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Expression of *Trichoderma reesei* Exo-Cellobiohydrolase I in Transgenic Tobacco Leaves and Calli

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Abstract

Expression of *Trichoderma reesei* exo-cellobiohydrolase I (CBHI) gene in transgenic tobacco was under the control of CaMV 35S promoter. In transgenic leaf tissues, CBHI activity up to 66.1 μmol/h/g total protein was observed. In transgenic calli, the highest CBHI activity was 83.6 μmol h/g total protein. Protein immunoblot analysis confirms the presence of CBHI enzyme in both transgenic calli and leaf tissues. CBHI expression levels accounted for about 0.11% and 0.082% of total protein in transgenic leaf tissues and calli, respectively. Furthermore, expression of CBHI gene did not affect normal growth and development of transgenic plants.

Index Entries: Bioreactor; cellulase; exo-cellobiohydrolase I; transgenic tobacco; *Trichoderma reesei*.

Introduction

Plant cell walls, the major reservoir of fixed carbon in nature, are composed of five different polymers: structural proteins; cellulose (insoluble fibers of β-1,4-glucans); hemicellulose (noncellulosic fibers including glucan, mannans, and xylans); pectins (mainly in the primary cell wall); and lignin (complex polyphenolics, mainly in the secondary cell wall). Cellulose is an unbranched, linear polymer that is composed of a great many glucose subunits, linked end to end with β-1,4-linkages, the major polysaccharide component in plant biomass, and the most abundant biopolymer in an ecosystem. Complete hydrolysis of the glycosidic bonds of cellulose yields a single product, glucose, and requires at least three different enzymes: β-1,4-endoglucanase, β-1,4-exoglucanase, and β-D-glucosidase

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(5,13). Endoglucanase or endo β-1,4-glucanohydrolase, (E.C. 3.2.1.4) mainly cleaves internal glycosidic bonds. Exoglucanase or cellobiohydrolase (CBH, β-1,4-D-glucancellobiohydrolase, E.C. 3.2.1.91) hydrolyzes cellobiosyl units from the nonreducing end of cellulose polymer chains, and β-glucosidase (E.C. 3.2.1.21) hydrolyses cellobiose to produce glucose. The gene of cellobiohydrolase I (CBH I) has been isolated from the filamentous fungus, *Trichoderma reesei (13,14,16)*. The mature CBHI is a glycoprotein of approximately 67 kDa and the coding region of the mature CBHI gene is 1485 bp *(14)*.

Typically, most industrially useful enzymes, such as cellulases, are produced using fermentative processes. However, the cost for fermentative production of cellulases is prohibitively high to be contemplated for "low-value" applications such as the conversion of lignocellulosic feedstock to ethanol. In contrast, alternative production of cellulases in transgenic plants is potentially economically feasible.

It is currently possible to express heterologous genes that encode high value products in plants, a technology being explored by several plant biotechnology companies and university laboratories. Transgenic plants offer the potential of being one of the most economical systems for large scale production of proteins for industrial, pharmaceutical, veterinary, or agricultural use *(3,8,11,19)*. Advantages of plant systems include the low cost of cultivation; easy scale-up; natural storage organs (e.g., seeds, tuberous roots); and established practices for efficient harvesting, transporting, storing, and processing of plant material *(19)*.

To date, high level production of cell-wall degrading enzymes, in general, has been pursued using only bacterial and fungal cultures. Only one type of thermostable biomass-degrading enzyme (xylanase from *Clostridium thermocellum*) has been produced in the transgenic tobacco *(6)*, at an expression level of up to 4.1% total soluble protein. The transgenic plants grown at greenhouse conditions were not affected by the foreign enzyme.

We are interested in using transgenic plants that efficiently synthesize heterologous enzymes and other proteins for both commercial and technical reasons. In this study, we have examined the expression of *T. reesei* CBHI in tobacco plants. Our results indicate that functional CBHI enzyme is produced in both transgenic tobacco whole plants and calli.

