In 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 as compared to corn (maize) grain ethanol due to the high costs in 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. We have recently reported the production of heterologous *Acidothermus cellulolyticus* (E1) endo-cellulase in endoplasmic reticulum and mitochondria of green tissues of transgenic corn plants, and 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. Our results show that the ratio of 1:4:1 (E1:CBH I:Cellobiase) 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.

With the 2003 awakening report that the United States 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 U.S. 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 the USDA-DOE documents the availability of U.S. lands for annual production of one billion tons of lignocellulosic matter in order to replace 30% of the foreign oil import to the U.S. by 2030 (http://www1.eere.energy.gov/biomass/pdfs/final_billionton_vision_report2.pdf).

Plant lignocellulosic biofuels are considered as excellent alternative 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 heavily 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 order to make the 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 mainly composed of crystalline cellulose, varying mixtures of hemicellulose and lignin. Pretreatment of the

lignocellulosic biomass is necessary prior to enzymatic hydrolysis because the access of enzymes to cellulose is restricted by lignin-hemicellulose interference. Pretreatments (e.g., 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 render 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. U33212) 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, we produced biologically active *Acidothermus cellulolyticus* E1, *Trichoderma reesei* CBH I, and bovine rumen *Butyrivibrio fibrisolvens* cellobiase in three different sub-cellular compartments of three different sets of transgenic corn plants. Then, we extracted plant-produced crude proteins containing each heterologous cellulase, mixed them together and added the mixture in certain ratios to Ammonia Fiber Explosion (AFEX) pretreated corn stover.[10] We found that under our conditions, certain ratio of the heterologous multicellulase mix was the 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, we found the composition of corn stover 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. X17220) 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 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

The 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 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 described before. [14] For crude protein extraction from T0 CBH I transgenic corn, 100 mg of leaf disks was ground in 4 volumes of ice-cold extraction buffer. The extract buffer contained 80 mM MES, pH 5.5, 10 mM 2-mercaptoethanol, 10 mM EDTA 0.1% sodium *N*-lauroylsarcosinate, 0.1% Triton X-100, 1 mM PMSF, 10 M leupeptin, and 1 *g*/ml each of aprotinin, pepstatin A, and chymostatin. The supernatant from the crude extract which was centrifuged at 15,000 *g* and 4 ^{o}C for 10 min was quantified using 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 mM sodium acetate pH 5.0 containing 1.0 mM 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 mM 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 SPECTRAmax M2 device (Molecular Devices Inc., CA) at of 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 mM *p*NPβG, 50 mM 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 M 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 0-100 nmol *p*NP were also included.

Percent heterologous E1 and cellobiase in plant crude protein extracts

178 The percentage of heterologous E1 in crude protein extract was measured in T0
179 transgenic plants based on densitometry analysis of Western blot X-ray film. The percentages of
180 the heterologous cellobiase in crude protein extract was measured via the standard curve
181 representing the biological activities of different dilutions of the purified *A. niger* cellobiase [22]
182 (80% pure; isolated from Novozyme™ 188).
183
184 *Estimation of heterologous cellulases per ton of dry mature corn stover versus corn silage*
185 Based on their plant crude protein extracts, two reports were used to estimate the amount
186 of heterologous cellulases per ton of dry mature corn stover versus corn silage. The first report is
187 from the Department of Animal Science at North Carolina State University
188 (http://www.agr.state.nc.us/drought/documents/InterpretingForageAnalysisReportsforcornstalks.
189 pdf). We calculated the amount of heterologous cellulases based on this report showing that 5%
190 of dry mature corn stover is proteins, and approximately 40 % of these proteins are water soluble
191 (total soluble proteins). The second report is from Manitoba Agriculture, Food and Rural
192 Initiatives (http://www.gov.mb.ca/agriculture/crops/specialcrops/bii01s02.html) which indicates
193 that about 9.4 % of corn silage is proteins.
194
195 *Optimization of ratio of E1 to CBH I for maximizing CMC conversion*
196 Different ratios of E1 to CBH I in T0 transgenic plants were used in order to find an ideal
197 ratio for carboxymethyl cellulose (CMC) conversion. The enzymatic hydrolysis experiment took
198 place in a vial containing 1% CMC (Sigma-Aldrich, St Louis, MO) substrate in a 15 ml reaction
199 buffer (7.5 ml of 100 mM sodium citrate buffer, pH 4.8). In addition, 60 µl (600 µg) tetracycline
200 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 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 were added to 1% glucan loading equivalent AFEX pretreated corn stover in microplates. After hydrolysis, 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 resistance 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 to glucose standards, and the actual percent 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 to measure accuracy.

