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Heterologous *Acidothermus cellulolyticus* 1,4-β-Endoglucanase E1 Produced Within the Corn Biomass Converts Corn Stover Into Glucose

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Abstract

Commercial conversion of lignocellulosic biomass to fermentable sugars requires inexpensive bulk production of biologically active cellulase enzymes, which might be achieved through direct production of these enzymes within the biomass crops. Transgenic corn plants containing the catalytic domain of *Acidothermus cellulolyticus* E1 endo-1,4-β glucanase and the *bar* bialaphos resistance coding sequences were generated after Biolistic® (BioRad Hercules, CA) bombardment of immature embryo-derived cells. *E1* sequences were regulated under the control of the cauliflower mosaic virus 35S promoter and tobacco mosaic virus translational enhancer, and E1 protein was targeted to the apoplast using the signal peptide of tobacco pathogenesis-related protein to achieve accumulation of this enzyme. The integration, expression, and segregation of *E1* and *bar* transgenes were demonstrated, respectively, through Southern and Western blotting, and progeny analyses. Accumulation of up to 1.13% of transgenic plant total soluble proteins was detected as biologically active E1 by enzymatic activity assay. The corn-produced heterologous E1 could successfully convert ammonia fiber explosion-pretreated corn stover polysaccharides into glucose as a fermentable sugar for ethanol production, confirming that the E1 enzyme is produced in its active form.

Index Entries: Ammonia fiber explosion; biomass conversion; cellulose; ethanol; transgenic maize; endoglucanase.

Introduction

According to a recent report from the Natural Resources Defense Council and the Institute for the Analysis of Global Security, the dependence of the United States on foreign petroleum both undermines its economic strength and threatens its national security *(1)*. Ethanol, obtained either

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from grain or from cellulosic materials, has the ability to decrease the need for petroleum fuel *(1)*. Accordingly, the ethanol fuel industry has been growing significantly in many countries throughout the world, and the United States ethanol production capacity reached nearly 13.4 billion L in 2004, up nearly by 1.15 billion L since 2003 *(2)*.

In the United States, ethanol is mostly produced from starch of corn kernels with a net energy balance *(2)*. However, it is believed that with proper management, roughly 1.18 billion mt of crop and forest residues and energy crops can become available in the United States *(3)* and over 1.5 billion mt/yr worldwide *(4)*, which mostly could be used for conversion into alcohol fuels. Some estimate the global availability at 9–45 billion mt of crop biomass annually *(5)*. In the United States, this translates into approx 411 billion L of petroleum in 1 yr *(4)*.

In addition to being cheap and widely available, lignocellulosic biomass has the added benefit of being renewable and thus sustainable *(4,5)*. A current goal for enhancing US economic security is to meet 10% of chemical feedstock demand by 2020 with plant-derived materials, or a fivefold increase over current usage levels *(6)*. Crops that have a high amount of lignocellulosic biomass, such as corn, rice, sugarcane, and fast growing perennial grasses have been recommended for conversion into alcohol fuels *(7,8)*.

Although production of fermentable sugars for alcohol fuels from plant biomass is an exciting and attractive idea, and substantial efforts have been put forth toward improving ethanol yield through this technology and reducing its production costs *(9,10)*, major roadblocks still stand in the way of widespread commercial implementation of this technology. These include prohibitive costs of pretreatment processing of the lignocellulosic matter and production of microbial cellulase enzymes used in the conversion of cellulosic matter into fermentable sugars *(11)*.

Enzymatic hydrolysis of cellulosic matter requires at least three groups of enzymes: 1,4-β-endoglucanase (E1; E.C. 3.2.1.4), cellobiohydrolase (E.C. 3.2.1.91), and β-D-glucosidase (E.C. 3.2.1.21) *(12,13)*. Currently these enzymes are expensively produced in microbial fermentation tanks *(7,14)*. Decades of research have been devoted to reducing microbial production costs of cellulases, resulting in significant decreases since 1980 *(7,15)*. Despite all efforts, enzyme production expenses are still high *(7)*. The latest model designed by the National Renewable Energy Laboratory (Golden, CO) and Genencor (Palo Alto, CA) is producing cellulases at around $0.03–0.05/L of ethanol (http://www.genencor.com/wt/gcor/ethanol).