Materials and Methods

Bacterial Strains, Plant Materials, Plant Transformation, and Plant Growth Conditions

Escherichia coli strains MC1000 and JM83 (*ara*, *leu*, *lac*, *gal*, *str*) were used as the recipients for routine cloning experiments. *Agrobacterium tumefaciens* LBA-4404 containing the Ach5 chromosomal background and a disarmed helper-Ti plasmid pAL-4404 *(7)* was used for transformation of tobacco plants (*Nicotiana tobacum* L. Cv petit Havana SR1). Transgenic plants were obtained by the co-cultivation method *(1)* using tobacco leaf disks grown aseptically on Murashige and Skoog (MS) agar medium

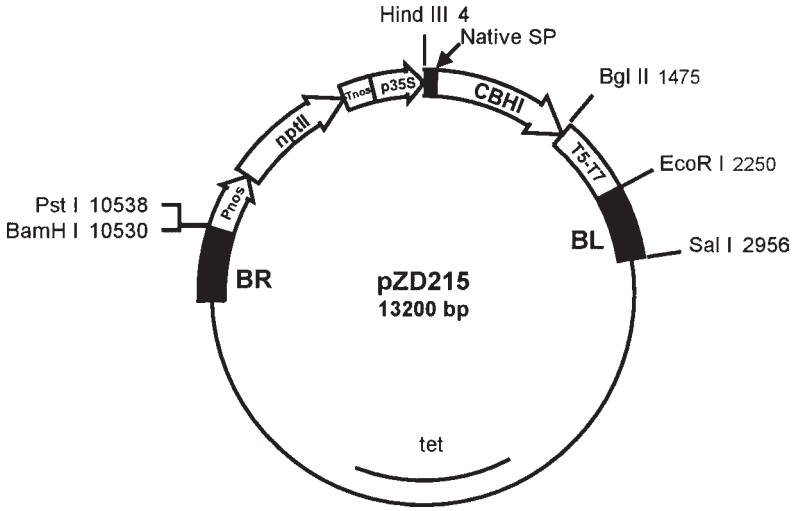

Fig. 1. Restriction endonuclease maps of plasmids used for tobacco transformation. The CBHI cDNA was inserted in a sense orientation within the T-DNA fragment of the binary vector pGA643 between the CaMV 35S promoter and the T7-T5 terminator. This vector also contains, within the T-DNA, the NPTII gene conferring resistance to kanamycin under the control of the nopaline synthase *(nos)* promoter and its terminator. BL, Left Border; BR, right border.

supplemented with 3% sucrose and appropriate amount of plant growth regulators *(10)*. Two milligrams/liter α-naphthaleneacetic acid and 0.5 mg/L 6-benzylaminopurine were used for callus induction and 0.5 mg L-1 6-benzylaminopurine for shoot induction. Kanamycin-resistant transformants were selected and grown on MS medium in a growth room, and seeds were collected after self-fertilization. Seeds were germinated on MS agar medium containing 100 mg/L of kanamycin, and healthy kanamycin-resistant plants were grown in a growth room under a 14 h light (25–28°C, 60% relative humidity)/10 h dark (22°C, 70% relative humidity) cycle. Irradiance, provided by a phytolamps and phytotubes, was 100–150 µmol quanta/ms at the plant canopy.

Recombinant DNA Techniques

Standard procedures were used for recombinant DNA manipulation *(12)*. The plasmid pB210-5A containing exo-cellobiohydrolase I (CBHI) isolated from fungus *Trichoderma reesei* cDNA library *(13)* was obtained from the National Energy Renewable Laboratory in Golden, Colorado. The 1500 bp *Pst*I/*Xho*I fragment (containing the entire CBHI coding region) was excised from pB210-5A. The excised fragment was treated with DNA polymerase I (Klenow enzyme) to produce blunt ends. And the fragment was inserted into the *Hpa*I restrict enzyme site in the poly-linker of pGA643 *(2)* in the sense orientation relative to the 35S cauliflower mosaic virus promoter and the terminator region of T5 and T7 genes of the octopine type Ti plasmid. This procedure yielded the Ti plasmid pZD215 (Fig. 1) used

directly in *Agrobacterium*-mediated transformation of leaf tissues or suspension cells.

