confirmed that the heterologous E1 converts cellulose into fermentable sugars.

RESULTS: Biologically active *A. cellulolyticus* E1, *Trichoderma reesei* 1,4-β-cellobiohydrolases I (CBH I) exo-cellulase and bovine rumen *Butyrivibrio fibrisolvens* cellobiase were expressed in corn plant endoplasmic reticulum (ER), apoplast (cell wall areas) and vacuole respectively. Results show that the ratio 1 : 4 : 1 (E1 : CBH I : cellobiase) of crude heterologous cellulases is ideal for converting ammonia fiber explosion (AFEX) pretreated corn stover into fermentable sugars.

CONCLUSIONS: Corn plants that express all three biologically active heterologous cellulases within their cellulosic biomass to facilitate conversion of pretreated corn stover into fermentable sugars is a step forward in the quest for alternatives to the present microbial cellulase mix production for cellulosic biofuels.

Keywords: E1; endo-cellulase; CBH I; 1,4-β-cellobiohydrolases I; cellobiase; corn; maize; fermentable sugar; biofuels; AFEX

INTRODUCTION

With the 2003 awakening report that the USA held 3% of the world's petroleum reserves, and consumed 25% of the world's petroleum consumption (http://www1.eere.energy.gov/vehiclesandfuels/facts/2004/fcvt_fotw336.html), the US government urged the agricultural and petrochemical industries to find and implement biofuels as alternatives to fossil fuels to reduce the nation's dependence on foreign oil. A report resulted in the 2005 publication of USDA-DOE documents that the U.S. has sufficient land to annually grow one billion tons of lignocellulosic matter for biofuels production purposes, reducing 30% of foreign oil import requirements by 2030 (http://www1.eere.energy.gov/biomass/pdfs/final_billionton_vision_report2.pdf).

Plant lignocellulosic biofuels are considered excellent alternatives to petroleum fuel, gasoline. Plants annually produce 180 billion tons of cellulose at the global level,[1] and as the most abundant biopolymer on earth, cellulose is indeed the most promising renewable energy source for biofuels production.

Despite the great potential of lignocellulosic biofuels, their production costs strongly depend on how cheap cellulase enzymes are produced and how efficiently lignocellulosic materials are broken down. At present, cellulase enzymes are produced in microbial bioreactors at approximate costs of $1.00 per gallon of ethanol,[2] which impedes the commercialization of cellulosic bioethanol. Therefore, the production costs of the microbially produced commercial cellulases need to be further reduced in

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order to make cellulosic biofuel technology competitive with corn grain ethanol.

At least three different cellulase enzymes are required to break down plant cell wall cellulose for cellulosic biofuel production. The plant secondary cell walls are composed mainly of crystalline cellulose, varying mixtures of hemicellulose and lignin. Pretreatment of the lignocellulosic biomass is necessary prior to enzymatic hydrolysis because access of the enzymes to cellulose is restricted by lignin–hemicellulose interference. Pretreatments (e.g. ammonia fiber explosion, AFEX) break the lignin seal, disrupt the crystalline structure of macro- and microfibrils and increase the pore volume and available surface area. These physicochemical changes allow the enzymes to penetrate into the lignocellulosic fibers, which renders them amenable to enzymatic hydrolysis.[3,4]

The three cellulases include endo- and exo-cellulases and cellobiases. The endo-cellulases such as β-1,4-glucanases (e.g. Cel5a; E1; EC 3.2.1.4, Accession no. U33 212) randomly cleave β-1,4-glucan along the polysaccharide chain and produce a new reducing and non-reducing end of the cellulose strand. After the reaction of an endo-cellulase, the smaller glucan chains are further hydrolyzed by exo-cellulases such as 1,4-β-cellobiohydrolases I or CBH I (Cel7a; EC 3.2.1.91. Accession no. E00389) which cleaves from the reducing ends, or the CBH II (Cel6a; EC 3.2.1.21, Accession no. M55080) which cleaves from the non-reducing ends of cellulose chains.[5]

The hydrolysis of cellulose due to synergistic action of endo- and exo-cellulases results in dimer glucose chains or cellobiose. The cellobiose can be further converted into the monomer glucose by cellobiases such as β-1,4-glucosidase 1 (EC 3.2.1.21, Accession no. M31120). The β-1,4-glucosidase 1 has been grouped into two glycosyl hydrolase sub-families, sub-family A and sub-family B. Sub-family A includes plant and non-rumen prokaryotic cellobiases. Sub-family B includes fungal cellobiases such as the one produced in *T. reesei, Aspergillus niger*, and *A. aculeatus*,[6,7] and rumen bacteria such as the anaerobic bovine symbiotic *Butyrivibrio fibrisolvens* used in our studies. Cellobiases also act as cellulase inducers and transcriptional regulators.[8] Cellobiase is only needed at about 100–1000 times lower amounts than endo and exo-cellulases for hydrolysis of cellulose.[9]

To reduce the costs of cellulases, biologically active *Acidothermus cellulolyticus* E1, *Trichoderma reesei* CBH I, and bovine rumen *Butyrivibrio fibrisolvens* cellobiase were produced in three different sub-cellular compartments of three different sets of transgenic corn plants. Then, plant-produced crude proteins containing each heterologous cellulose were extracted, mixed together and the mixture added in specified ratios to AFEX-pretreated corn stover.[10] It was found that under these conditions, a specific ratio of the heterologous multicellulase mix was most effective for cellulose conversion into glucose. In this research, we accomplish production of all three heterologous cellulases in corn plants in a cost-effective manner and suggest the feasible application of the plant-produced heterologous multicellulase mix in biofuel industries.