An alternative strategy might be to use biomass crops as biofactories to produce these enzymes on a large scale, and store them in safe cell compartments until used. Plants are already being used successfully for molecular farming *(16)* of enzymes *(17,18)* and other proteins *(19)*, carbohydrates *(20,21)*, and lipids *(22)*. Plant-based production of enzymes has several critical advantages compared with microbial fermentation or bioreactors.

For example, plants can directly use the energy of the sun. Furthermore, proteins produced in plants mostly display correct folding, glycosylation activity, reduced degradation, and increased stability *(16)*. In addition, the infrastructure and know-how are mostly in place for plant genetic transformation, growing, harvesting, transporting, and processing the corn plant matter *(16)*.

To this end, the thermostable *E1* transgene from *Acidothermus cellulolyticus (23,24)* has successfully been expressed in several plants including *Arabidopsis (12)*, potato *(25)*, and tobacco *(13,26)*. However, none are considered a significant biomass crop for producing adequate amounts of enzymes needed for lower costs of commercial ethanol production. Corn, on the other hand, is an ideal crop for enzyme production, because it produces a large amount of biomass, is an annual crop, and is already grown extensively in the United States.

In this study, we present the successful production and accumulation of E1 in biomass of transgenic corn plants at a relatively high level. This is the first report on the conversion of biomass to fermentable sugars through the use of *A. cellulolyticus* E1 enzyme constitutively produced within the most acknowledged biomass crop, corn.

Materials and Methods

Transformation Vectors

A combination of pMZ766-$E1_{CAT}$ *(12)* and pBY520 *(27)*, or pMZ766-$E1_{CAT}$ and pDM302 *(28)* was used in transformation research. Vector pMZ766-$E1_{CAT}$ encodes the catalytic domain of E1 from *A. cellulolyticus*, targeted to the apoplast with the signal peptide from tobacco pathogenesis-related protein 1a (Pr1a), under regulation of the cauliflower mosaic virus (CaMV) 35S promoter, the tobacco mosaic virus translational enhancer (Ω), and the polyadenylation signal from nopaline synthase gene (3′ *nos*) (Fig. 1A). Vector pDM302 contains the *bar* coding sequences under the control of the rice actin 1 (Act1) promoter and nos terminator (Fig. 1B). Vector pBY520 contains the barley *HVA1* coding sequences regulated by the Act1 promoter and potato proteinase inhibitor II terminator, as well as the *bar* coding sequences regulated by the CaMV 35S promoter and nos terminator (Fig. 1C).

Corn Transformation, Acclimation, and Transfer to Greenhouses

Highly proliferating, immature embryo-derived type II embryogenic callus *(29)* was used in transformation experiments. Two to four hours before bombardment, callus was transferred in 2 cm circles in the center of a Petri dish containing an osmotic *(30)* or conditioning medium. Conditioned callus was bombarded with ethanol washed tungsten particles combined with a total of 10 μg of 1 : 1 mixture of pMZ766-$E1_{CAT}$ and either pDM302 or pBY520, according to the manufacturer's protocol