Molecular Analysis of Putative Transgenic Plants and Their Progeny

The genomic DNA was extracted as adapted from the rapid CTAB DNA isolation technique described by Stewart and Via *(15)*. Each fresh leaf tissue sample (~25 mg) was placed in a 1.5 mL microcentrifuge tube. The microcentrifuge tubes were frozen in liquid nitrogen for 5–10 min and then kept in a –80°C freezer (up to 5 mo). Leaf tissues were ground to a powder with a 1000-µL pipet tip that was bent upward. The 600 µL of 65°C extraction buffer (3% [w/v] CTAB, 1.42 *M* NaCl, 100 m*M* Tris-HCl, pH 8.0, 20 m*M* EDTA, 2% [w/v] polyvinylpyrrolidone (PVP-40; Sigma Chemical Co.), 5 m*M* ascorbic acid, 2% [v/v] β-mercaptoethanol) were added to the microcentrifuge tubes and further ground. The samples were then heated at 65°C for 15 min. Homogenate was extracted with 600 µL phenol-chloroform-isoamyl alcohol (24:24:1) for 1 min and centrifuged at 1000*g* at 22°C for 5 min to separate phases. The upper, aqueous, DNA-containing phase was transferred to a fresh microcentrifuge tube, diluted with 1/10 volume 3 *M* NaOAc, and precipitated with 0.7 volume of isopropanol for 8 min at –80°C. The DNA was pelleted by centrifugation at 15,000*g* for 15 min. The DNA pellet was washed once with 70% ethanol, precipitated by centrifugation at 15,000*g*, and then air-dried. The DNA pellet was resuspended with 50 µL TE.

Polymerase chain reaction (PCR) was performed in a total sample volume of 50 µL consisting of 1X PCR buffer, 200 µ*M* each of dNTPs, 1 µ*M* each of the two primers, 1 unit of Taq DNA polymerase and 1 µg leaf extract. The PCR conditions were 1 cycle at 94°C for 5 min (for initial denaturation); 30 cycles of three steps as follows: 95°C for 1 min, 52°C for 2 min, and 72°C for 2 min; followed by 10 min at 72°C for final extension. Finally, the reaction mixture was maintained at 10°C for 30 min before an aliquot of 15 µL was used for gel electrophoresis.

Plant Protein Extraction, Electrophoresis, and Protein Gel-Blot Analysis

Total soluble protein in samples was isolated using two different methods: phenol extraction or rapid total protein isolation. The procedure of phenol extraction of total protein was slightly modified from the method described by van Etten et al. *(17)*. One-gram leaf samples were harvested, frozen in liquid nitrogen, and ground to powder with a precooled mortar and pestle. The fine powder was re-suspended in 2 mL/g grinding medium (4°C, 250 m*M* Tris-HCl, pH 7.5, 700 m*M* sucrose, 100 m*M* KCl, 50 m*M* [ethylenediamine tetraacetic acid] (EDTA)-Na_2, 2% [v/v] β-mercaproethanol, 2% [w/v] SDS, 2% [w/v] insoluble polyvinyl polypyrrolidone (PVP). Four mL of water-saturated phenol was added to the mixture and grinding was continued to total maceration. Another 3 mL grinding medium was added and the mixture was ground further until it was completely thawed. The

extract mixture was vortexed vigorously for 2 min and centrifuged at room temperature for 20 min at 30,000*g*. The phenol phase was re-extracted 2–3 times by adding an equal volume of grinding buffer (without insoluble PVP). The proteins in the phenol phase were precipitated by the addition of 5 vol of –20°C methanol solution containing 100 m*M* ammonium acetate and 10 m*M* β-mercaptoethanol. The mixture was then centrifuged at 15,000*g* for 5 min. The resulting pellet was re-suspended and washed twice by vortexing with 1 mL of cold methanol solution and twice with 1 mL of cold 100% acetone. After centrifugation the pellet was air-dried for 5–10 min. Finally, the pellet was dissolved in protein sample buffer (50 m*M* Tris-HCl, pH 7.0, 2% [w/v] SDS, 10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol).

