Research Article

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Green tissue-specific production of a microbial endo-cellulase in maize (*Zea mays* L.) endoplasmic-reticulum and mitochondria converts cellulose into fermentable sugars

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Abstract

BACKGROUND: Commercial conversion of lignocellulosic biomass to fermentable sugars for biofuels and chemical byproducts uses relatively expensive bulk production of biologically active cellulase enzymes, which could alternatively be achieved by using solar energy for direct production of these enzymes within feedstock crop cellulosic biomass.

RESULTS: The *Acidothermus cellulolyticus* endo-cellulase E1 has been produced in transgenic maize plants. This heterologous enzyme was specifically targeted for accumulation into two sub-cellular compartments, endoplasmic reticulum (ER) or mitochondria of plant leaves and stalks. Furthermore, successful use of this maize-produced heterologous cellulase in converting cellulose into fermentable sugars for biofuels, has been confirmed.

CONCLUSIONS: Green-specific expression of cellulases in maize plants can avoid public controversies associated with production of transgene products in maize seeds and/or pollen. Sub-cellular targeting of cellulases may result in better expression of transgene products because these compartments, specially ER, normally contain molecular chaperones that enhance protein folding and there the biological activity. Also, using solar energy to produce cellulases within crop cellulosic biomass can replace the costly process of cellulase production in microbial bioreactors, and therefore, save costs.

Keywords: transgenic maize; corn; cellulase; sub-cellular targeting; biomass conversion; fermentable sugar

INTRODUCTION

Utilization of agricultural cellulosic biomass waste for biofuel production has drawn much interest in many science and engineering disciplines as a major supplement to 'corn ethanol'.[1,2] Ethanol produced from the starch of maize kernels has a net energy balance of about 25%.[3] However, the use of maize kernels for ethanol has greatly increased food and feed prices, and brought concerns about competition in the USA and countries that import maize products from the USA. Therefore, corn ethanol might not offer a long-term solution to US transportation fuel needs.[4]

It is believed that roughly 1.18 billion metric tons of lignocellulosic matter from crops, forest residues and energy crops could become available in the USA,[5] most of which could be used for conversion into alcohol fuels. Some estimate the global availability at 10–50 billion metric tons of crop biomass annually,[6] or approximately 411 billion liters of ethanol per year.[7]

Although production of fermentable sugars for alcohol fuels from plant lignocellulosic biomass is an exciting idea and substantial efforts have been made towards improving 'cellulosic ethanol',[8,9] major roadblocks still stand in the way of widespread commercial implementation of this technology. The roadblocks include the prohibitive costs of pretreatment processing and costs associated with production of microbial cellulase enzymes used in the conversion of cellulosic matter into fermentable sugars.[1,10]

Enzymatic hydrolysis of cellulosic matter requires at least three groups of cellulase enzymes. These cellulases include endo-cellulase or β-1,4-endoglucanase (E1; E.C. 3.2.1.4), exo-cellulase or cellobiohydrolase (E.C. 3.2.1.91), and cellobiase or β-D-glucosidase (E.C. 3.2.1.21). Currently these enzymes are produced expensively in microbial bioreactors.[10,11] Although decades of research have been devoted to reducing microbial production costs of cellulases, resulting in significant cost decreases since 1980,[12] expenses associated with production of cellulases are still high.[1] A recent report calculates that the cost of the above cellulases is approximately $0.50 per gallon of ethanol produced from lignocellosic biomass.[13] That accounts for about half of the

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maize grain ethanol production cost and is prohibitively expensive for cost-effective ethanol production. The latest projection for cost effective ethanol production made by the National Renewable Energy Laboratory (NREL) requires that cellulases be produced at $0.10 per gallon of ethanol.[14]

An alternative strategy to microbial bioreactors is to use biomass crops as biofactories for large-scale production of these enzymes.[1,15] Plants are already being used successfully for molecular farming of enzymes,[16–18] other proteins,[19] carbohydrates[20,21] and lipids.[22] The major advantage of heterologous production of cellulases within feedstock crop biomass over their production in microbial bioreactors is that plants use solar energy while bioreactors require high energy input,[1] typically from non-renewal sources.

