PLANT EXPRESSED CELLULASES FOR THE PRODUCTION OF BIOFUELS

By

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Abstract

SYNGENTA has pioneered the concept of plant expressed enzymes for biofuel production, and is continuing to develop crops tailored for next generation biofuel production by expressing optimised enzymes *in planta* for the conversion of cellulose to fermentable sugars. Agricultural waste streams such as corn stover, corn cobs and sugarcane bagasse are attractive potential feed stocks for advanced second generation biofuels due to their comparatively low cost, abundance and availability. A sugarcane mill processing 7 million t/y will generate close to 2 million t/y of wet bagasse. Given that about one quarter of this material is cellulose, then more than 12 000 t/y of enzymes are needed at current enzyme loadings to convert the cellulose to fermentable sugar. Producing this much enzyme by microbial fermentation requires twelve 100 000 L/d fermentors operating at 100 g enzyme/L ferment for a year. In an alternative approach, Syngenta has demonstrated the production of several classes of cellulases in maize and shown that they are nearly as active as their microbially produced counterparts. Furthermore, maize- expressed cellulases were tested on pre-treated sugarcane bagasse. To make sugarcane production and delivery of cellulases a reality, Syngenta recently formed the Syngenta Center for Sugarcane Biofuel Development (SCSBD) in collaboration with the Queensland University of Technology, Brisbane, Australia to develop efficient sugarcane transformation technologies. By providing technologies like these, Syngenta is helping to make second generation biofuels economically viable, while reducing agricultural waste and increasing the value realised from a hectare of land.

Introduction

Many governments have passed legislation promoting biofuel production. For example, the European Union has specified that 5.75% of its overall transport fuel should come from renewable sources by 2010, and that this level should rise to 10% by 2020, while the United States passed the Energy Independence and Security Act (EISA) in December of 2007 that sets a target of 136 billion L of ethanol by 2025 with 80 billion L coming primarily from cellulosic ethanol.

Initiatives like these, along with Brazil's ethanol mandate have driven fuel ethanol production from 30 billion L in 2000 to over 65 billion L in 2008 (Renewable fuels association, 2009; Berg, 2001). With steep projected growth in the bioethanol markets, Syngenta has pioneered the concept of plant-expressed enzymes to make converting plant materials to fermentable sugars more efficient and cost effective.

Advanced traits are needed for 'second generation' biofuels. Cellulosic ethanol (ethanol from cellulose) is viewed as truly 'renewable' but is currently expensive due to high capital and

enzyme costs. One analysis of industry data showed that enzymes cost $0.59/L cellulosic ethanol (Bon and Ferrara, 2007). Even with a selling price of $1.00/L ethanol, this cost is too high for successful commercialisation. By comparison, enzyme costs for the corn dry-grind process were about forty times cheaper, estimated at $0.014/L ethanol (Galbe *et al*., 2007).

The main reason for the higher cost is that more enzyme (25 kg/t) is required to convert cellulose to ethanol than is required for starch to ethanol processes (1.9 kg/t) (Somerville, 2007; McAloon *et al*., 2000). Reducing the cost of the enzyme is critical to making this a practical technology. Syngenta estimates that the enzyme cost can be reduced to under $0.02/ L ethanol by producing the enzymes *in planta*.

In this scenario, the main cost component would be separation of the enzymes from the biomass if an enzyme unfriendly process (e.g. acid/steam hydrolysis) is used, though other process models might include ammonia-fibre-explosion (AFEX), or an enzymatic pre-treatment process.

Efficient conversion of cellulose to fermentable sugars typically requires four fungal enzymes: two cellobiohydrolases (CBHI and CBHII), endoglucanase (EG), and beta-glucosidase. Here we report on the expression of the cellobiohydrolase enzymes in *Zea mays*.

Results

Gene cassettes were made to target accumulation of CBHI or CBHII to the cytoplasm, endoplasmic reticulum (ER), or apoplast and transformed into maize. Studies evaluated subcellular targeting in both seed, and vegetative tissues.

Figure 1 shows the results for individual T1 transformants expressing CBHI in seed. Subcellular targeting of CBHI was found to affect both the numbers of transformants showing activity and the level of activity seen. Targeting the protein to the ER produced more plants with high activity levels than did cytoplasmic, or apoplastic targeting.