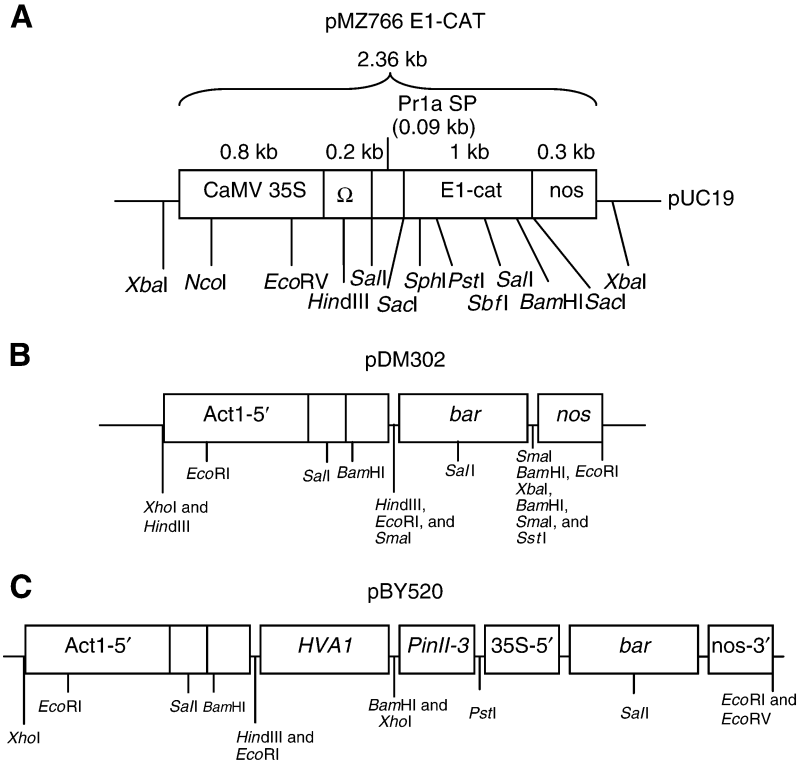

Fig. 1. **(A)** Transformation vector pMZ766E1-$_{CAT}$. CaMV 35S, cauliflower mosaic virus 35S promoter; Ω, tobacco mosaic virus Ω translational enhancer; Pr1a SP, the sequence encoding the Pr1a signal peptide; E1-cat, catalytic domain of 1,4-β-glucanase E1 (E1-$_{cat}$, EC 3.2.1.4) from the eubacterium *A. cellulolyticus*; nos, polyadenylation signal of nopaline synthase. **(B)** Transformation vector pDM302. Act1-5′, rice actin 1 promoter; *bar*, bialaphos (herbicide)-resistance gene; nos. **(C)** Transformation vector pBY520. Act1-5′, rice actin 1 promoter; *HVA1*, a barley late embryogenesis abundant protein gene; PinII-3′, potato proteinase inhibitor II terminator; 35S, CaMV 35S promoter; *bar*; and nos.

(PDS 1000/He Biolistic® gun, BioRad, Hercules, CA) at a pressure of 7579 kPa. The pDM302 or pBY520 containing the *bar* selectable marker gene was used to provide the herbicide resistance.

The bombarded callus was kept on the same conditioning medium for 24 h, transferred to callus proliferation medium *(31,32)* for 5 d, and then placed on selection medium containing 2.0 mg/L bialaphos wherein they were maintained for 6–8 wk with 2-wk subcultures into fresh medium. All cultures were maintained in the dark up to this point. The detected bialaphos-resistant surviving callus clones were placed in regeneration medium *(33)* and exposed to light (60 μmol quanta/m^2/s from cool-white 40 W Econ-o-watt fluorescent lamps; Philips Westinghouse, Somerset, NJ) for 4–6 wk. Plantlets were transferred further to rooting medium containing

2.0 mg/L bialaphos selectable herbicide *(33)*, and maintained for 2–4 wk under the aforementioned light conditions.

Rooted plantlets of eight to ten centimeters in height were transferred to pots containing soil, and pots were covered with plastic bags and kept under light to mimic the tissue culture conditions. Small holes were made daily in each bag, for 10–14 d acclimating the plants to greenhouse conditions before plants were transplanted into 7.6 L pots and transferred to a long day (16 h/d light) greenhouse.

DNA Analyses

Genomic DNA was extracted from leaf tissue with C-TAB as described *(34)*. For polymerase chain reaction (PCR), the oligonucleotide primers 5′-GCG GGC GGC GGC TAT TG-3′ and 5′-GCC GAC AGG ATC GAA AAT CG-3′ were designed, synthesized, and used to amplify a 1.0 kb fragment spanning the catalytic domain of the *E1* gene. The PCR products were analyzed by electrophoresis in 0.8% agarose gels containing ethidium bromide, and visualized under ultraviolet light.

For Southern blot analyses, 5 µg of genomic DNA and 10.0 pg plasmid DNA (pMZ766-E1$_{CAT}$) were digested with *Hin*dIII or *Sac*I and fractionated on a 1% agarose gel. *Hin*dIII was chosen because it appeared to be a unique site in the construct, and *Sac*I because it cuts out the coding sequence of the catalytic domain (Fig. 1). The DNA was depurinated, denatured, and neutralized, and the gel was blotted onto a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. The PCR DIG Probe Synthesis Kit (Roche Applied Science, Penzberg, Germany, Cat no. 11 636 090 910) was used according to the kit's instructions to generate a probe labeled with digoxigenin–dUTP, representing the E1-$_{CAT}$ coding region. Probe hybridization and immunological detection were carried out using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Cat no. 1 585 614) with the instructions therein. Blots were exposed to X-ray film and developed in a Kodak RP X-OMAT Processor (Kodak).