Previously, the composition of corn stover was found to include 34.4% glucan and 22.8% xylan.[11] Theoretically, production of a few heterologous cellulases should have no effect on corn stover composition. Furthermore, the composition of corn stover is nearly identical in AFEX-pretreated and untreated corn stover.[4,12]

MATERIALS AND METHODS

Co-transformation vectors

There are five transformation vectors included in our experiments (Fig. 1). The pE1ER contains the *A. cellulolyticus* E1 gene[13] included in ImpactVector™. This vector has been designed based on the green-specific Rubisco promoter and the signal peptide sequences to target E1 into the ER as described.[14]

The pDM302 (Accession no. X17 220) contains the bar gene encoding phosphinothricin acetyltransferase (PAT) as a selectable marker. The gene regulated by the rice actin 1 (Act1) promoter and nos terminator.[15]

The pApo is a binary vector targeting the CBH I gene[16] into apoplast. This vector was constructed using the *T. reesei* CBH I gene. The gene was obtained from digestion of the pMZ766-CBH I with XbaI enzyme and the released CBH I gene cassette was then ligated into pCAMBIA3303. This vector contains the CaMV 35S promoter, the tobacco mosaic virus translational enhancer (Ω), the tobacco pathogenesis-related protein 1a (Pr1a) signal peptide for apoplast targeting, the six histidines, enterokinase recognition site (EK) and the polyadenylation signal from nopaline synthase gene (3′ nos).

The pBGVac, or pUC1813,[17] contains the bglA gene[18] encoding *B. fibrisolvens* H17c β-glucosidase, the ER leading sequence, the vacuole-targeting signal peptide (VT), and the CaMV 35S promoter and terminator.

The pGreen[19] is a binary vector containing the bar selectable marker gene regulated by the CaMV 35S promoter and nos terminator, and the FLOWERING LOCUS C (FLC) gene regulated

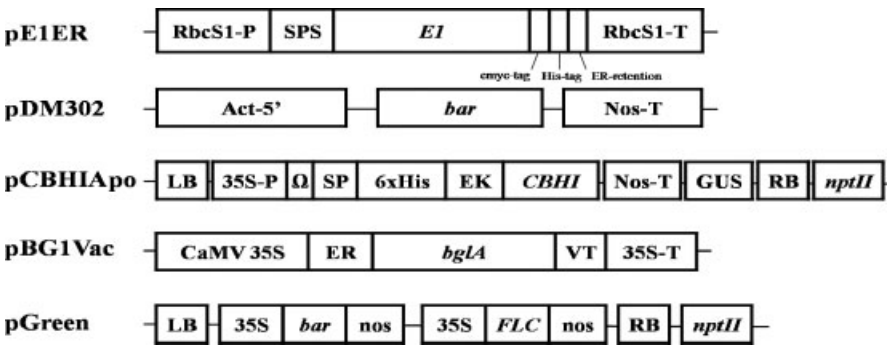

Figure 1. Schematic drawing of the plasmid vectors E1, CBH I and cellobiase (*bg1A*). Plasmids include pE1ER: Plasmid containing the *A. cellulolyticus* E1[13] targeted into ER regulated by green tissue-specific rubisco promoter; pDM302: plasmid containing the bar selectable marker gene regulated by rice actin promoter and introns; pCBH-IApo: plasmid containing the *T. reesei* CBH I[46] targeted into apoplast, and six histidine tags were included to purify the protein to send to our industry partner; pBG1Vac: plasmid containing the *Butyrivibrio fibrisolvens* cellobiase[18] targeted into vacuole; and pGreen[19]: plasmid containing the bar and the FLOWERING LOCUS C (FLC) genes, each regulated by 35S promoter.

by the CaMV 35S promoter and nos terminator. This vector also contains T-DNA left and right borders and carries the nptII gene for bacterial resistance to Kanamycin.

Corn genetic transformation and production of transgenic progenies

Highly proliferating, immature-embryo-derived Hi II embryogenic corn calli were co-bombarded via the Biolistic™ gun with a 1 : 1 ratio of the pE1ER, pCBH-IApo or pBGVac, and either the pDM302[15] or pGreen constructs[19] containing the bar herbicide resistance selectable marker gene. *In vitro* culture, phosphinothricin (PPT) resistant callus was selected based on our standard procedures.[20] The herbicide resistant plants were acclimated in a growth chamber, and then transferred to a greenhouse until maturity. Fertile first generation transgenic plants were self-pollinated and seeds were harvested 35–45 days after pollination, when they were dry.

Transgene integration and transcription analyses

PCR analyses were performed on both first (T0) and second (T1) generation transgenic plants to confirm the presence of transgenes. Northern blotting was performed to confirm transcription of transgenes. Total RNA was isolated from putatively transgenic and wild-type control untransformed plants using Trizol reagent following the manufacturer's instructions (Invitrogen, CA). RNA gel blot analysis was carried out following modifications of our previous procedure.[20]

Preparation of crude plant protein extracts and western blotting

Proteins were extracted from wild-type control untransformed and T0 E1 transgenic leaf tissues as previously described.[14] For crude protein extraction from T0 CBH I transgenic corn, 100 mg of leaf disks was ground in four volumes of ice-cold extraction buffer. The extract buffer contained 80 mmol L^{-1} MES, pH 5.5, 10 mmol L^{-1} 2-mercaptoethanol, 10 mmol L^{-1} EDTA 0.1% sodium *N*-lauroylsarcosinate, 0.1% Triton X-100, 1 mmol L^{-1} PMSF, 10 mol L^{-1} leupeptin, and 1 g mL^{-1} each of aprotinin, pepstatin A, and chymostatin. The supernatant from the crude extract, which was centrifuged at 15 000 *g* and 4 °C for 10 min, was quantified using the Bradford method.[21]

The Invitrogen NuPAGE® Bis-Tris Discontinuous Buffer System with a 10% NuPAGE® Novex Bis-Tris Pre-Cast Gel was used for Western blotting of T0 transgenic plants according to the manufacturer instruction (Invitrogen, CA).