For rapid isolation of total protein from calli, 500 mg calli was harvested, frozen in liquid nitrogen, and ground to fine powder with a pre-chilled mortar and pestle. The fine powder was re-suspended in 1 mL protein sample buffer (50 m*M* Tris-HCl, pH 7.0, 2% SDS, 10% [v/v] glycerol, 5% [v/v] β-mercaptoethanol, 5% [w/v] insoluble PVP, 1 µL/mL proteinase inhibitors [Aprotinin, pepstatin A, chymostatin, leupeptin, PMSF]) and boiled for 5 min. The samples were centrifuged at 10,000*g* for 10 min. The upper aqueous phase was transferred into new microcentrifuge tubes. The total protein amount in each sample from the previous two different protein purification methods was determined by Pierce's Micro BCA method. Bovine serum albumin (BSA) was used as protein standard.

Fifty micrograms of total proteins for each sample was separated by electrophoresis on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and electrophoretically transferred onto nitrocellulose membranes (BA-S85; Schleicher and Schuell, Keene, NH) *(4)*. The protein was reacted with affinity-purified rabbit anti-CBHI polyclonal antibody (in 1:500 dilution). The antibody was detected by color-development kit (Bio-Rad, Hercules, CA) for leaf tissues or CDP-Star chemiluminescence kit (Tropix, Bedford, MA) for calli and a goat anti-rabbit secondary antibody (IgG) conjugated with alkaline phosphatase (Pierce, Rockford, IL). Membranes were performed color development or exposed to x-ray film, respectively. To confirm equivalent protein loading, a second set of protein gel was stained with Coomassie blue.

The amount of CBHI expressed in leaf tissues was estimated by densitometry analysis. The protein blot bands were scanned with Hewlett Packard ScanJet 6100C Scanner (Hewlett Packard Inc., Palo Alto, CA). The imaging data were analyzed with DENDRON 2.2 program (Solltech Inc., Oakdale, IA). The known amount of CBHI from *T. reesei* was used as standard for estimating the CBHI production in transgenic leaf tissues or transformed calli.

Exo-Cellobiohydrolase I Assay

Exo-cellobiohydrolysing activity was measured using the fluorescence substrate, 4-methylumbelliferal-β-D-cellobioside *(9)*. CBHI-containing samples from transgenic tobacco leaf tissues were prepared in phos-

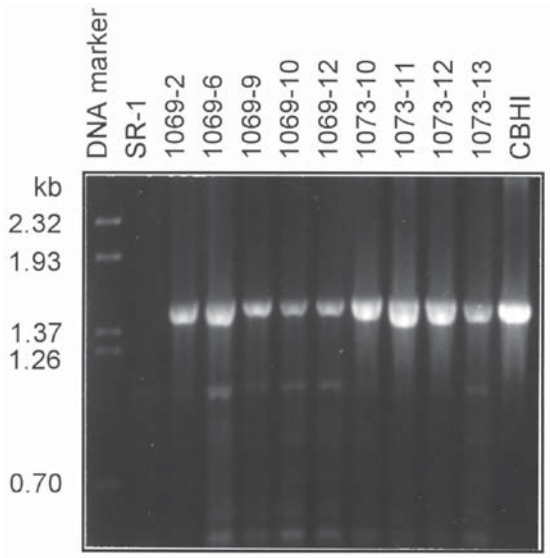

Fig. 2. PCR analysis of the CBHI gene region amplified from genomic DNA of selected CBHI transgenic plants and wild-type SR1 tobacco plants. Molecular weight markers are indicated on the left in kb. Lane 12 is PCR product with the template of pB210-5A plasmid DNA.

phate/citrate (PC) buffer (50 m*M* phosphate, 12 m*M* citrate, pH 6.0). Similarly, kanamycin resistant calli were harvested and sonicated in phosphate/citrate buffer using a 4710 series ultrasonic homogenizer (Cole Parmer Instrument Co., Chicago, IL). Fifty-microliter samples of the original extraction buffer were pipeted into Eppendorf tubes containing 100 µL of 1 m*M* 4-methylumbelliferyl β-D-cellobioside and incubated at 55°C for 1 h. The reaction was stopped by adding 100 µL of 0.15 *M* glycine/NaOH at pH 10.3. The resulting production of 4-methylumbelliferone from the reaction was determined using a TK100 DNA fluorimeter. 4-methylumbelliferone was used for standard.