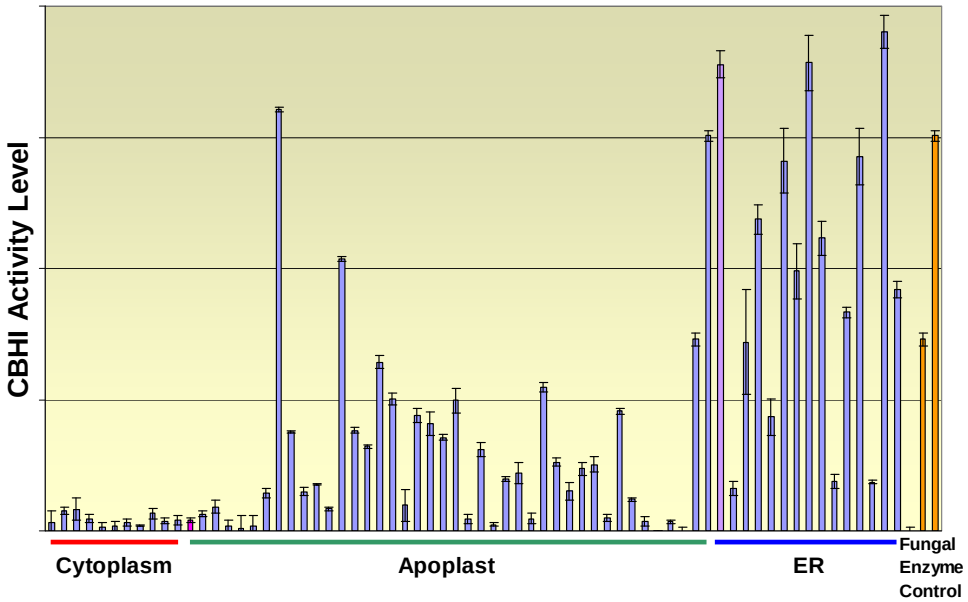

Fig. 1—Relative activity levels of *Trichoderma reesei* CBHI from transgenic maize seed. Expression was sub-cellularly targeted to cytoplasm, apoplast or ER. Each bar represents relative activity from a single transgenic event. Relative activity (arbitrary units) was determined by extracting protein from 25 ground T1 maize seed and measuring activity of 1 mg of total soluble protein (TSP) on *p*-nitrophenyl-□-D-lactopyranoside, a chromogenic soluble substrate. Fungal controls were made by dosing 200 mg ground wild type flour with 0, 181, or 362 □g/g of purified *Trichoderma reesei* CBH I protein purchased from Megazyme (Megazyme, International, Ireland) prior to protein extraction.

Mature maize leaves were collected from two high activity T2 events and the protein levels were determined. Results shown in Figure 2 indicate that 7%–10% of total soluble protein (TSP) in the T2 generation was CBHI.