Total Soluble Proteins Extraction

Total soluble proteins (TSP) were extracted from leaf tissues as described *(13)*. Briefly, 100.0 mg fresh leaf tissue was ground in the sodium acetate-grinding buffer and precipitated with saturated ammonium sulfate. Extracts were quantified using the Bradford method *(35)* using a standard curve generated from bovine serum albumin. For the large-scale TSP extraction (to check the activity on biomass), an automatic solvent extractor (Dionex, Sunnyvale, CA) was used. To a total of 9.0 g pulverized transgenic corn residue, 60 mL grinding buffer was added and used by the machine to extract TSP. The extracted TSP were precipitated by adding an equal volume of saturated ammonium sulfate and allowing to stand overnight at 4°C. The precipitated TSP

were collected by centrifugation and concentrated by resuspending in 5 mL grinding buffer. This TSP concentrate was measured for activity (described under "MUCase Activity Assay") and used without any further dilution.

MUCase Activity Assay

E1 activity was assessed as described *(13)*. Briefly, a series of soluble protein dilutions ranging from 10^{-1} to 10^{-3} were developed, representing concentrations of 0.1–10.0 ng/μL. In a 96-well plate, 10 μL samples (representing 1–100.0 ng TSP) were mixed with 100 μL reaction buffer containing 4-methylumbelliferone β-D-cellobioside (MUC). The fluorophore 4-methyl umbelliferone, as the product of E1 hydrolization of the substrate β-D-cello bioside, was measured as follows. Plates were covered with adhesive lids and incubated at 65°C for 30 min. The reaction was stopped with the addition of the stop buffer, and the fluorescence was read at 465 nm using SPECTRAmax M2 device (Molecular Devices Inc., Sunnyvale, CA) at an excitation wavelength of 360 nm. After subtracting background fluorescence contributed by the control, activity of each sample was calculated using a standard curve representing 4–160 pmol methylumbelliferone and compared with the activity of pure E1 reported in Ziegelhoffer et al. *(13)*.

Western Analysis

For Western blotting, the Invitrogen NuPAGE® Bis-Tris Discontinuous Buffer System with a 10% NuPAGE Novex Bis-Tris Pre-Cast Gel was used (Invitrogen, Carlsbad, CA). One microgram TSP was run on the gel and blotted onto a nitrocellulose membrane (Amersham Hybond™ ECL™; Amersham-Pharmacia Biotech) according to the manufacturer's instructions. The membrane was blocked with 1x PBS, 5% nonfat dry milk, 0.1% Tween-20 and incubated with primary antibody (mouse anti-E1, 1 μg/mL) and secondary enzyme conjugate antimouse IgG : HRPO (BD Transduction Laboratories™, BD Biosciences, San Jose, CA; 1 : 2000). The Pierce SuperSignal® West Pico Chemiluminescent Substrate was used for detection 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.

Progeny Analyses

Fertile T_0 plants expressing the highest percentages of E1 were self-pollinated or cross-pollinated. In some cases, transgenic ears were pollinated with wild-type pollen because of lack of sufficient transgenic pollen. Plants were allowed to mature and seeds were harvested after dry-down when the abscission layer had formed 35–45 d after pollination. T_1 seeds were germinated in vitro on 2.0 mg/L bialaphos selection medium *(33)* to determine segregation ratios of the offspring. Then, PCR analysis using the aforementioned primers was used to examine the presence of the *E1* gene in the progeny.

Table 1
Maize Transgenic Lines, Enzymatic Activity, and Percentage E1 Produced in Transgenic Plant TSP

Plant	1	2	3	4	5	6	7	8	–C
E1 (%)	1.16	0.35	0.27	0.26	0.18	0.05	0.03	0.02	0
Activity (nmol/ μg/min)	0.464	0.1408	0.109	0.104	0.072	0.02	0.012	0.008	0

Pretreatment of Biomass

Milled corn stover (about 1 cm in length) was pretreated using the ammonia fiber explosion (AFEX) technology *(36)*. In more detail, the crop biomass was transferred to a high-pressure reactor (PARR Instrument Col, IL) with 60% moisture (kg water/kg dry biomass) and liquid ammonia ratio 1.0 (kg of ammonia/kg of dry biomass) was added. The temperature was slowly raised and the pressure in the vessel increased. The temperature was maintained at 90°C for 5 min before explosively releasing the pressure. The instantaneous drop of pressure in the vessel caused the ammonia to vaporize, causing an explosive decompression and considerable fiber disruption. The pretreated material was kept under a hood to remove residual ammonia and was stored in a freezer until further use.