Results and Discussion

Molecular and Protein Analysis of Transgenic Plants and Calli

In order to confirm integration of foreign gene into genomic DNA of transgenic plants and calli, the genomic DNA was isolated from kanamycin-resistant primary transformant plants and calli. One microgram genomic DNA was used for PCR screening for the presence of the CBHI gene with primers specified to the coding sequence of CBHI gene. The resulting PCR products are shown in Fig. 2. Similarly, the calli were also determined by PCR. More than 90% of transformed calli and plants that were assayed carried the full length CBHI coding sequence. Those transgenic plants and calli that were confirmed carrying CBHI coding sequence were further analyzed for expression of CBHI enzyme using protein immunoblotting.

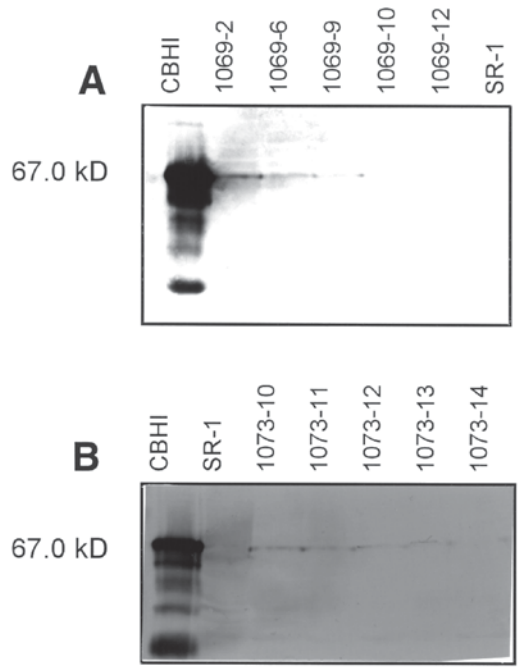

Fig. 3. Protein gel immunoblot analysis of CBHI protein expressed in leaf tissues of different transgenic tobacco plant lines. Total proteins were extracted from different transgenic tobacco lines and wild type SR1 as indicated. Proteins were separated on the SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, reacted with the affinity-purified rabbit anti-CBHI antibody, and detected with color development kit as described in methods. Panel **(A)**, Lane 1 contains 1 µg of affinity-purified CBHI protein from *T. reesei*, whereas all other lanes contain 50 µg total protein from a tobacco-leaf extract. Lanes 2, 3, 4, 5, and 6 represent five independent transgenic tobacco lines: 1069-2, 1069-6, 1069-9, 1069-10, and 1069-12 leaf extract, respectively. (The 1069 is *Agrobacterium tumefaciens* strain number used for transformation and the –2, –6, –9, –10, or –12 is the independent transformed events.) Lane 7 is wild-type SR-1 tobacco-leaf extract for a negative control. Panel **(B)**, Lane 1 is a 0.27 µg of affinity-purified CBHI protein from *T. reesei* used for a positive control. Lanes 2–7 contain 50 µg of total protein from a tobacco leaf extract. Lane 2 is a wild-type SR-1 tobacco-leaf extract. Lanes 3, 4, 5, 6, and 7 represent independent transgenic tobacco lines 1073-10, 1073-11, 1073-12, 1073-13, and 1073-14 leaf extract, respectively. (The 1073 is *Agrobacterium tumefaciens* strain number used for transformation and the –10, –11, –12, –13, or –14 is the independent transformed events.)

The CBHI expression in selected transgenic tobacco-leaf tissues and calli was examined by protein immunoblotting. Results for leaf tissues (Fig. 3A, B) and calli (Fig. 4) clearly indicate production of CBHI enzyme in transgenic plants and calli as compares with untransformed controls (SR-1 plants and NT-1 calli).