Biological activities of heterologous E1, CBH I and cellobiase

The biological activities of heterologous E1 and CBH I were measured in T0 transgenic plants following our previous research.[2] Briefly, 10 µL of a set of diluted crude protein containing each heterologous cellulase extract was mixed with 100 µL reaction buffer (50 mmol L^{-1} sodium acetate pH 5.0 containing 1.0 mmol L^{-1} of substrate MUC, 4-methylumbelliferone β-D-cellobioside) in 96-well plates. Plates were covered and incubated at 65 °C in the dark for 30 min. Then, 100 µL of stop buffer (100 mmol L^{-1} glycine, pH 10.3) was added and the fluorophore 4-methylumbelliferone (MU; the product of E1 or CBH I hydrolysis of the substrate MUC) was measured by reading the fluorescence at 465 nm using a SPECTRAmax M2 device (Molecular Devices Inc., CA) at 360 nm excitation wavelength. After subtracting the background, the activity of each sample was calculated using a MU standard curve, which contributed to deactivated enzyme extract.

The biological activity of heterologous cellobiase of T0 plants was measured via the modification of our standard procedure,[14] measuring the hydrolysis of *p*-nitro-phenyl-β-D-glucopyranoside (*p*NPβG), The incubation mixture included 2 mmol L^{-1} *p*NPβG, 50 mmol L^{-1} sodium phosphate buffer (pH 6.5) and 30 µL crude protein in a total volume of 100 µL. The reaction was conducted at 40 °C for 15 min and stopped by the addition of 300 µL 1.0 mol L^{-1} Na_2CO_3. The amount of *p*-nitrophenol (*p*NP) released was determined using a spectrophotometer via measuring the absorbance of the solution at 415 nm. Standard solutions between 0 and 100 nmol *p*NP were also included.

Percentage heterologous E1 and cellobiase in plant crude protein extracts

The percentage of heterologous E1 in crude protein extract was measured in T0 transgenic plants based on densitometry analysis of Western blot X-ray film. The percentages of the heterologous cellobiase in crude protein extract was measured via the standard curve representing the biological activities of different dilutions of the purified *A. niger* cellobiase[22] (80% pure; isolated from Novozyme™ 188).

Estimation of heterologous cellulases per ton of dry mature corn stover versus corn silage

Based on their plant crude protein extracts, two reports were used to estimate the amount of heterologous cellulases per ton of dry mature corn stover versus corn silage. The first report is from the Department of Animal Science at North Carolina State University (http://www.agr.state.nc.us/drought/documents/InterpretingForageAnalysisReportsforcornstalks.pdf). We calculated the amount of heterologous cellulases based on this report showing that 5% of dry mature corn stover is proteins, and approximately 40% of these proteins are water soluble (total soluble proteins). The second report is from Manitoba Agriculture, Food and Rural Initiatives (http://www.gov.mb.ca/agriculture/crops/specialcrops/bii01s02.html) which indicates that about 9.4% of corn silage is proteins.

Optimization of ratio of E1 to CBH I for maximizing CMC conversion

Different ratios of E1 to CBH I in T0 transgenic plants were used in order to find an ideal ratio for carboxymethyl cellulose (CMC) conversion. The enzymatic hydrolysis experiment took place in a vial containing 1% CMC (Sigma-Aldrich, St Louis, MO) substrate in a 15 mL reaction buffer (7.5 mL of 100 mmol L^{-1} sodium citrate buffer, pH 4.8). In addition, 60 µL (600 µg) tetracycline and 45 µL (450 µg) cycloheximide were added to each vial to prevent the growth of microorganisms during incubation and hydrolysis reaction. The reaction was supplemented with *A. niger* cellobiase (Novozyme™ 188) to convert the cellobiose to glucose. Distilled water was added to bring the total volume in each vial to 15 mL. All reactions were performed in duplicate to test reproducibility. The hydrolysis reaction was carried out at 50 °C with a shaker speed of 90 rpm. About 1 mL of each sample was taken out from the hydrolysis reaction after 72 h of hydrolysis, and filtered using a 0.2 µm syringe filter and kept frozen. The amount of glucose produced in the enzyme blank and substrate blank were subtracted from the respective

hydrolyzed glucose levels. The equivalent glucose concentration was quantified using a glucose analyzer (YSI 2700 SELECT™ Biochemistry Analyzer, Yellow Springs, OH) using glucose as the standard.

Optimization of ratio of E1 to CBH I to cellobiase for maximizing AFEX pretreated corn stover conversion

The DNS assay was employed to quantify the reducing sugar produced as the result of enzymatic hydrolysis, determining the optimum ratio of all three heterologous enzymes produced in T0 transgenic plants on conversion of AFEX pretreated corn stover into fermentable sugars.[23] DNS is a colorimetric reagent used in standard assays to detect reducing sugars. For conversion, 1% glucan loading equivalent AFEX pretreated corn stover was hydrolyzed using the microplate hydrolysis conditions as described elsewhere.[11] Also, different ratios of E1 : CBH I : cellobiase were produced by diluting of crude proteins of different transgenic plants. Each of the different crude cellulase mix ratios was added to 1% glucan loading equivalent AFEX pretreated corn stover in microplates. After hydrolysis, a 50 µL sample supernatant from each vial was taken and placed in each well of a 96-well plate, 100 µL DNS was added to each well, and the color was developed at 100 °C for 30 min.[11,22] Heat resistant sticky film lid was used to cover the 96-well plate prior to heating to avoid evaporation. The reading was done with 100 µL sub-samples using a UV spectrophotometer at 540 nm. The readings were compared with glucose standards, and the actual percentage AFEX pretreated corn stover conversion into glucose equivalents was calculated. In these assays, the enzyme and substrate blanks were included, and all reactions were done in triplicate.