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. 2a) in leaves of PCR positive plants. The production of heterologous E1 protein was confirmed via Western blotting using monoclonal E1 antibody (Fig. 2a).

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. 2b). In addition, PCR confirmed the presence, and Western blotting confirmed the production of heterologous CBH I protein in tobacco plants (Fig. 2c).

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. 1d).

Biological activities of heterologous cellulases

Biological activity of each of the heterologous cellulases is shown in Fig. 3. In Fig. 3a, 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 mg of plant total soluble protein (TSP or crude protein extract) added into one mM of 4MUC in one minute. Fig. 3a 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 TSP/min).

Enzymatic activity of CBH I was measured in leaves of transgenic corn and transgenic tobacco plants (Fig. 3b and 3c). In Fig. 3b, one unit of CBH I activity is defined by measuring the amount of 4MU released from reaction of one mg of crude protein added into one mM of 4MUC in one hour. 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 as compared to their wild-type control plant leaf. In Fig. 3c, we used one unit of CBH I activity as defined by measuring the amount of 4MU released from reaction of one picomole (pmol) of crude protein added into one mM of 4MUC in one hour. Transgenic tobacco leaf (line 1-3) shows 25 times

273 greater activity than its wild-type control tobacco plant leaf (Fig. 3c). Overall, the activity of
274 heterologous CBH I was much lower in transgenic corn than transgenic tobacco.
275 In Fig. 3d, enzymatic activity of cellobiase was measured in leaves of transgenic corn. In
276 Fig. 3d, one unit of cellobiase activity is defined by measuring the amount of pNP released from
277 reaction of one mg of crude protein added into one mM of *p*NPβG in one minute. Fig. 3d
278 confirms that the wild-type control plant leaf had no activity while different independent
279 transgenic corn cellobiase lines show different levels of activities, with line 3-1 showing the
280 highest (5.475nmol pNPU/min).
281 We must indicate that the units for measuring the tobacco (Fig. 3c) and corn (Fig. 3b)
282 heterologous CBH I are very different. While corn heterologous CBH I was measured in nmol,
283 tobacco heterologous CBH I was measured in pmol due to its low activity.
284
285 *Carboxymethyl cellulose (CMC) conversion using heterologous cellulases*
286 CMC substrate conversion into low molecular weight reducing sugars was performed
287 using the corn crude protein containing heterologous E1 or cellobiase. Fig. 4a shows that the four
288 corn E1 transgenic lines tested have significantly higher CMC conversion as compared to the
289 wild-type control corn plant. Fig. 4a shows that the crude protein containing corn-produced
290 heterologous E1 tested displays higher CMC conversion capacities, and Fig. 4b shows that the
291 crude protein containing heterologous cellobiase displays higher cellobiose conversion as
292 compared to the wild-type control crude protein.
293
294 *Multicellulase enzyme mix ratio optimization for CMC and AFEX-pretreated corn stover*
295 *conversion*
296

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

304 The ultimate goal of producing hydrolytic enzymes in plants is to use them in actual
305 cellulosic biomass conversion. Therefore, various combinations of corn-produced E1, CBH I and
306 cellobiase enzyme isolates were tested on AFEX pretreated corn stover representing 1% glucan
307 in 24 h hydrolysis reaction. Fig. 5b shows the amount of reducing sugars estimated by
308 dinitrosalicylic acid (DNS) assay, and the best ratio of E1:CBH I:cellobiase tested appears to be
309 a 1:4:1, with release of nearly 1 g/L glucose equivalents. Although the biological activities of
310 CBH I was relatively low, the conversion activity of the three plant-produced crude heterologous
311 enzymes at 1:4:1 ratio shows similar conversion effectiveness as compared to the commercial
312 enzyme Spezyme CP (SCP), meaning that the heterologous enzyme mixtures have the potential
313 to substitute or at least be used as supplements to commercially available cellulase mixtures.