Conversion Analyses

E1 biomass conversion ability was assessed by measuring the reaction of TSP extracted from line 2 (Fig. 3, Table 1) E1-expressing corn leaves, with soluble cellulose (carboxymethyl cellulose [CMC]), crystalline cellulose (Avicel®, FMC Biopolymer, Philadelphia, PA), and material containing both amorphous and crystalline cellulose, i.e., AFEX-pretreated corn stover *(36)*.

The enzyme hydrolysis was performed in a sealed scintillation vial. A reaction medium, made up of 7.5 mL of 0.1 *M*, pH 4.8 sodium citrate buffer, was added to each vial. In addition, 60 μL (600.0 μg) tetracycline and 45 μL (450.0 μg) cycloheximide were added to prevent the growth of microorganisms during the hydrolysis reaction. The corn stover substrate was hydrolyzed at a glucan loading of 1% (w : v) biomass. The TSP from the plant producing the E1 was concentrated to 1.8%. Two hundred and fifty milliliter of TSP containing 1.8% of E1 protein was used in the enzymatic hydrolysis experiment containing 1% substrate in a 15 mL reaction volume. The reaction was supplemented with 64 pNPGU/g glucan (Novo 188 from Sigma) to convert the cellobiose to glucose. Distilled water was then 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 sample was collected at 72 h of hydrolysis, filtered using a 0.2 mm 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. Hydrolyzate was quantified using Waters high-performance liquid chromatography by running the sample in Aminex HPX-87P (Biorad) column, against sugar standards.

Results and Discussion

E1 Expression in Transgenic Corn

Recently, the United States Government urged the agricultural and petrochemical industries to find and implement alternatives to fossil fuels to reduce dependence on the foreign oil. One of the specific recommendations has been the development of polysaccharide degrading enzymes within the crop plants *(37,38)*. To date, *A. cellulolyticus* the catalytic domain of E1 has been successfully produced in *Arabidopsis (12)* and tobacco *(13,26)* and the full-length E1 peptide in potato *(25)*. Also, the *Thermomonospora fusca* E2 was produced in alfalfa *(39)*. The apoplast is an ideal compartment for expressing foreign proteins because it is spacious compared with other cellular compartments, thus it has the ability to accumulate large quantities of foreign proteins *(12)*, and for expressing E1-$_{CAT}$ in particular because its pH matches that of *A. celloulolyticus*, i.e., pH 5.5–5.6. The catalytic domain of E1 has been shown to have more activity than the full-length peptide *(13)*. In the present study, corn Hi-II callus was genetically transformed with the catalytic domain of the *E1* gene, and over 100 herbicide-resistant transgenic plantlets were produced. Of these, 73 regenerated plants survived to the greenhouse stage. Integration of the *E1* coding sequence confirmed through PCR showed that 31 of these (data not shown) plants carried the *E1* transgene. Southern blotting further verified the integration of the *E1* transgene in these plants (Fig. 2).

Among 31 PCR positive transgenic plants, 16 showed biological activity as compared with control untransformed plants, as shown in percent in biologically active E1 in plant leaf extract TSP (Table 1). Percentages of E1 in TSP ranged from 0.01 to 1.16%. The assay was able to detect enzyme activity levels as low as 0.01% E1, which was accurately confirmed in the sample through Western blotting.

Nine plants were chosen for further study owing to having the highest levels of enzymatic activity. Western blotting confirmed the translation of E1, also showing differences in the production levels (Fig. 3).

pDM302 containing the *bar* selectable marker was used in plant numbers 1, 2, 3, and 6. pBY520 was used in plants number 4, 5, 7, 8, and 9 (Fig. 3). In general, the signal strength observed in the Western blot corresponded with the percentage E1 observed in activity assays (Fig. 2).