Sub-cellular production of cellulases, especially when produced in ER, generally results in correct protein folding, glycosylation, and higher biological activity, reduced protein degradation and increased stability compared to their production in the cytosol.[1] In addition, the infrastructure and expertise for plant genetic transformation, sub-cellular targeting, growing, harvesting, transporting and processing the maize crop are already in place.[20]

To this end, the thermostable E1 transgene from *Acidothermus cellulolyticus*[23,24] has successfully been constitutively expressed in *Arabidopsis*,[25] potato,[26] tobacco,[27,28] duckweed,[29] rice[30] and maize.[31] Also, in order to optimize E1 production, more work on transcriptional, post-transcriptional and post-translational modification of E1 protein sub-cellular compartments has been carried out in tobacco plants.[32] Sub-cellular targeting of E1 cellulase and cellobiohydrolase in transgenic maize seeds have also recently been reported.[33]

In this study, E1 was produced in green tissues of maize ER and mitochondria in a green tissue-specific manner, and the heterologous E1 successfully converted cellulose into fermentable sugar, glucose.

MATERIALS AND METHODS

Transgene vectors and maize transformation

The E1 gene sequences were included in two constructs called ImpactVector™ (Fig. 1). These vectors have been designed based on light-regulated Rubisco promoter, and signal peptide sequences to target E1 into ER or mitochondria. The catalytic domain of endo-1,4-β-glucanase E1 from *A. cellulolyticus* was PCR-amplified with primers 5′-CCATGGCGGGCGGCGGCTATTGG-3′ and 5′-AGATCTGCGCCGACAGGATCGAAAATCG-3′ attached to Nco I and Bgl II (underlined) restriction enzymes, respectively. The PCR-amplified E1 gene was first cloned into pGEM Teasy vector (Promega, Wisconsin) and sequenced with T7 and SP6 primers to confirm the correct gene sequences. The amplified E1 gene was then cloned into each of these two vectors with Nco I and Bgl II enzymes. Each of the two vectors carrying the E1 gene was mixed in a 1 : 1 ratio with pDM302,[34] which contains the bar herbicide resistance marker gene regulated by rice actin promoter for production of transgenic plants to be selected for resistance to herbicide PPT (phosphinothricin). Then, the vector mixtures were co-bombarded into highly proliferating immature-embryo-derived type II maize callus[35] following a routine maize transformation and whole plant regeneration protocol.[31]

PCR and Northern blot analyses

Genomic DNA was extracted from putatively transformed leaf tissues with C-TAB as described.[36] For PCR, the oligonucleotide primers 5′-GCGGGCGGCGGCTATTG-3′ and 5′-GCCGACAGGATCGAA AATCG-3′ were designed, synthesized and used to amplify a 1.0 kb fragment spanning the catalytic domain of the endo-1,4-β-glucanase gene.

For Northern blot analysis, total RNA was extracted from leaves of transgenic plants using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Total RNAs (20 μg each) were fractionated on a 1.2% agarose gel containing formaldehyde and blotted onto a nylon membrane. Then, E1 gene-specific probe was labeled with [α-^{32}P] dCTP using the Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA, USA). Hybridization of RNA blots was carried out overnight with PerfectHyb Plus hybridization buffer (Sigma-Aldrich, St Louis) at 62 °C. Subsequently, blots were washed with 2 × SSC (1 × SSC is 0.15 mol L^{-1} NaCl plus 0.015 mol L^{-1} sodium citrate) twice for 10 min, then 0.5 × SSC and 0.5% sodium dodecyl sulfate twice for 20 min at 62 °C. Then, the blots were exposed to X-ray film at −80 °C and developed in a Kodak RP X-OMAT processor.

Total soluble protein extraction

Total soluble proteins (TSP) were extracted from wild type untransformed and E1 transgenic leaf tissues as described.[27] Briefly, 100 mg fresh leaf tissues were ground with 0.3 mL of the grinding buffer (50 mmol L^{-1} sodium acetate, 10 mmol L^{-1} EDTA, pH 5.0) and centrifuged at 20 000 *g* at 4 °C for 20 min. The supernatant was precipitated using 70% saturated ammonium sulfate, and the subsequent pellet was resuspended with grinding buffer. Extracts were quantified via a spectrophotometer following the Bradford method using a standard curve generated from bovine serum albumin (BSA).