314 Since the heterologous multicellulase enzyme mix shows efficient conversion of
315 pretreated corn stover, it is worthwhile to have estimations of heterologous cellulase productions
316 in mature corn stover dry matter versus corn silage. Table 1 represents the amount of
317 heterologous cellulases which could have been produced per ton dry mature corn stover versus
318 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 grams per ton of dry mature corn stover and 752 grams 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 grams per ton of dry mature corn stover and at 1165 grams per ton on 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 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 have 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 report, 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

342 fact that ER is the first site for protein synthesis and is known to contain a series of molecular
343 chaperones such as the ER Luminal Binding Protein (BiP) needed during protein folding,
344 assembly and preventing the transport of immature protein molecules.[27-29]

345 We have targeted *T. reesei* CBH I into corn apoplast because this sub-cellular
346 compartment is a free diffusional space outside of the plasma membrane meaning that it has the
347 ability to accumulate large quantities of foreign proteins. The filamentous fungus *T. reesei* is
348 considered to be the most efficient cell wall degrading microbe, encoding for only 10 cellulolytic
349 enzymes including cellobiohydrolases. [30, 31] About 80-85% (40 g/L) of genetically modified *T.*
350 *reesei* extracellular proteins is cellobiohydrolases, among which 50-60% are CBH I [32]. In fact,
351 due to its importance, CBH I enzyme quantity has been increased up to 1.5 fold via genetic
352 engineering of *T. reesei.*[5]

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

358

359 *Using biologically active crude heterologous cellulases for saccharifying cellulosic biomass*

360 It would have been ideal to use mixtures of pure E1, CBH I and cellobiase as positive
361 controls in Fig. 3. However, we only had pure E1 available in our laboratory. Fig. 3 shows the
362 biological activities of heterologous E1, CBH I and cellobiase. We used commercial pure
363 microbial E1 (provided by National Renewable Energy Laboratory; NREL) as positive control in

Fig. 3a. We also used a commercially available pure E1- CBH I mixture (SCP) and an impure commercial microbial cellobiase as positive control (Fig. 5b).

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. 3b) and tobacco (Fig. 3c) 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. 3a) or cellobiase (Fig. 3d) 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. 3a, we show the biological activity of E1 in nmol 4MU/mg TSP per minute. However, in Fig. 3b, we show the biological activity of E1 in nmol 4MU/mg TSP per hour 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 amount of crude plant proteins, substrates and incubation time. There is an inconsistency between data presented in Table 1 and Fig. 3. In Fig. 3a, 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 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 as compared to 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 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%202007%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 grams of E1 and 622 grams of cellobiase per ton of dry mature corn stover (third column) and up to 752 grams of E1and 1165.6 grams 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 cellulase 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

433 microbial cytosol as bacteria do not contain sub-cellular compartments. Cellulosome contains
434 nine different cellulases on the same structural base which is a "scaffolding protein" containing
435 cellulose binding domains. [41] The idea of assembling several cellulases as gene stacking on a
436 structural base could be applicable to crop plants should the optimum ratio be achieved.