T0 transgenic E1, CBH I and cellobiose were self-bred for production of T1 plants, and seeds were collected for further analyses. PCR analyses were performed to confirm the transfer of each transgene into its next generation.

RESULTS

Plant genetic engineering followed by confirmation of transgene integration and expression

Herbicide resistant transgenic corn plants were produced from immature embryo-derived cell lines biolistically co-bombarded with each of the three constructs (pE1ER, pCBH-IApo, and pBG1Vac) containing the cellulase genes and one of the two constructs containing the bar gene (pDM302 and pGreen). We also produced several CBH I independent transgenic tobacco plants via the *Agrobacterium* transformation system because most independent transgenic CBH I corn lines died prior to the completion of our studies due to our greenhouse conditions. Polymerase chain reaction (PCR) analysis of herbicide resistant plants confirmed the presence of E1 gene in plants (data not shown), and Northern blotting confirmed the E1 transcription (Fig. 2(a)) in leaves of PCR positive plants. The production of heterologous E1 protein was confirmed via Western blotting using monoclonal E1 antibody (Fig. 2(a)).

A total of 30 mature independent CBH I transgenic corn lines were produced. Prior to death of some of these plants, PCR analysis of CBH I confirmed the presence and Northern blotting confirmed the transcription of CBH I transgene in corn plants (Fig. 2(b)). In addition, PCR confirmed the presence, and Western blotting confirmed the production of heterologous CBH I protein in tobacco plants (Fig. 2(c)).

A total of 35 mature independent corn cellobiase transgenic lines were produced. PCR analysis confirmed the presence, and Northern blotting confirmed the transcription of cellobiase transgene in corn plants (Fig. 1(d)).

Biological activities of heterologous cellulases

Biological activity of each of the heterologous cellulases is shown in Fig. 3. In Fig. 3(a), enzymatic activity of E1 was measured in leaves of transgenic corn plants. One unit of E1 activity is defined by measuring the amount of 4MU released from reaction of one

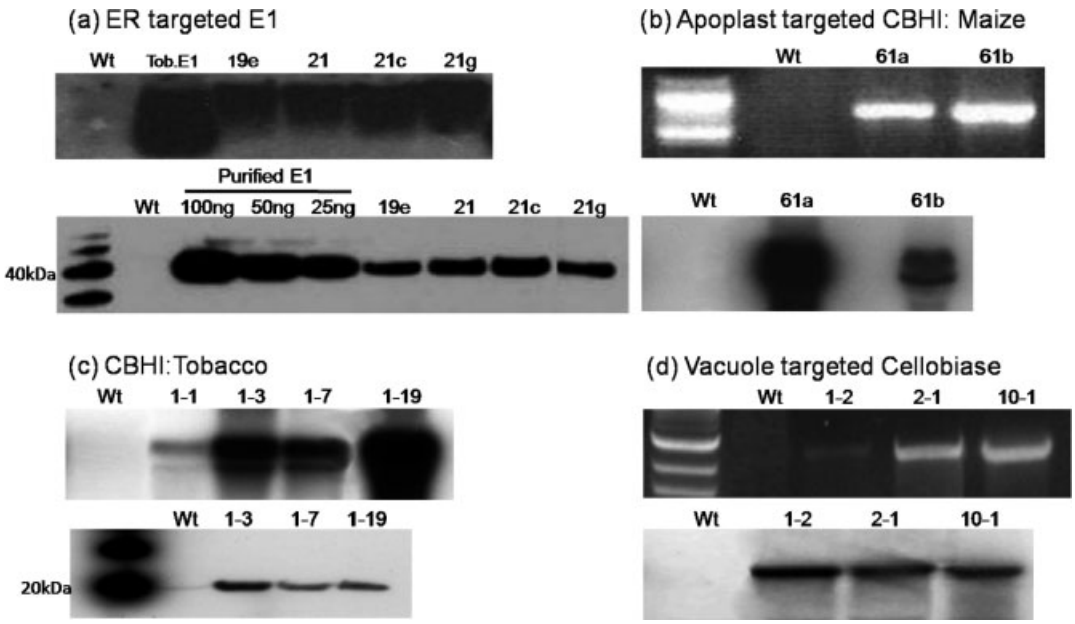

Figure 2. Molecular analyses of E1, CBH I and cellobiase. In all experiments, Wt means wild-type untransformed control plant leaf. (a) E1 Northern blot analysis (top. Tob. E1; E1 heterologous tobacco) and Western blot analysis (bottom) with three different purified E1 concentrations (100 ng, 50 ng, 25 ng) compared with the heterologous E1. (b) CBH I PCR analysis (top) and Northern blot analysis (bottom). (c) Tobacco heterologous CBH I Northern blot analysis (top) and Western blot analysis with 6xhistidine antibody (bottom). (d) Cellobiase PCR analysis (top) and Northern blot analysis (bottom).