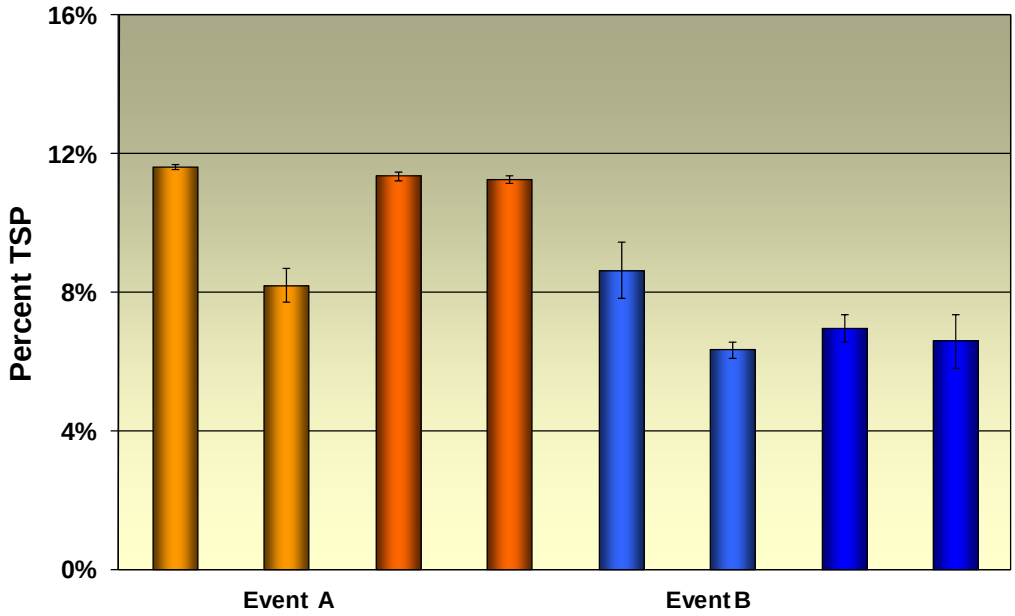

Fig. 2—Estimated fraction of TSP that was CBHI (Syngenta proprietary) in T2 maize leaves. Two events are shown: orange bars are for event A and blue bars are for event B. The first two bars for each event are T2 siblings from the same T1 parent, while the second two bars are T2 siblings from a different T1 parent for the same event. Both events were ER targeted and vegetatively expressed CBHI. Activity of 1 mg of TSP on 4-methylumbelliferyl-β-D-lactoside was measured and the percentage of active CBHI was calculated by dividing the rate of the reaction by the specific activity of the same microbially expressed protein to give the amount of active CBHI. This was divided by the total amount of TSP and expressed as a percent. Error bars represent standard deviations of extraction replicates n = 4 (one biological sample).

To ensure that the plant-made enzymes are as effective as their microbial counterparts, the plant-produced enzymes were tested in head to head reactions in enzyme cocktails on pre-treated sugarcane bagasse.

For CBHI, enzyme cocktails were prepared containing EG, CBHII and either a plant produced or microbially produced version of CBHI loaded on an equal mg protein basis and then used to digest pre-treated sugarcane bagasse (acid/steam pre-treatment process). The results shown in Figure 3 indicate that, in a cocktail, the purified plant produced CBHI performed as well as the purified microbial protein.

For CBHI, the test was repeated with the plant produced version of the protein loaded on an equal activity basis on a soluble, fluorescent substrate 4-methylumbelliferyl-β-D-lactoside (MUL) with similar results. Similar tests were done for CBHII with CBHII loaded on an equal activity on phosphoric acid swollen cellulose (PASC); however, CBHI was left out of the cocktail so that the difference between the plant and microbial versions of CBHII could be more clearly seen.

Based on the PASC results, plant produced CBHII was loaded at 90% that of purified microbial CBHII (on a mass basis) due to its higher specific activity on PASC.

The results in Figure 4 indicate that the plant produced CBHII had 64%–77% the activity of the microbial version on pre-treated cane bagasse at this loading.

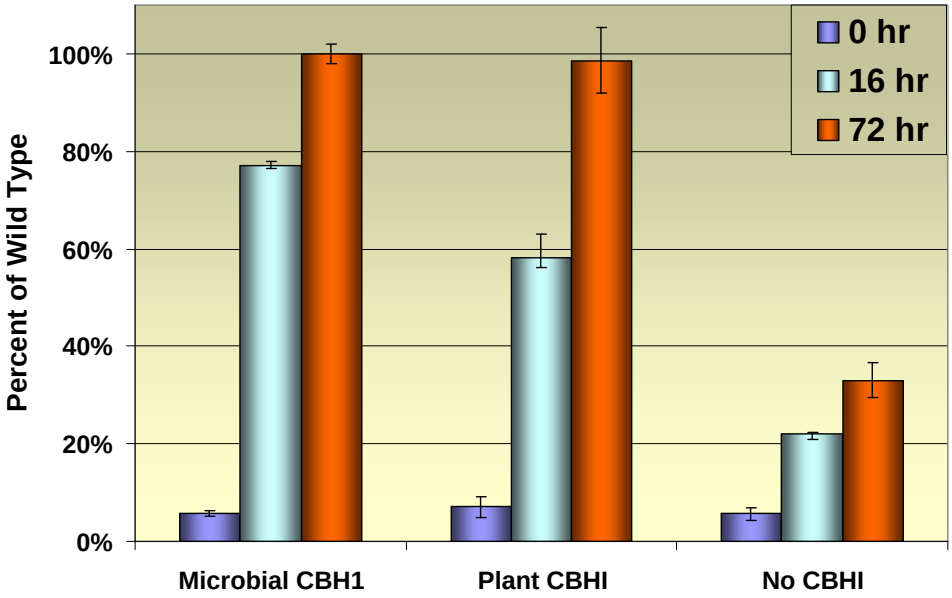

Fig. 3—Performance of a purified, seed expressed, ER targeted *Trichoderma reesei* CBHI on acid/steam pre-treated sugarcane bagasse. Results are expressed as a percentage of the performance of a purified, microbially produced CBHI (Syngenta proprietary). All reactions contained CBHII and an EG. Samples of the reactions were taken after incubating at 40°C with agitation at 300 rpm for zero h, 16 h and 72 h and the percent hydrolysis was determined. Enzyme loadings were 12 mg of each enzyme per gram cellulose except for the negative control where CBHI was omitted. All reactions were done in triplicate. Error bars are standard error (n = 3).