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

453

454 *Quest for alternatives to production of microbial cellulases*

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

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

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

470

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REFERENCES

1. Emons AM and Mulder BM, How the deposition of cellulose microfibrils builds cell wall architecture. Trends in plant science **5**:35-40 (2000).
2. Mei C, Park S-H, Sabzikar R, Callista Ransom CQ and Sticklen M, Green tissue-specific production of a microbial endo-cellulase in maize (Zea mays L.) endoplasmic-reticulum and mitochondria converts cellulose into fermentable sugars. John Wiley & Sons, Ltd., pp. 689-695 (2009).
3. Sticklen M, Feedstock genetic engineering for biofuels. Crop science **47**:2238-2248 (2007).
4. Chundawat SP, Vismeh R, Sharma LN, Humpula JF, da Costa Sousa L, Chambliss CK, Jones AD, Balan V and Dale BE, Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. Bioresour Technol **101**:8429-8438 (2010).
5. Miettinen-Oinonen A, Paloheimo M, Lantto R and Suominen P, Enhanced production of cellobiohydrolases in *Trichoderma reesei* and evaluation of the new preparations in biofinishing of cotton. Journal of biotechnology **116**:305-317 (2005).
6. Rojas A and Romeu A, A sequence analysis of the beta-glucosidase sub-family B. FEBS letters **378**:93-97 (1996).
7. Murray P, Aro N, Collins C, Grassick A, Penttila M, Saloheimo M and Tuohy M, Expression in *Trichoderma reesei* and characterisation of a thermostable family 3 beta-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*. Protein expression and purification **38**:248-257 (2004).
8. Fowler T and Brown RD, Jr., The bgl1 gene encoding extracellular beta-glucosidase from *Trichoderma reesei* is required for rapid induction of the cellulase complex. Molecular microbiology **6**:3225-3235 (1992).
9. Hood EE, Love R, Lane J, Bray J, Clough R, Pappu K, Drees C, Hood KR, Yoon S, Ahmad A and Howard JA, Subcellular targeting is a key condition for high-level accumulation of cellulase protein in transgenic maize seed. Plant biotechnology journal **5**:709-719 (2007).
10. Vega-Sachez ME and Ronald PC, Genetic and biotechnological approaches for biofuel crop improvement. Current Opinion in Biotechnology **21**:218-224 (2010).
11. Chundawat SP, Balan V and Dale BE, High-throughput microplate technique for enzymatic hydrolysis of lignocellulosic biomass. Biotechnol Bioeng **99**:1281-1294 (2008).
12. Chundawat SP, Venkatesh B and Dale BE, Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. Biotechnol Bioeng **96**:219-231 (2007).
13. Tucker MP, Mohagheghi A, Grohmann K and Himmel ME, Ultra-Thermostable Cellulases From Acidothermus cellulolyticus: Comparison of Temperature Optima with Previously Reported Cellulases. Nat Biotech **7**:817-820 (1989).
14. Mei C, Park SH, Sabzikar R, Qi C and Sticklen M, Green tissue-specific production of a microbial endo-cellulase in maize (*Zea mays* L.) endoplasmic-reticulum and mitochondria converts cellulose into fermentable sugars. J Chem Technol Biotechnol **84**:689-696 (2008).

15. McElroy D, Zhang W, Cao J and Wu R, Isolation of an efficient actin promoter for use in rice transformation. The Plant cell **2**:163-171 (1990).
16. Klarskov K, Piens K, Stahlberg J, Hoj PB, Beeumen JV and Claeyssens M, Cellobiohydrolase I from Trichoderma reesei: identification of an active-site nucleophile and additional information on sequence including the glycosylation pattern of the core protein. Carbohydr Res **304**:143-154 (1997).
17. Yao JQ, Genetic transformation of tobacco with a beta-glucosidase gene to induce constitutive systemic acquired resistance against tobacco mosaic virus. PhD Dissertation Western Michigan University, Kalamazoo, MI (2004).
18. Lin LL, Rumbak E, Zappe H, Thomson JA and Woods DR, Cloning, sequencing and analysis of expression of a *Butyrivibrio fibrisolvens* gene encoding a beta-glucosidase. Journal of general microbiology **136**:1567-1576 (1990).
19. Hellens RP, Edwards EA, Leyland NR, Bean S and Mullineaux PM, pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant molecular biology **42**:819-832 (2000).
20. Biswas GCG, Ransom C and Sticklen M, Expression of biologically active *Acidothermus cellulolyticus* endoglucanase in transgenic maize plants. Plant Science **171**:617-623 (2006).
21. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**:248-254 (1976).
22. Gao D, Chundawat SP, Krishnan C, Balan V and Dale BE, Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. Bioresour Technol **101**:2770-2781.
23. Cai YJ, Chapman SJ, Buswell JA and Chang S-t, Production and Distribution of Endoglucanase, Cellobiohydrolase, and beta -Glucosidase Components of the Cellulolytic System of *Volvariella volvacea*, the Edible Straw Mushroom. Appl Environ Microbiol **65**:553-559 (1999).
24. Reggi S, Marchetti S, Patti T, De Amicis F, Cariati R, Bembi B and Fogher C, Recombinant human acid beta-glucosidase stored in tobacco seed is stable, active and taken up by human fibroblasts. Plant molecular biology **57**:101-113 (2005).
25. Kiran NS, Polanska L, Fohlerova R, Mazura P, Valkova M, Smeral M, Zouhar J, Malbeck J, Dobrev PI, Machackova I and Brzobohaty B, Ectopic over-expression of the maize {beta}-glucosidase Zm-p60.1 perturbs cytokinin homeostasis in transgenic tobacco. J Exp Bot **57**:985-996 (2006).
26. Sakon J, Adney WS, Himmel ME, Thomas SR and Karplus PA, Crystal structure of thermostable family 5 endocellulase E1 from *Acidothermus cellulolyticus* in complex with cellotetraose. Biochemistry **35**:10648-10660 (1996).
27. Wrobel RL, GR OB and Boston RS, Comparative analysis of BiP gene expression in maize endosperm. Gene **204**:105-113 (1997).
28. Sticklen MB, Expediting the biofuels agenda via genetic manipulations of cellulosic bioenergy crops. Biofuels, Bioproducts and Biorefining **3**:448-455 (2009).
29. Randall JJ, Sutton DW, Hanson SF and Kemp JD, BiP and zein binding domains within the delta zein protein. Planta **221**:656-666 (2005).
30. Wang L, Zhang Y and Gao P, A novel function for the cellulose binding module of cellobiohydrolase I. Science in China **51**:620-629 (2008).