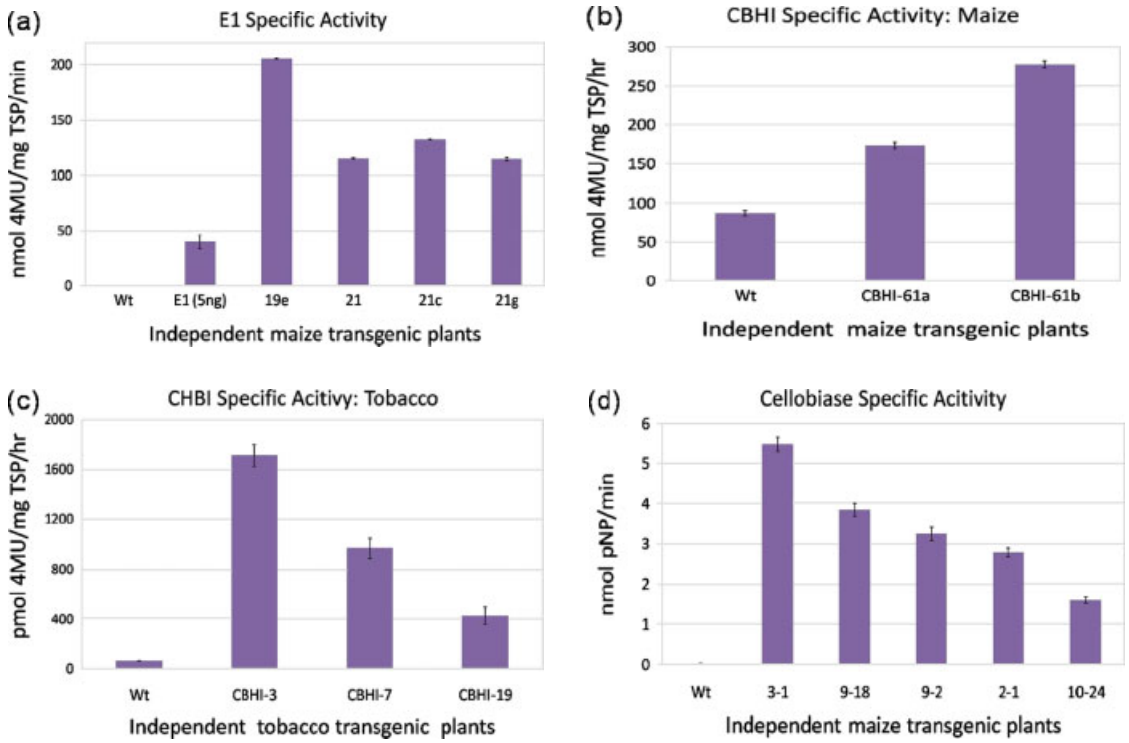

Figure 3. Heterologous E1, CBH I and cellobiase enzymatic activity assays. (a) Corn heterologous E1 activity. (b) Corn heterologous CBH I activity. (c) Tobacco heterologous CBH I activity. (d) Corn heterologous cellobiase activity. TSP means plant total soluble protein or crude protein extract. Mean ± standard deviation ($P < 0.05$, $n = 3$).

mg of plant total soluble protein (TSP or crude protein extract) added into 1 mmol L^{-1} of 4MUC in 1 min. Figure 3(a) confirms no activity in the wild-type control leaf while leaves from different independent transgenic E1 lines show different levels of activities, with line 19e showing the highest (205 nmol 4MU mg^{-1} TSP min^{-1}).

Enzymatic activity of CBH I was measured in leaves of transgenic corn and transgenic tobacco plants (Fig. 3(b) and 3(c)). In Fig. 3(b), one unit of CBH I activity is defined by measuring the amount of 4MU released from reaction of 1 mg of crude protein added into 1 mmol L^{-1} of 4MUC in 1 h. Although wild-type control plant leaf shows a small amount of CBH I activity, transgenic corn leaves (61a and 61b) show 1.5 to 2.5 times greater activity compared with their wild-type control plant leaf. In Fig. 3(c) we used one unit of CBH I activity as defined by measuring the amount of 4MU released from reaction of 1 pmol of crude protein added into 1 mmol L^{-1} of 4MUC in 1 h. Transgenic tobacco leaf (line 1–3) shows 25 times greater activity than its wild-type control tobacco plant leaf (Fig. 3(c)). Overall, the activity of heterologous CBH I was much lower in transgenic corn than transgenic tobacco.

In Fig. 3(d), enzymatic activity of cellobiase was measured in leaves of transgenic corn. In Fig. 3(d), one unit of cellobiase activity is defined by measuring the amount of pNP released from reaction of 1 mg of crude protein added into 1 mmol L^{-1} of *p*NPβG in 1 min. Figure 3(d) confirms that the wild-type control plant leaf had no activity while different independent transgenic corn cellobiase lines show different levels of activities, with line 3-1 showing the highest (5.475 nmol pNPU min^{-1}).

We must indicate that the units for measuring the tobacco (Fig. 3(c)) and corn (Fig. 3(b)) heterologous CBH I are very different. While corn heterologous CBH I was measured in nmol, tobacco heterologous CBH I was measured in pmol due to its low activity.

Carboxymethyl cellulose (CMC) conversion using heterologous cellulases

CMC substrate conversion into low molecular weight reducing sugars was performed using the corn crude protein containing heterologous E1 or cellobiase. Figure 4(a) shows that the four corn E1 transgenic lines tested have significantly higher CMC conversion as compared to the wild-type control corn plant. Figure 4(a) shows that the crude protein containing corn-produced heterologous E1 tested displays higher CMC conversion capacities, and Fig. 4(b) shows that the crude protein containing heterologous cellobiase displays higher cellobiose conversion as compared to the wild-type control crude protein.