MUCase activity assay

E1 activity was assessed as described.[27,37] Briefly, a series of soluble protein dilutions ranging from 10^{-1} to 10^{-2} were conducted. In a 96-well plate, 10 μL of a series of diluted E1 extracts were 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). Plates were covered with adhesive lids and incubated at 65 °C in the dark for 30 min. Then, the reaction was stopped with the addition of 100 μL of stop buffer (0.1 mol L^{-1} glycine, pH 10.3). The fluorophore 4-methylumbelliferone (MU), as the product of E1 hydrolysis of the substrate MUC was measured as follows. The fluorescence was read at 465 nm using a SPECTRAmax M2 device (Molecular Devices Inc., Sunnyvale, CA) at an excitation wavelength of 360 nm. The activity of each sample was calculated using a MU standard curve after subtracting background fluorescence contributed by deactivated enzyme extract.[27]

Western analysis

The Invitrogen NuPAGE® Bis-Tris Discontinuous Buffer System with a 10% NuPAGE® Novex Bis-Tris Pre-Cast Gel (Invitrogen, Carlsbad, California) was used for Western blotting. 5 μg of total soluble proteins was run on the gel and blotted onto a nitrocellulose membrane (Amersham Hybond™ ECL™, Amersham-Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The membrane was blocked with 1X PBS, 5% non-fat dry milk, 0.1% Tween-20 at room temperature for 1 h, and then incubated with primary antibody (mouse anti-E1, 1 μg mL^{-1}) at 4 °C overnight. The membrane was washed three times with 1X PBS containing 0.1% Tween-20, each time for 10 min and incubated with secondary enzyme

conjugate anti-mouse IgG : HRPO (BD Transduction Laboratories™, BD Biosciences, San Jose, CA; 1 : 2000) at room temperature for 1 h. The membrane was washed three times with 1X PBS containing 0.1% Tween-20, each time for 10 min. The Pierce SuperSignal® West Pico chemiluminescent substrate was used for detection of fluorescence signals following the manufacturer's protocol (Pierce Biotechnology, Rockford, IL). The blot was exposed to X-ray film for 1 min and developed in a Kodak RP X-OMAT processor.

CMCase activity analysis and production of glucose

The ability of heterologous E1 to convert cellulose into glucose was assessed by measuring the reaction of TSP extracted from leaves of E1-expressing maize plants with soluble cellulose (carboxymethyl cellulose, CMC) substrate.[37,38] The enzyme hydrolysis was performed in a sealed scintillation vial. A reaction buffer (7.5 mL of 0.1 mol L^{-1}, pH 4.8 sodium citrate buffer) was added to each vial. In addition, 60 µL (600 µg) tetracycline and 45 µL (450 µg) cycloheximide were added to prevent the growth of microorganisms during incubation and hydrolysis reaction. 1–3 mg of TSP was used in the enzymatic hydrolysis experiment containing 1% CMC substrate in a final 15.0 mL reaction volume.

Two concentrations of commercial E1 (100 ng and 200 ng) were also used as positive control. In addition, a total of 38 µL of commercial β-glucosidase (Novyzyme 188, >250 U g^{-1}) was included in each sample to avoid inhibition of reaction production by cellobiose.[38]

All reactions were performed in three replications. The hydrolysis reaction was carried out at 50 °C with a shaker speed of 90 rpm. About 1.0 mL of each sample was collected after 72 h of hydrolysis, boiled for 20 min and centrifuged at 14 000 rpm for 10 min. The supernatant was filtered through a 0.2 µm membrane and kept frozen until analysis. The amount of glucose produced in the enzyme blank and substrate blank were subtracted from the respective hydrolyzed glucose levels. Hydrolyzate was quantified using YSI 2300 Stat Plus Glucose and Lactate Analyzer (YSI Life Sciences, Yellow Springs, OH) with glucose as a standard.

RESULTS AND DISCUSSION

Regeneration and molecular analyses of E1 transgenic plants

Mature plants were produced from immature embryo-derived calli which were co-bombarded with each of the two E1 targeting gene constructs (Fig. 1) and the construct containing the bar herbicide resistance selectable marker gene.[34]

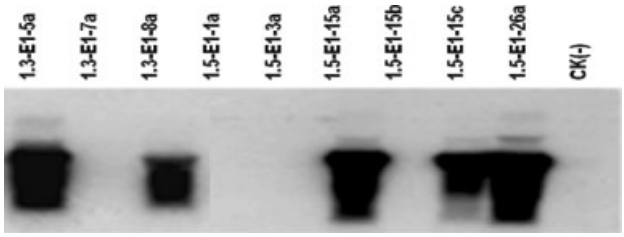

Figure 2. Northern blot analysis of representative of maize transformants.

PCR analysis of herbicide resistant plants confirmed the integration of E1 transgene in herbicide resistant plants (data not shown) and Northern blotting of transgenic plants confirmed the transcription of E1 transgene (Fig. 2).