31. Claeyssens M, van Tilbeurgh H, Kamerling JP, Berg J, Vrsanska M and Biely P, Studies of the cellulolytic system of the filamentous fungus *Trichoderma reesei* QM 9414. Substrate specificity and transfer activity of endoglucanase I. The Biochemical journal **270**:251-256 (1990).
32. Durand H, Clanet M and Tiraby G, Genetic improvement of *Trichoderma reesei* for large scale cellulase production. Enzyme and microbial technology **10**:341-346 (1988).
33. Huber DJ and Nevins DJ, Exoglucanases from *Zea mays* L. seedlings: their role in β-D-glucan hydrolysis and their potential role in extension growth. Planta **155**:467-472 (1982).
34. Hatfield R and Nevis DJ, Purification and properties of an endoglucanase isolated from the cell walls of *Zea mays* seedlings. Carbohydrate Research **148**:265-278 (1986).
35. Chinedu SN, Nwinyi CO and Okochi VI, Properties of endoglucanase of *Penicillium chrysogemum* PCL501. Australian Journal of Basic and Applied Sciences **2**:738-746 (2008).
36. Shieh MW and Cosgrove DJ, Expansins. Journal of plant research **111**:149-157 (1998).
37. Li Y, Darley CP, Ongaro V, Fleming A, Schipper O, Baldauf SL and McQueen-Mason SJ, Plant expansins are a complex multigene family with an ancient evolutionary origin. Plant physiology **128**:854-864 (2002).
38. Cosgrove DJ, Li LC, Cho HT, Hoffmann-Benning S, Moore RC and Blecker D, The growing world of expansins. Plant & cell physiology **43**:1436-1444 (2002).
39. Brotman Y, Briff E, Viterbo A and Chet I, Role of swollenin, an expansin-like protein from Trichoderma, in plant root colonization. Plant physiology **147**:779-789 (2008).
40. Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyyssonen E, Bhatia A, Ward M and Penttila M, Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. European journal of biochemistry / FEBS **269**:4202-4211 (2002).
41. Doi RH and Kosugi A, Cellulosomes: plant-cell-wall-degrading enzyme complexes. Nat Rev Microbiol **2**:541-551 (2004).
42. Riedel K and Bronnenmeier K, Intramolecular synergism in an engineered exo-endo-1,4-beta-glucanase fusion protein. Molecular microbiology **28**:767-775 (1998).
43. Verma D, Kanagaraj A, Jin S, Singh ND, Kolattukudy PE and Daniell H, Chloroplast-derived enzyme cocktails hydrolyse lignocellulosic biomass and release fermentable sugars. Plant Biotechnol J **8**:332-350 (2010).
44. Kirk TK, Carlson JE, Ellstrand N, Kapuscinski AR, Lumpkin TA, Magnus DC, Nester EW, Peloquin JJ, Snow AA, Sticklen M and Turner PE, Biological confinement of genetically engineered organisms. National research council of the national academies, Washington, DC, pp. 79-81 (2004).
45. Sticklen M, Is Large-Scale Production of Biofuel Possible? . Bioscience http://www.actionbioscience.org/newfrontiers/sticklen.html (2010).
46. Klarskov K, Piens K, Stahlberg J, H PB, Van Beeumen J and Claeyssens M, Cellobiohydrolase I from *Trichoderma reesei*: Identification of an active-site nucleophile and additional information on sequence including the glycosylation pattern of the core protein. Carbohydrate Research **304**:143-154 (1997).