The protein blots were scanned with HP ScanJect 6100C Scanner and analyzed with the DENDRON program. The relative amount of proteins in gels was estimated based on the density of blots in scanned imaging data using the DENDRON program. By comparing the relative amount of

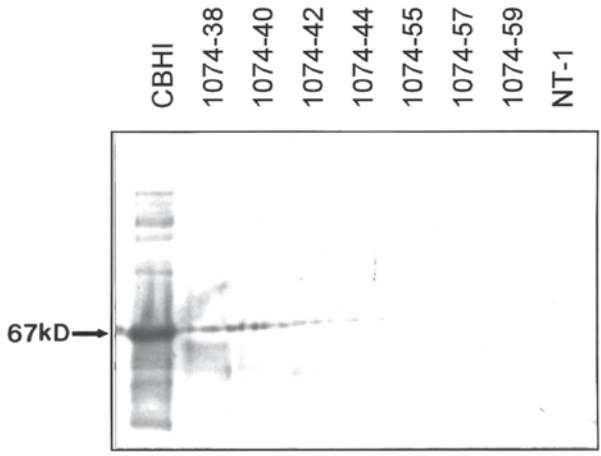

Fig. 4. Protein gel immunoblot analysis of CBHI protein expressed in transgenic tobacco calli. Equal amounts of protein were separated on the SDS-polyacrylamide gel and analyzed by protein gel immunoblot as described in the legend of Fig. 3. Lane 1 is a 0.27 μg of affinity-purified CBHI protein derived from *T. reesei*, whereas Lanes 2–9 contains 50 μg of total protein from a tobacco-leaf extract. Lanes 2, 3, 4, 5, 6, 7, and 8 represent independent transgenic tobacco lines 1074-38, 1074-40, 1074-42, 1074-44, 1074-55, 1074-57, and 1074-59 leaf extract, respectively. Lane 9 is the wild-type SR1 tobacco-leaf extract.

plant produced CBHI protein to the known amount of *T. reesei*-derived CBHI, the maximum CBHI expression in transgenic leaf tissues (line 1069-2) was estimated at 0.11% of total protein (Fig. 3A). Similarly, transformed calli showed that the maximum level of CBHI expression is 0.08% total protein (Fig. 4).

Transgenic Tobacco Transformed with CBHI Exhibits Hydrolyzing Activity in Leaves and Calli

In order to further determine correct processing of CBHI enzyme in tobacco-leaf tissues and calli, the 4-methylumbelliferyl-β-D-cellobioside assay was performed on proteins extracted from transgenic leaves and calli with phosphate-citrate protein extraction buffer. Levels of cellobiohydrolysing activity measured in transgenic plants, used in earlier immunoblot assays, are shown in Fig. 5. All transformed plants tested possessed at least two times or greater activity than that of control SRI tobacco plants. The highest CBHI activity achieved was observed in transformant 1073-12, which reached a level of 66.1 μmol/h/g total protein, approximately 10 times higher than that of the negative control (6.11 ± 4.3 μmol/h/g total soluble proteins).

The CBHI activity in selected transgenic calli was also determined using a similar method. More than 80% of transgenic calli examined possessed higher CBHI activity than that of untransformed control NT1 tobacco calli (data not shown). The CBHI activities of 16 independent transgenic

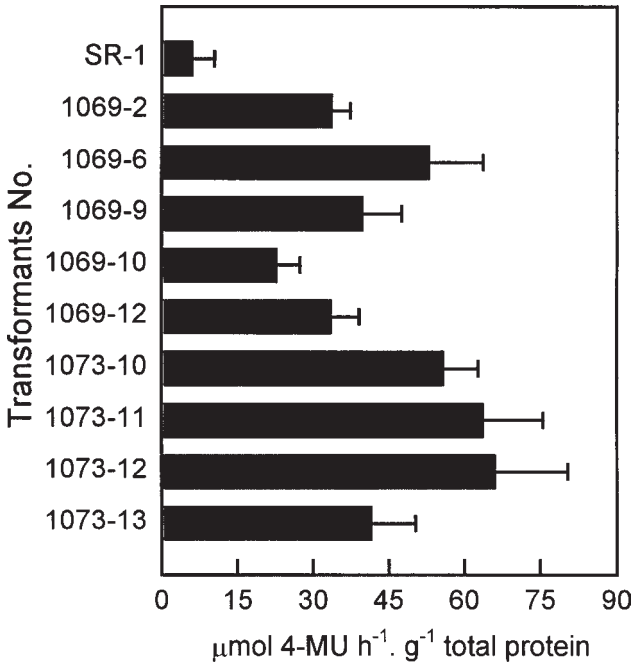

Fig. 5. Measurements of CBHI activity in selected transgenic tobacco plants using the 4-methylumbelliferon assay. Each bar represents the mean ± one standard deviation of 4–5 replicates.