MUCase activity assay

Figure 3 shows that the MUCase activity assays confirmed no activity in control untransformed plant TSP. However, E1 transgenic plant TSP showed different levels of MUCase activities. Furthermore, Fig. 3 confirms that the ER targeted E1 cellulase had a higher level of MUCase activity than the E1 produced in mitochondria.

Western blot analysis

Western blot analysis using the monoclonal anti-E1 antibody and plant TSP confirmed the production of E1 with the correct size band in the ER and mitochondrial cellulase targeted plants (Fig. 4). The maximum amount of the heterologous E1 protein accumulated in the ER was 2.0% and in mitochondria was 0.2% of the plant TSP (Table 1).

It is well known that ER is the first site for protein synthesis and it contains a series of protein folding enzymes including the ER Luminal Binding Protein (BiP) needed during protein translation[39] and transport and secretion of different proteins.[40] It is possible that E1 protein produced in maize ER has a higher level of E1 cellulase production than mitochondria due to the presence of endogenous proteins such as BiP.

Although not performed here, electron microscopy of the leaves of E1-producing plants labeled with monoclonal anti-E1 antibody could have assisted in showing the relative amount of accumulation of this cellulase in ER versus mitochondria in plants,

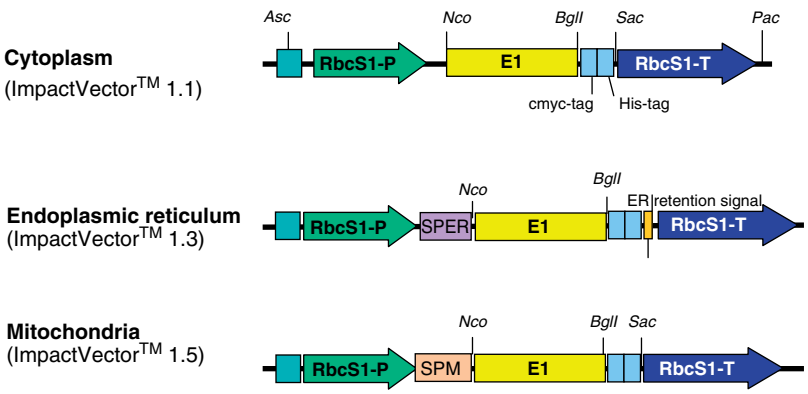

Figure 1. Transgene constructs developed for green-specific transgene expression, and ER or mitochondria targeting of heterologous E1 in maize. RbcS1-P: rubisco small subunit promoter, SPER: ER signal peptide, SPM: Mitochondrial signal peptide, c-myc: antibody tag for protein identification, His-tag: for protein purification, and RbcS1-T: rubisco small subunit terminator.