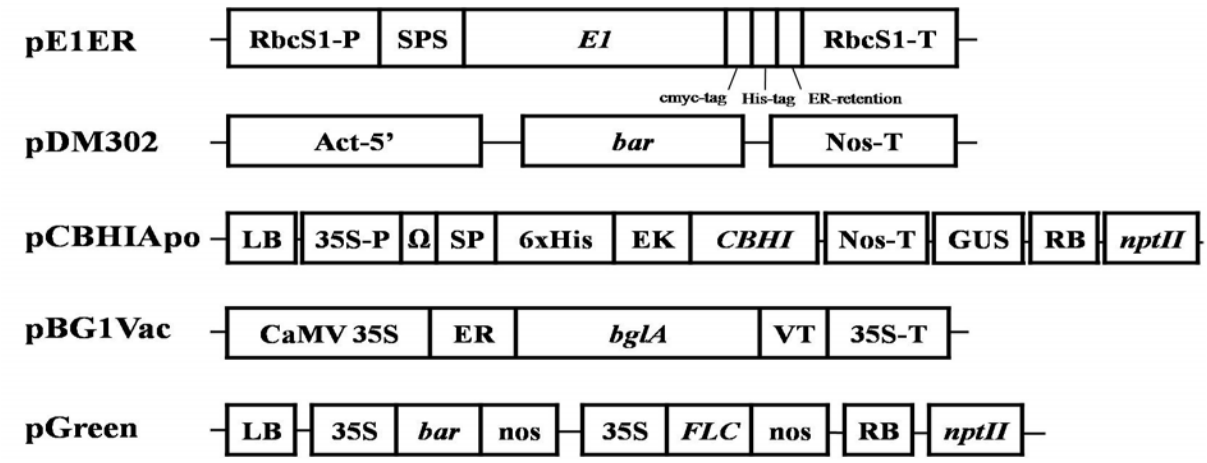

Figure 1. Schematic drawing of the plasmid vectors E1, CBH I and cellobiase (*bg1A*). Plasmids include pE1ER: Plasmid containing the *A. cellulolyticus* E1 [13] targeted into ER regulated by green tissue specific rubisco promoter; pDM302: plasmid containing the bar selectable marker gene regulated by 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.

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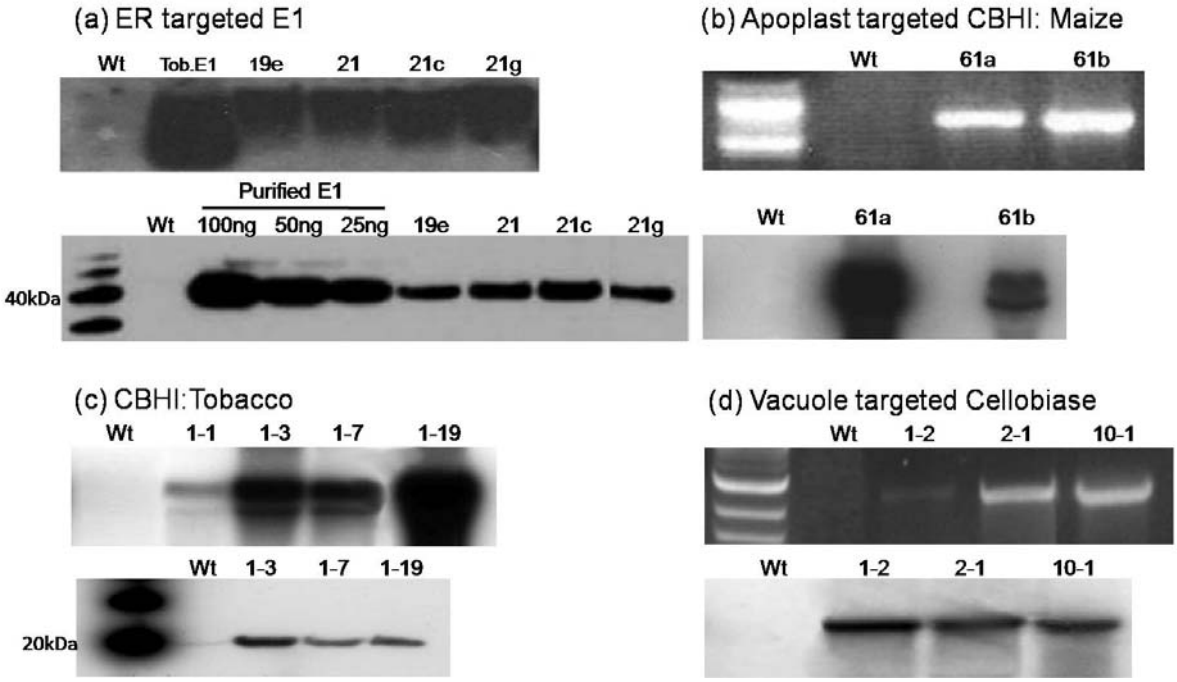