Multicellulase enzyme mix ratio optimization for CMC and AFEX-pretreated corn stover conversion

It has been well documented that different cellulases work together synergistically to decrystallize and hydrolyze cellulose, and also much more CBH I enzyme is required for optimal conversion. Therefore, different ratios of E1 : CBH I (1 : 4, 1 : 10 and 1 : 15) based on total protein concentration were used in the hydrolytic conversion of soluble cellulose CMC to glucose. The total proteins were extracted from E1 and CBH I transgenic tobacco plants, respectively. Figure 5(a) shows that the ratio of 1 : 4 of E1 : CBH I was the most effective ratio in cellulose-to-glucose conversion.

The ultimate goal of producing hydrolytic enzymes in plants is to use them in actual cellulosic biomass conversion. Therefore, various combinations of corn-produced E1, CBH I and cellobiase enzyme isolates were tested on AFEX pretreated corn stover representing 1% glucan in 24 h hydrolysis reaction. Figure 5(b) shows the amount of reducing sugars estimated by dinitrosalicylic acid (DNS) assay, and the best ratio of E1 : CBH

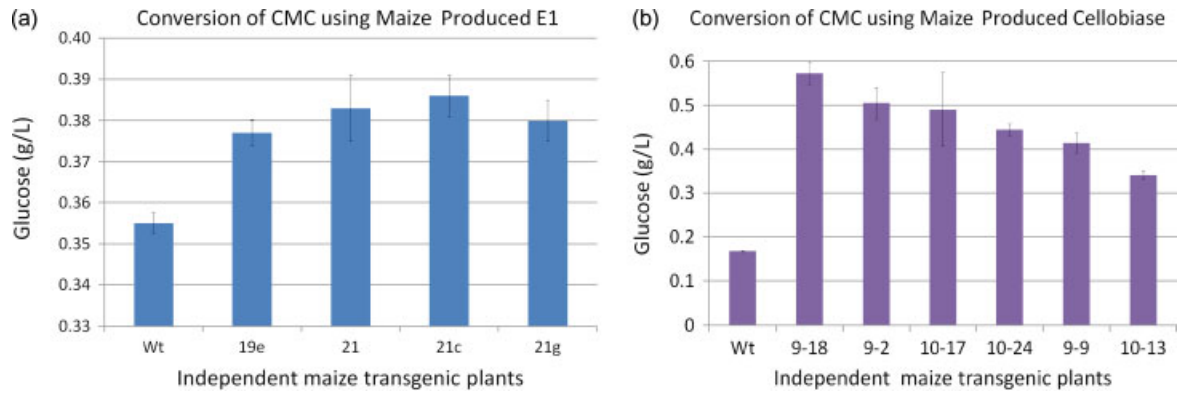

Figure 4. Glucose conversion assays of heterologous E1 using CMC (a) and cellobiase using cellobiose (b) as substrate. Mean ± standard deviation ($P < 0.05$, $n = 3$). Note: Fig. 4(b) is a modified version of Fig. 5 of a previous article.[10].

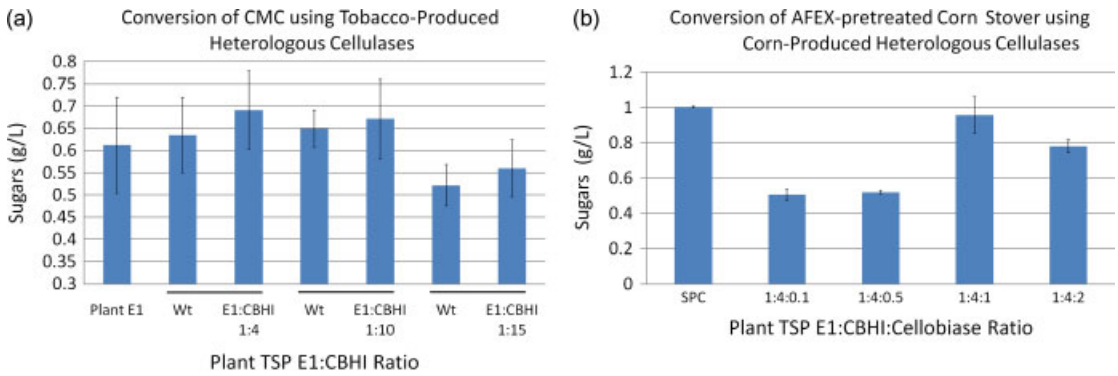

Figure 5. Heterologous multicellulase ratio optimization. (a) E1 and CBH I ratio optimization using CMC substrate incubated at 50 °C and 90 rpm shaking for 72 h. In this graph, plant E1 means tobacco-produced heterologous E1 used as positive control. Commercial Novozyme™ 188 (*A. niger* cellobiase) was added to heterologous E1 or E1 : CBH I crude protein mixtures because accumulation of cellobiose inhibits the conversion of CMC into fermentable sugars; (b) SCP means commercial Spezyme CP (a mixture of endo and exo-glucanase) mixed with commercial β-glucosidase (Novozyme™ 188). The E1 : CBH I : cellobiase ratio optimization was performed via DNS assay using AFEX-pretreated corn stover representing 1% glucan as described in the methods section.

I : cellobiase tested appears to be 1 : 4 : 1, with release of nearly 1 g L^{-1} glucose equivalents. Although the biological activities of CBH I was relatively low, the conversion activity of the three plant-produced crude heterologous enzymes at 1 : 4 : 1 ratio shows similar conversion effectiveness to the commercial enzyme Spezyme CP (SCP), meaning that the heterologous enzyme mixtures have the potential to substitute or at least be used as supplements to commercially available cellulase mixtures.

Since the heterologous multicellulase enzyme mix shows efficient conversion of pretreated corn stover, it is worthwhile to have estimations of heterologous cellulase productions in mature corn stover dry matter versus corn silage. Table 1 represents the amount of heterologous cellulases which could have been produced per ton of dry mature corn stover versus corn silage.