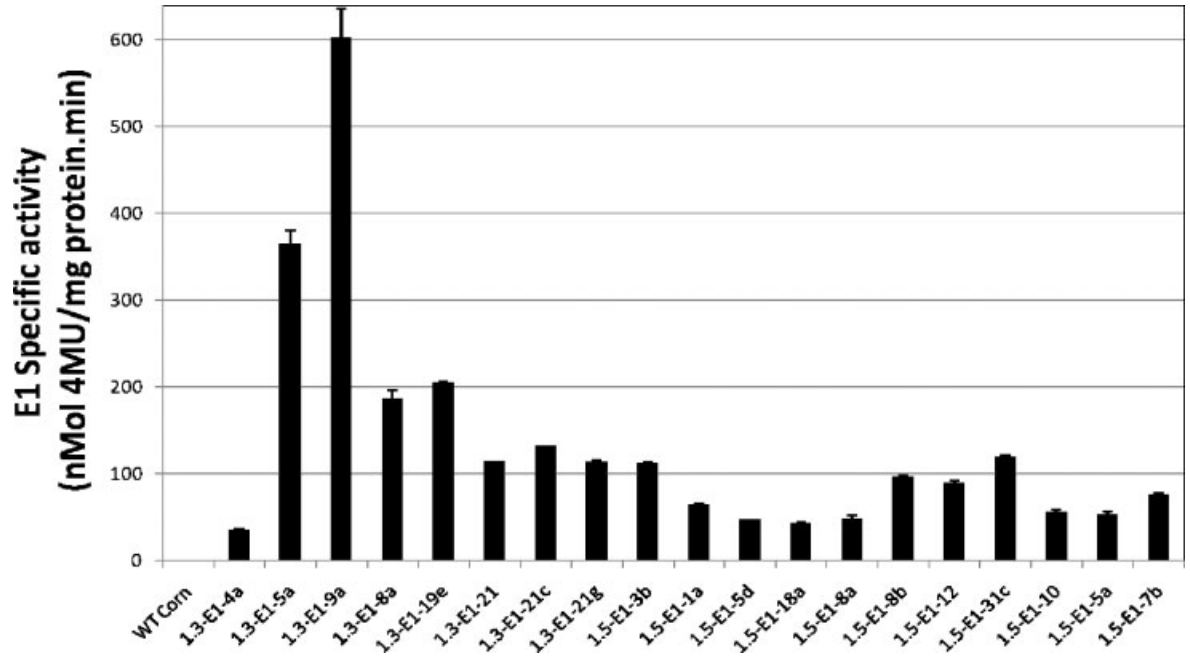

Figure 3. Enzymatic activity of herterologous E1 targeted to maize ER versus mitochondria.

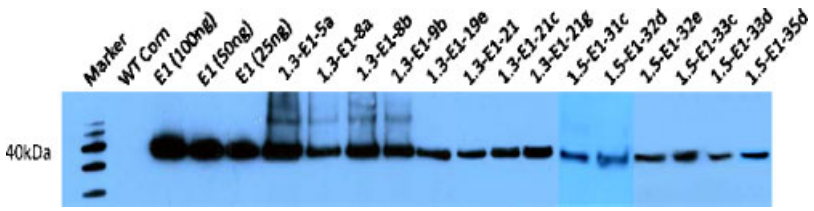

Figure 4. Western blot analysis showing the presence of the heterologous E1 enzyme targeted to ER versus mitochondria. 1 μg of total soluble protein was used for the the Western blot analysis.

as was demonstrated for the accumulation of E1 in transgenic maize leaves.[14]

CMCase activity analysis and production of glucose

When the hydrolytic conversion of the CMC substrate to glucose was compared for TSP of ER-targeted and mitochondria-targeted transgenic plants, the ER-targeted enzyme converted much higher levels of CMC than that of mitochondria. Also, the TSP of ER- and mitochondria-targeted E1 plants converted much higher levels of CMC than the TSP from wild-type untransformed plants (Fig. 5). Figure 5 shows that the wild-type non-transgenic plant TSP also converted CMC into fermentable sugar, glucose. This might be due to possible plant endogenous cellulases and/or other enzymes that assist in conversion of cellulose into glucose. This figure also shows that the heterologous E1 showed relatively higher CMCase activity than 200 ng of commercial E1 used as positive control.

Previously, E1 was produced in apoplast of rice and maize consecutively at 5% and 2% TSP[30,31] respectively and this heterologous E1 could successfully convert Ammonia Fiber Explosion (AFEX)-pretreated maize stover into fermentable sugars.[30,37] In the present studies, it was found that the ER and mitochondria are also great compartments for accumulation of E1 in maize. Results on ER targeting of E1 agree with results obtained by Hood *et al.*,[36] which indicate that the amount of E1 catalytic domain was high in ER in maize seeds. An ER-directed signal peptide in N-terminus and ER retention signal in a C-terminus was reported for accumulation of the highest levels of active *Trichoderma reesei* cellobiohydrolase I in transgenic maize seeds.[41]

The E1 is a disulfide bond protein.[42] The ER-specific protein called protein disulfide isomerase (PDI) is a protein that is specifically involved in folding of disulfide bond proteins.[43] The role of PDI is specifically to catalyze disulfide bond formation and isomerization.[44] Therefore, the ER PDI may have played a role in oxidative folding of the E1 cellulase.

Production of polysaccharide degrading enzymes within crop biomass may reduce the costs of production of these enzymes for biomass conversion into fermentable sugars. The enzymes could be extracted at the site of hydrolysis and fermentation to gain substantial reduction in the costs.[1]

A cellulase enzyme could be simultaneously produced in multiple sub-cellular compartments of the same plants for higher enzyme production level.[1,17] This strategy was tested by targeting of a heterologous xylanase enzyme into *Arabidopsis* chloroplasts or/and peroxisome, resulting in higher enzyme production when targeted to both chloroplast and peroxisome of the same plants.[45] Work is in progress on multiple sub-cellular targeting of E1 by cross-breeding of apoplast, ER and mitochondria-targeted E1 transgenic plants (unpublished).