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631 **Figure 2**. Molecular analyses of E1, CBH I and cellobiase. In all experiments, Wt means wild-
632 type untransformed control plant leaf. (a) produced E1 Northern blot analysis (top. Tob. E1; E1
633 heterologous tobacco) and Western blot analysis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

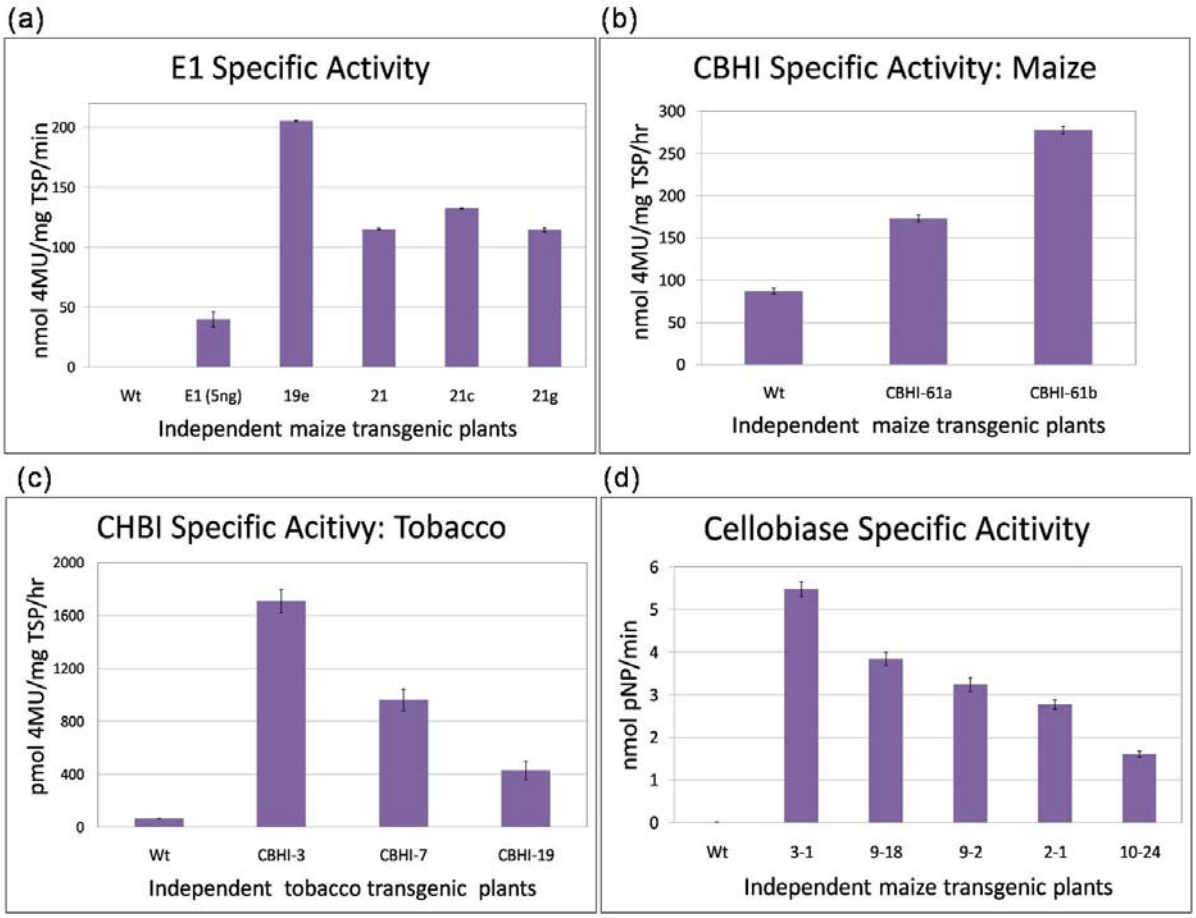
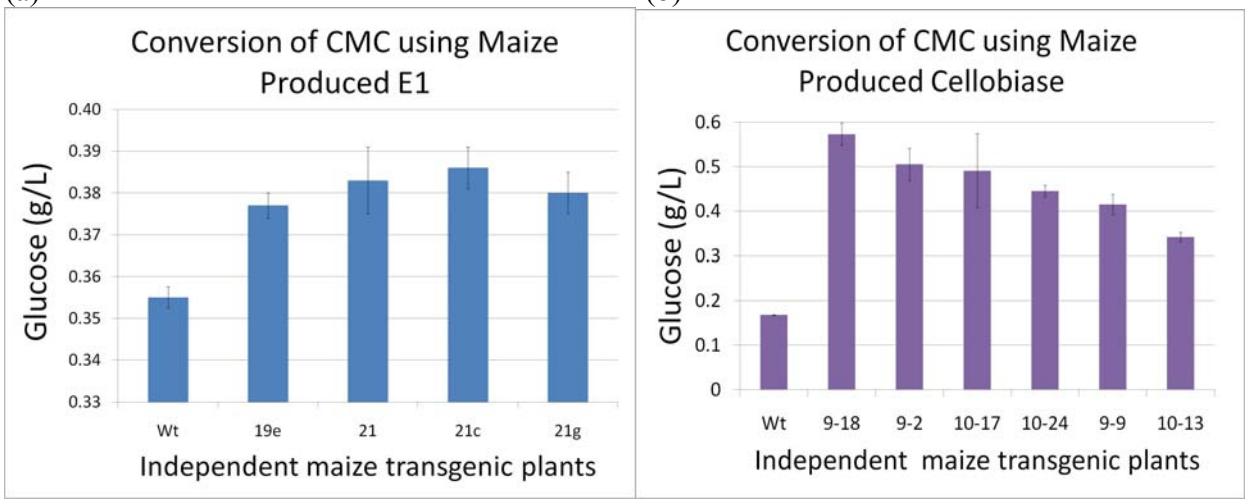