To obtain second-generation (T_1) transgenic seeds, the plants were self- or cross-pollinated in the greenhouse. The most successful crosses

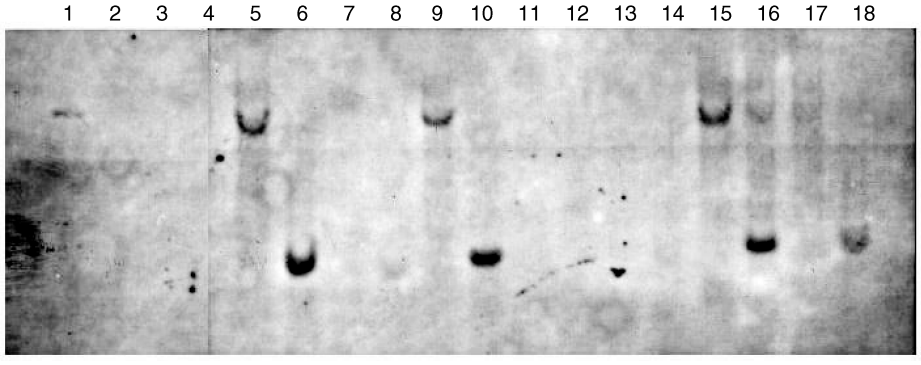

Fig. 2. Southern blot of transgenic maize plants. Genomic DNA from maize plants probed with the E1-$_{CAT}$. Lane 1, 10.0 pg of *Hin*dIII digested pMZ766-E1$_{CAT}$; lane 2, 10.0 pg of *Sac*I digested pMZ766-E1$_{CAT}$; lanes 3 and 4, untransformed maize control; lanes 5–18, seven pMZ766-E1$_{CAT}$ transformants; (5, 7, 9, 11, 13, 15, and 17) DNA digested with *Hin*dIII; (6, 8, 10, 12, 14, 16, and 18) DNA digested with *Sac*I.

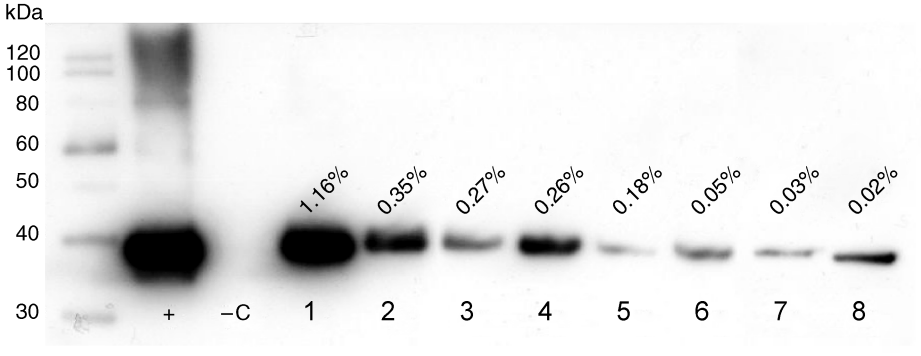

Fig. 3. Western blot of transgenic maize plants expressing E1. Western blot of 1.0 μg TSP from transgenic maize plants expressing E1. Lanes: +, positive tobacco control *(39)*; –C: negative maize control (untransformed); 1–9: transgenic maize plants. Invitrogen Magic Mark™ Western Standard used for size markings. Percentages of E1 as determined by enzyme activity assay are displayed above bands for reference, and also in Table 1.

included plant 2 crossed with plant 8; plant 3 crossed with nontransgenic control; plant 7 crossed with plant 8; and plant 8 crossed with plant 9.

When T_1 seeds were germinated on bialaphos selective medium, a 3 : 1 ratio was observed suggesting that the gene was transmitted as a single copy in a normal Mendelian fashion (data not shown). PCR analysis confirmed the transmission of the *E1* gene to the progeny (data not shown).

Conversion Analyses

The hydrolytic conversion of corn stover using the plant-produced E1 was confirmed by adding transgenic corn stover TSP to three types of substrates: CMC, Avicel, and AFEX-pretreated corn stover. The conversion of