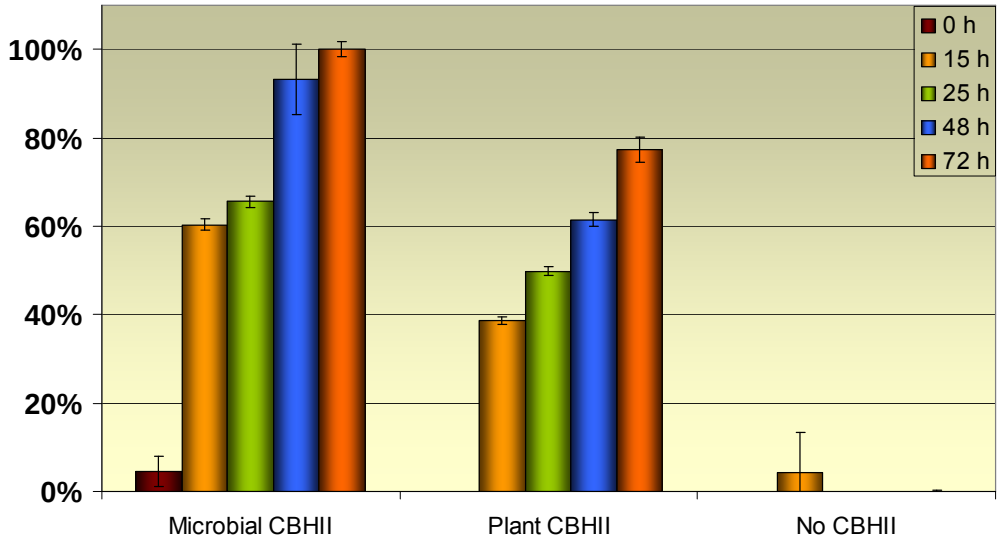

Fig. 4—Performance of purified, vegetatively expressed, ER targeted, CBHII on acid/steam pre-treated sugarcane bagasse. Results are expressed as a percentage of the performance of a purified, microbially produced version of the same enzyme. EG and the microbially produced CBHII were used at 7 mg/g cellulose, while the purified plant CBHII loadings were matched based on activity on PASC. Plant produced CBHII was used at 6.4 mg/g cellulose based on its higher PASC (specific) activity. Samples of the reactions were taken after incubating at 40°C with agitation at 300 rpm for zero h, 15 h, 25 h, 48 h and 72 h and the percent hydrolysis was determined. All reactions were done in triplicate. Error bars are standard error (n = 3).

Discussion and conclusions

The work presented here shows that it is possible to produce cellobiohydrolases at high levels (7–10% of TSP) in plants, and that these CBH enzymes have good activity against pre-treated sugarcane bagasse. If the expression levels obtained in maize can be achieved in sugarcane, it will be possible to use cane itself to produce enough enzyme for converting the cellulose in bagasse to ethanol.

A cane mill processing 7 million t/y of cane produces about 980 000 t/y of dry bagasse. Since only about half of dry bagasse is cellulose, the current enzyme requirements are close to 12 000 t/y of enzyme.

Preliminary data from maize suggests that we can meet this requirement and produce enough extra enzyme to account for the small performance differences seen in the plant produced enzyme. This is important information for a process model because these plant produced CBH enzymes were tested on pre-treated cane bagasse that is believed to be an important target for the cellulosic ethanol industry.

While the enzymes used in this study were produced in maize, the Syngenta Center for Sugarcane Biofuel Development at Queensland University of Technology, Brisbane, Australia, is developing efficient cane transformation technologies focusing on cane produced cellulases (Sainz and Dale, 2009).

By showing that these enzymes can be produced at relevant levels and are active on important substrates, Syngenta is contributing to making this new technology a reality which might lead to the creation of new products for the SC industry.