It would also be ideal to increase the level of expression of heterologous cellulases in plants so one could mix wild-type cellulosic material with transgenic feedstock cellulosic biomass for

Table 1. Percentage of heterologous E1 in transgenic plants TSP calculated based on Western blot analysis

	Samples	μg mL^{-1}	ng (compared with purified E1s)	TSP (ng)	E1%/TSP	E1 g ton^{-1} maize stalks
1	1.3-E1-5a	1	60	3000	2.0	400
2	1.3-E1-8a	1.02	10	3000	0.3	67
3	1.3-E1-8b	0.99	25	3000	0.8	167
4	1.3-E1-9b	1	20	3000	0.7	133
5	1.3-E1-19e	1.33	5	3000	0.2	33
6	1.3-E1-21	2.4	5	3000	0.2	33
7	1.3-E1-21c	2.4	10	3000	0.3	67
8	1.3-E1-21g	2.5	20	3000	0.7	133
9	1.5-E1-31c	1.6	5	6000	0.1	17
10	1.5-E1-32d	1.3	5	6000	0.1	17
11	1.5-E1-32e	1.62	5	3000	0.2	33
12	1.5-E1-33c	1.38	5	3000	0.2	33
13	1.5-E1-33d	1.51	5	3000	0.2	33
14	1.5-E1-35d	1.41	5	6000	0.1	17

Crude proteins in maize leaves and stalks are normally about 5%.[46]

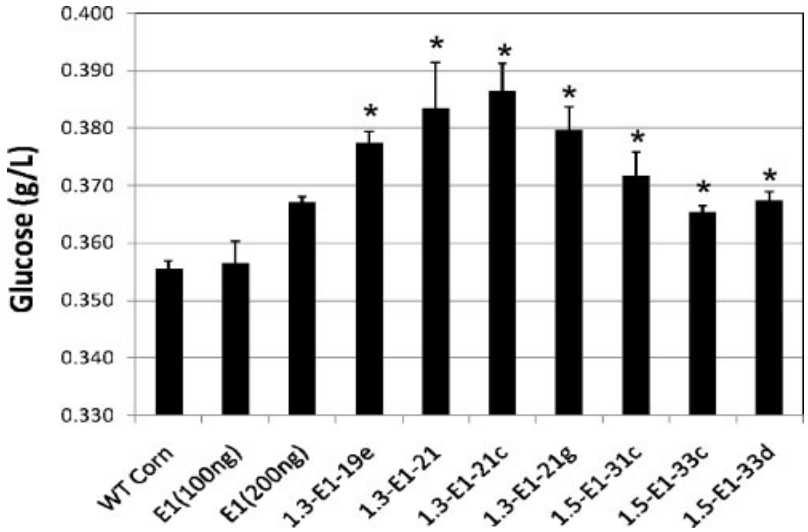

Figure 5. Conversion of cellulose-to-glucose (g L^{-1}) using maize heterologous E1 TSP compared with that of commercial E1 enzyme. The substrate used in the experiment was CMC. The enzymatic hydrolysis was performed for 72 h, at 50 °C with 90 rpm shaking. * Indicates that transgenic samples are significantly different ($P = 0.05\%$) from the wild type control.

conversion into biofuels. Several factors can affect the foreign gene expression level and protein accumulation. These include different genes obtained from different organisms, sites of transgene insertions, different vectors, and codon usage in higher plants.[33] In addition, as concluded here, sub-cellular targeting of proteins can play an important role in the level of production of heterologous proteins in transgenic plants.[1]

E1 Cellulase in maize leaves and stalks as a value-added heterologous biobased product

Table 1 shows that E1 heterologous cellulase could be produced at up to 2% plant TSP (i.e. plant total crude soluble proteins). Based on a previous report,[46] total crude protein in maize stover is 5%. Considering that about 40% of plant crude protein is soluble, transgenic maize reported here could produce heterologous E1 cellulase at up to 400 g ton^{-1} maize stover. In another report,[47] the total crude protein in maize silage was said to be 9.4%, meaning that the transgenic plants reported here could produce up to 752 g heterologous E1 cellulase ton^{-1} maize silage. These projections mean that heterologous E1, as a value added cellulose, may have increased the value of maize stover and maize silage. Therefore, the technology reported here may reduce the cost of production of cellulosic biofuels.

Production of heterologous enzymes using rubisco promoter could avoid production of these enzymes in plant seeds and pollen, and therefore reduce public concerns about the transfer of heterologous cellulases to cross-breedable plant species.[48]

Michigan State University has named the ER-targeted heterologous E1 reported here, 'Spartan Corn I' with the hope that its technology would be a step forward towards less expensive production of cellulases for cellulosic ethanol.

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