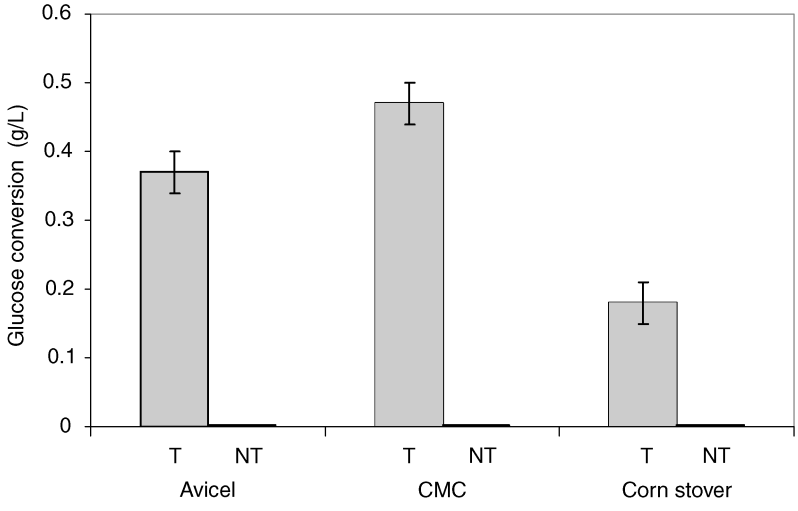

Fig. 4. Conversion of cellulose-to-glucose (g/L) using E1 produced from transgenic maize. The substrates used in the experiment were Avicel, CMC, and AFEX treated corn stover. The enzymatic hydrolysis was done for a period of 72 h, at 50°C with 90 rpm shaking. T, transgenic TSP; NT, nontransgenic TSP.

cellulose to glucose ranged from 0.18 to 0.47 g/L when transgenic plant TSP concentrate was used on various substrates (Avicel, CMC, and corn stover; Fig. 4). As expected, we saw higher sugar release up to 0.47 g/L (after 72 h) when the transgenic plant TSP was reacted with CMC compared with 0.37 g/L released (after 72 h) with Avicel (Fig. 4).

An intermediate amount of glucose was released, up to 0.18 g/L (after 72 h), when the E1 of TSP reacted with pretreated corn stover. Overall, the conversion results (Fig. 4) clearly show that there is a significant amount of E1 enzymatic activity in the TSP extracted from corn stover, because we could obtain a higher sugar yield as we increased the amount of TSP (results not shown).

It has been well documented that different cellulases work together synergistically to decrystallize and hydrolyze cellulose. For example, exoglucanases act on cellulose chain ends of crystalline cellulose, endo-1,4-β-glucanase E1 acts on the interior portions of the cellulose chain of amorphous cellulose, and β-glucosidase converts cellobiose released by cellulases to glucose *(40)*. All of the aforementioned enzymes, when derived from thermophilic microorganisms, have interesting characteristics such as stability at high temperature and extreme pH *(41)*. In recent years, use of thermostable enzymes in industry has been increasing wherein high temperatures are favored to reduce microbial contamination. The heterologous E1 produced in this study is from the thermophilic *A. cellulolyticus*. One advantage of using the *E1* gene from a thermophyllic microbe is that the plant in vivo temperature is low, and therefore the E1 enzyme accumulated in the plant apoplast would not harm the plant cell wall integrity.

Although *A. cellulolyticus* E1 enzyme is thermostable, the reaction in this study was performed at 50°C, because β-glucosidase, which has higher cellobiase activity at this temperature, was included in the reaction.

Based on a previous study, AFEX pretreatment destroys as much as two-thirds of the activity of plant-produced heterologous E1 *(36)*. As most of the pretreatment methods including AFEX follow stringent conditions, we extracted the transgenic TSP concentrate containing the biologically active E1 enzyme, and then added to the corn stover after pretreatment, followed by enzymatic hydrolysis. Production of polysaccharide degrading enzymes within the 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. The production of transgenic plant TSP is quick and very easy, and the E1 remains biologically active under freezer conditions for several months.

Although in this study we added the transgenic plant TSP to the AFEX-pretreated corn stover for conversion of cellulosic matter into glucose, it could be added to any other pretreated (acid and/or heat) lignocellulosic matter of any other crops (rice straw, switchgrass, wheat straw, and so on.). However, at the commercial level, it might be best to extract and lyophilize the enzymes for low-cost storage and easy transportation. The E1 and/or other polysaccharide degrading enzymes could be simultaneously produced and targeted to different cellular compartments of the same plants for a maximum enzyme production level *(38)*. This strategy was recently tested by single targeting of a xylanase heterologous enzyme to *Arabidopsis* chloroplasts and peroxysome as compared with its dual targeting to chloroplast and peroxysome *(42)*.

Acknowledgments

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