Using densitometry analysis, the heterologous E1 protein production was estimated to be up to 2% of transgenic corn leaf crude protein. Based on our calculations, the heterologous E1 could be produced up to 400 g per ton of dry mature corn stover and 752 g per ton of corn silage.

The heterologous cellobiase protein produced was estimated up to 3.11% of transgenic plant leaf crude protein extract. Based on our calculations, the heterologous cellobiase could be produced up to 622 g per ton of dry mature corn stover and at 1165 g per ton of corn silage.

DISCUSSION

Corn-produced heterologous multi-cellulases as a value-added biobased product

The demands for cellulosic biofuels as petroleum alternatives have surged within the last few decades. Despite efforts made to date to increase the productivity of cellulase-producing microbes through genetic engineering, the high costs of microbial cellulase enzyme production still impede the commercialization of cellulosic ethanol industries. The production of microbial E1and CBH I in different plants has already been reported,[3] and human and corn cellobiase genes have been expressed in tobacco.[24,25]

A. cellulolyticus E1 is thermostable, which helps it to endure the relatively high temperature of pretreatment processes (example; AFEX pretreatment), and shows high specific affinity to cellulose derivatives such as CMC[26] which was used in our studies for E1 enzymatic activity tests.

In this work, we have targeted the *A. cellulolyticus* E1 into corn ER. Our recent report[14] indicated that the ER targeting is suitable for the accumulation of heterologous E1 because of the fact that ER is the first site for protein synthesis and is known to contain a series of molecular chaperones such as the ER Luminal Binding Protein (BiP) needed during protein folding, assembly and preventing the transport of immature protein molecules.[27–29]

We have targeted *T. reesei* CBH I into corn apoplast because this sub-cellular compartment is a free diffusional space outside of the

Table 1. Estimation of heterologous cellulase productions in dry mature corn stover versus corn silage

Heterologous cellulase	Transgenic lines	% cellulase in crude protein extract	Approximate heterologous cellulases (g)/ton dry mature corn stover	Approximate heterologous cellulases (g)/ton corn silage
E1	5a	2.0	400	752
	19e	0.2	33	75.2
	21	0.2	33	75.2
	21c	0.3	67	112.8
	21g	0.7	133	263.2
Cellobiase	3-1	3.11	622	1165.6
	9-18	2.2	436	827.2
	9-2	1.8	368	676.8
	2-1	1.6	314	601.6
	10-24	0.9	182	338.4

plasma membrane meaning that it has the ability to accumulate large quantities of foreign proteins. The filamentous fungus *T. reesei* is considered to be the most efficient cell wall degrading microbe, encoding for only 10 cellulolytic enzymes including cellobiohydrolases.[30,31] About 80–85% (40 g L^{-1}) of genetically modified *T. reesei* extracellular proteins is cellobiohydrolases, among which 50–60% are CBH I.[32] In fact, due to its importance, CBH I enzyme quantity has been increased up to 1.5-fold via genetic engineering of *T. reesei*.[5]

We have targeted the third heterologous cellulase, cellobiase, into corn vacuoles because vacuoles occupy 30–90% (depending on plant maturity) of the cell volume, and therefore more heterologous proteins may accumulate in mature transgenic plants. We selected the cellobiase gene from bovine rumen *B. fibrisolvens* H17c[18] because its enzyme assists in enabling the conversion of cellulosic matter of silage feed into energy in rumen.

Using biologically active crude heterologous cellulases for saccharifying cellulosic biomass

It would have been ideal to use mixtures of pure E1, CBH I and cellobiase as positive controls in Fig. 3. However, we only had pure E1 available in our laboratory. Figure 3 shows the biological activities of heterologous E1, CBH I and cellobiase. We used commercial pure microbial E1 (provided by National Renewable Energy Laboratory; NREL) as positive control in Fig. 3(a). We also used a commercially available pure E1-CBH I mixture (SCP) and an impure commercial microbial cellobiase as positive control (Fig. 5(b)).

Corn plants contain exo-glucanase genes and therefore exhibit background exo-glucanase activities.[33] It is also possible that wild-type tobacco plants have exo-cellulase activities. These might be the reasons that the wild-type corn (Fig. 3(b)) and tobacco (Fig. 3(c)) plants have shown some exo-glucanase biological activities. Also, corn contains endo-glucanase[34] and β-glucosidase (cellobiase) genes.[25] The reason that the wild-type corn plants did not show any biological activity of E1 (Fig. 3(a)) or cellobiase (Fig. 3(d)) might be because either these two genes were not on to produce these enzymes when we harvested the plant leaves for analysis, or the amount of activity of these endogenous cellulases were not sufficient for detection. The activity assay for detecting E1 and CBH I were the same.

In Fig. 3(a), we show the biological activity of E1 in nmol 4MU mg^{-1} TSP min^{-1}. However, in Fig. 3(b), we show the biological activity of E1 in nmol 4MU mg^{-1} TSP h^{-1} because the heterologous E1 had much more activity as compared to the heterologous CBH I, and therefore less time is needed for the analysis of the heterologous E1. For the activity assay, we used EDTA in our extraction buffer for production of E1 and CBH I crude proteins. Considering that EDTA is known to partially inhibit the biological activities of cellulases,[35] the biological activity of heterologous cellulases produced in plants in our studies might have been much more, should we have used an alternative to EDTA in our extraction buffer.

To calculate the biological activity of each heterologous cellulase in unit, we used equal amounts of crude plant proteins, substrates and incubation time. There is an inconsistency between data presented in Table 1 and Fig. 3. In Fig. 3(a), the 21g column (the column related to crude protein of independent transgenic corn line) should have been higher than the 19e column because we used a higher percentage of E1 in 21g. This inconsistency might be due to the fact that non-measureable factors such as expansins and other cell wall loosening proteins in crude protein extracts of different independent transgenic lines might have been different in 21g compared with 19e.