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LA PRODUCTION DES BIOCARBURANTS DANS LES PLANTES QUI EXPRIMENT LA CELLULASE

Par

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MOTS CLÉS: Cellulases, Expression dans la Plante, Biocarburants, Éthanol, Bagasse.

Résumé

SYNGENTA a été le pionnier dans la conception de la production des biocarburants dans les plantes qui expriment des enzymes. Elle continue à développer de nouvelles générations de plantes pour la production des biocarburants en exprimant des enzymes *in planta* pour la transformation de la cellulose en sucres fermentescibles. Les déchets agricoles, composés de tiges et d'épis de maïs ainsi que la bagasse de la canne à sucre, sont considérés comme d'éventuelles sources attrayantes pour la production des biocarburants de deuxième génération en raison de leur coût relativement faible, leur abondance et leur disponibilité. Une usine qui peut transformer 7 millions tonnes de canne par an peut générer près de 2 millions tonnes de bagasse fraîche. Etant donné que près d'un quart de cette matière est composée de la cellulose, plus de 12 000 tonnes d'enzymes par an sont nécessaires pour transformer la cellulose en sucres fermentescibles. La production de cette quantité d'enzyme par la fermentation microbienne nécessite douze fermenteurs avec une capacité de 100 000 L par jour et fonctionnant à 100 g d'enzyme/L de ferment durant toute l'année. Syngenta a démontré la possibilité de produire différents types de cellulases dans le mais et elles sont aussi actives que leurs homologues microbiennes. De plus, les cellulases exprimées chez le mais ont été testées sur de la bagasse pré-traitée de la canne à sucre. Dans le but de développer des technologies efficaces pour la production et la transformation de la canne à sucre et de rendre possible la production de la cellulase, Syngenta a récemment crée le 'Syngenta Center for Sugarcane Biofuel Development' (SCSBD), en partenariat avec l'Université de Technologie de Queensland, en Brisbane, Australie. Avec de telles technologies, Syngenta contribue à faire de la deuxième génération de biocarburants une opération économiquement viable, tout en réduisant les déchets agricoles et en augmentant les bénéfices obtenus à partir d'un hectare de terre.

EXPRESIÓN DE CELULASES EN PLANTAS PARA LA PRODUCCIÓN DE BIOCOMBUSTIBLES

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PALABRAS CLAVE: Celulasas, Expresión en Plantas, Biocombustibles, Etanol, Bagazo.

Resumen

SYNGENTA ha sido pionera en el concepto de la expresión de enzimos en plantas para producir biocombustibles, y continúa el desarrollo de cultivos diseñados para la siguiente generación de biocombustibles al expresar enzimas optimizadas *in planta* para la conversión de celulosa en azúcares fermentables. Los flujos de residuos agrícolas como el rastrojo de maíz, las mazorcas de maíz y el bagazo de la caña de azúcar son atractivas materias primas con potencial en la segunda generación avanzada de biocombustibles debido a su costo comparativamente bajo, abundancia y disponibilidad. Un molino de caña de azúcar procesando 7 millones t/a generará cerca de 2 millones t/a de bagazo húmedo. Dado que cerca de un cuarto del material es celulosa, entonces más de 12 000 t/a de enzimos serían necesarios, a niveles actuales de producción, para convertir celulosa en azúcar fermentable. La producción de tanto enzimo por fermentación microbiológica requiría 100 000 L/d de fermentadores operando a una capacidad de 100 g de enzimo/L de fermento durante un año. En un enfoque alternativo, Syngenta ha demostrado la producción de diversas clases de celulasas en el maíz y mostró que son casi tan activas como sus contrapartes producidas de manera microbiana. Además, las celulasas expresadas en maíz fueron evaluadas en bagazo de caña de azúcar tratadas previamente. Para hacer realidad la producción de caña de azúcar y la entrega de enzimos, Syngenta recientemente formó el Centro Syngenta para el Desarrollo de Biocombustibles de la Caña de Azúcar (SCSBD, por sus siglas en Inglés), en colaboración con la Universidad de Tecnología de Queensland, Brisbane, Australia, a fin de desarrollar tecnologías eficientes de transformación de caña de azúcar. Al proveer de tales tecnologías, Syngenta está ayudando a hacer económicamente viables los biocombustibles de segunda generación, mientras reduce el desecho agrícola e incrementa el valor derivado de un hectárea de tierra.