Figure 3. Heterologous E1, CBH I and cellobiase enzymatic activity assays. (a) Corn heterologous E1 activity. (b) Corn heterologous CBH I activity. (c) Tobacco heterologous CBH I activity. (d) Corn heterologous cellobiase activity. TSP means plant total soluble protein or crude protein extract. Mean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

644 (a) (b)



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

(a) (b)

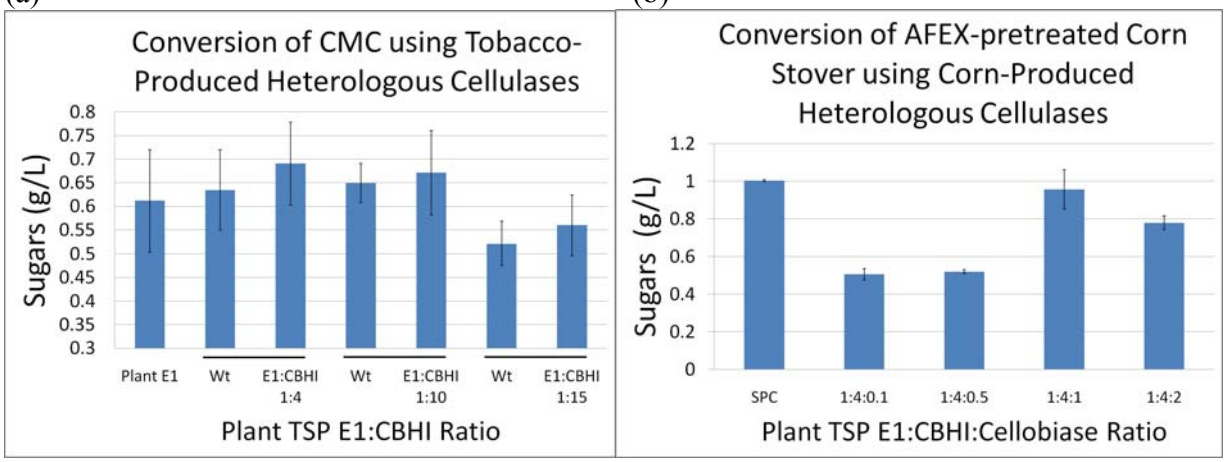

Figure 5. Heterologous multicellulase ratio optimization. (a) E1 and CBH I ratio optimization using CMC substrate incubated at 50 ºC and 90 rpm shaking for 72 h. In this graph, plant E1 means tobacco-produced heterologous E1 used as positive control. Commercial Novozyme™ 188 (*A. niger* cellobiase) was 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

662 **Table 1.** Estimation of heterologous cellulase productions in dry mature corn stover versus corn
663 silage.
664

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

665