calli are plotted in Fig. 6. Among all tested calli, transformant 1074-38 possessed the highest level of CBHI activity at 83.6 μmol/h/g total soluble protein, which is approximately 15 times higher than that of control NT1 calli (5.3 μmol/h/g total soluble proteins). The CBHI activity of most transformed calli was at least four times higher than that of NT1 calli.

Because plant cell-wall polymers are abundant and renewable structures, we are attempting to exploit available enzymatic-degradation capabilities to expand the utility of this biomass resource. Plant cell-wall degrading enzymes may be used to produce hydrolytic products or residual fibers that may be subsequently further converted to specific end products with other related enzymes. Large-scale production of enzymes at low cost is crucial for biological conversion of cellulose to ethanol. Herbers and Sonnewald *(6)* demonstrated that transgenic tobacco plants accumulate the protein xylanase at levels of up to 4.1% total soluble protei n within the apoplastic space of leaf tissues. Other research efforts have yielded plant-based expression levels of up to 14.4% total soluble protein *(18)*. The results here further demonstrate that tobacco plants can be used as bioreactors for the production of the hydrolytic plant cell wall-degrading enzyme CBHI from *T. reesei* without deleterious effects on plant growth and reproduction at growth room temperatures conditions (20–25°C). The *T. reesei* CBHI accumulating in plant cells is active at 55°C during functional analysis. Mean-

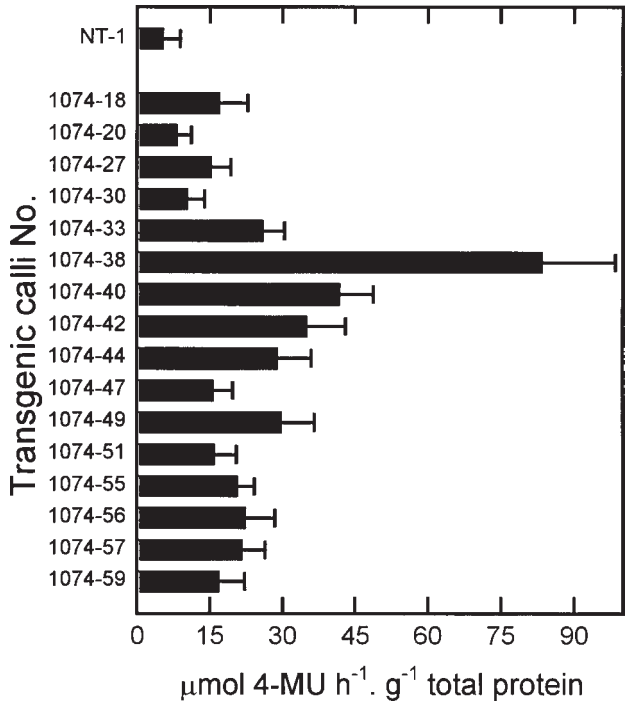

Fig. 6. Measurements of CBHI activity in selected transgenic tobacco calli using the 4-methylumbelliferon assay. Each bar represents the mean ± one standard deviation of 4–5 replicates.

while, the transgenic plants did not show any abnormal phenotype at growth room temperatures as compared with control tobacco plants.

In summary, the thermostable enzyme CBHI can be produced in transgenic plants. We are currently investigating methods to optimize the expression of *T. reesei* CBHI as well as *Acidothermus cellulolyticus* E1 in transgenic tobacco through postranscriptional modifications. The results of this effort will provide us important information for creating bioreactors for the production of valuable industrial or feed-related enzymes.

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