Crude heterologous cellulase mix ratio

At present, a naturally produced mixture of endo-glucanase, exo-glucanase and cellobiase is extracted from microbes and added to pretreated corn stover for enzymatic hydrolysis. When NREL mixed pure microbial E1 and CBH I and added the mixture to the pretreated corn stover in different ratios, a ratio of 1 : 17 (E1-CBH I) resulted in the highest level of fermentable sugars produced (communication with Dr Michael Himmel of NREL). Therefore, one of our research goals was to find the optimal ratio of plant-produced heterologous cellulases on AFEX-pretreated corn stover for production of fermentable sugars.

We learned that a ratio of 1 : 4 of the crude E1 to CBH I was needed for production of the highest level of glucose. Crude cellulases are advantageous over using purified cellulases because plant crude proteins contain other useful molecules that cause cell wall loosening. For example, expansins[36–38] break hydrogen bonding between cellulose microfibrils or between cellulose and other cell wall polysaccharides without having any hydrolytic activity.[39] Both the amino acid sequence and the role of plant expansins are similar to those of *T. reesei* swollenin which is reported to weaken filter paper (cellulose) and disrupt other cellulosic materials such as cotton fibers.[40]

In our studies, we produced three different cellulases in three sets of independent transgenic plants, and then mixed all three plant crude proteins in a ratio of 1 : 4 : 1 (E1 : CBH I : cellobiase) for conversion of AFEX-pretreated corn stover into fermentable sugars because this ratio was most effective under our experimental conditions.

Field level estimation of corn-produced heterologous cellulases

We extrapolated the amount of heterologous cellulases that could be produced in the field per ton of mature dry corn stover (http://www1.eere.energy.gov/biomass/pdfs/Biomass%202 007%20Overview_Web.pdf) versus corn silage (http://www.gov.mb.ca/agriculture/crops/specialcrops/bii01s02.html) based on data produced from our greenhouse studies (Table 1). With these calculations, transgenic corn reported here could have produced up to 400 g of E1 and 622 g of cellobiase per ton of dry mature corn stover (third column) and up to 752 g of E1and 1165.6 g of heterologous cellobiase per ton of corn silage (fourth column).

Single cellulases gene transfer versus gene stacking

We chose to produce each cellulase enzyme in one set of transgenic corn plants instead of using transgene stacking because we wished to assure the possible effect of each transgene on plant health. We have started cross-breeding of these cellulose-producing corn plants to combine transgenes (to be reported elsewhere). Gene stacking in transgenic plants might be a good option, should one be able to control the ratio of production of heterologous cellulases produced in the same plant or to balance the ratio by adding certain cellulases. The idea of gene stacking comes from bacterial cellulosome. Cellulosome is a large extracellular enzyme complex in certain anaerobic bacteria which break down cellulose. Unlike our transgenic plants that carry different heterologous cellulases in different sub-cellular compartments, cellulosomes are produced in microbial cytosol as bacteria do not contain sub-cellular compartments. Cellulosome contains nine different cellulases on the same structural base which is a 'scaffolding protein' containing cellulose binding domains.[41] The idea of assembling several cellulases as gene stacking on a structural base could be applicable to crop plants should the optimum ratio be achieved.

Expression of multi-gene assembly also works when genes are translationally fused and transferred to chloroplast genome. Plant chloroplasts can be genetically engineered with several coding sequences controlled only under one promoter, a phenomenon that cannot occur in nuclear transgenesis as presented here. The authors hope that the problems associated with chloroplast transgenesis of cereal crops including corn will soon be resolved, because translationally fused cellulases might be even more efficient for cell wall degradation than the heterologous cellulase mix produced in our studies. For example, when the fusion cellulase (CelYZ) produced from fusion of artificial heterologous endo 1,4-glucanase (CelZ) and exo1,4-glucanase (CelY) genes, regulated by tetA promoter/operator was successfully produced in *Escherichia coli*, the hydrolytic activity of such fusion protein was 3–4-fold higher than the sum of the activity of the combined CelZ and CelY due to the intra-molecular synergism of the fused cellulases in hydrolysis of crystalline cellulosic matter.[42] This means that it would have been more beneficial, if we were able to produce the heterologous fused cellulase mix in corn chloroplasts, extract the fusion cellulase, and add to pretreated lignocellulosic matter for enzymatic hydrolysis, a cocktail of 12 heterologous hydrolytic enzymes were produced in tobacco via chloroplast transgenesis.[43]

Quest for alternatives to production of microbial cellulases

According to a National Research Council report of the US National Academies[44] the chloroplast transgenesis platform has the major advantages of (1) relatively higher heterologous protein production, (2) reducing or preventing transgene flow via pollen grain transfer in most flowering plants due to maternal inheritance of chloroplast genome, and (3) plastid genome is normally transferred via heterologous recombination allowing the site-specific insertion of transgenes in chloroplast genome, helping with reducing 'unintended phenotypic effects of transgenes'.

The nuclear transgenesis presented here and that of chloroplast transgenesis for production of multiple heterologous cellulases in tobacco[43] are expected to advance the field of cellulosic biofuels by reducing the costs associated with production of cellulases in microbial systems. This is because plants use the free solar energy for protein production while microbial bioreactors require chemical energy inputs.

The research presented here is indeed a step forward in the quest for commercialization of biomass crop-produced heterologous cellulases as an alternative or supplement to current microbial-based cellulase production for cellulosic biofuels.[45]

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