Novel Cellulase Profile of *Trichoderma reesei* Strains Constructed by *cbh1* Gene Replacement with *eg3* Gene Expression Cassette

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Abstract To construct strains of the filamentous fungus *Trichoderma reesei* with low cellobiohydrolases while high endoglucanase activity, the P_{cbh1}-eg3-T_{cbh1} cassette was constructed and the coding sequence of the cellobiohydrolase I (CBHI) gene was replaced with the coding sequence of the *eg3* gene by homologous recombination. Disruption of the *cbh1* gene was confirmed by PCR, Southern dot blot and Western hybridization analysis in two transformants denoted as L13 and L29. The filter paper-hydrolyzing activity of strain L29 was 60% of the parent strain Rut C30, and the CMCase activity was increased by 33%. This relatively modest increase suggested that the *eg3* cDNA under the control of the *cbh1* promoter was not efficiently transcribed as the wild type *cbh1* gene. However our results confirmed that homologous recombination could be used to construct strains of the filamentous fungus *Trichoderma reesei* with novel cellulase profile. Such strains are of interest from the basic science perspective and also have potential industrial applications.

Key words *Trichoderma reesei*; gene replacement; gene disruption; *cbh1*; *eg3*

The filamentous fungus *Trichoderma reesei* is considered to be the most efficient cellulase producer, and has a long history in the production of hydrolytic enzymes, which was widely used in the food and feed industries and recently also used in the textile, pulp and paper industries [1,2]. With the development of molecular manipulation technology and filamentous fungi transformation systems, it is possible to genetically modify the filamentous fungi strains with recombinant DNA technology.

The cellulolytic system of *T. reesei* is composed of two cellobiohydrolases (CBHI and CBHII) and at least five endoglucanases (EGI, EGII, EGIII, EGIV and EGV) [3,4]. The best strain can secrete up to 40 g protein per liter of culture, most of which is cellubiohydrolase I [5]. Thus, the promoter of *cbh1* gene is considered to be one of the strongest promoters in *T. reesei*. Endoglucanase III is classified as a family A cellulase and considered to be the endoglucanase with the highest catalytic efficiency among all the endoglucanases in *T. reesei* [6,7]. The textile and paper industries need some of the cellulase components to modify the properties of the fabric surface without reducing the integrity of fibers. Unfortunately, CBHI does affect fibers’ integrity [8]. Thus industry requires novel strains with altered cellulase profile more appropriate for fabric surface modification. The development of genetic engineering in filamentous fungi provides us with the possibility of reconstructing the ratio of cellulase components produced by the organism.

In our previous work, we cloned the strong promoter and terminator of cellobiohydrolase I gene *cbh1* from cellulose-producing filamentous fungus *T. reesei* strain QM9414. Using these sequences we constructed a pUC19 based expression vector, pTRIL, with multiple cloning sites *Pst*I, *Apa*II, *Kpn*I, *Sma*I, *Sac*I and *Xho*I between the promoter and terminator of *cbh1* [9]. Here we report the use of the pTRIL plasmid to construct the P_{cbh1}-eg3-T_{cbh1} expression cassette and the use of this cassette to generate *T. reesei* strains with decreased cellobiohydrolases activity but elevated endoglucanase activity via homologous recombination. Our results will further help the understanding of the gene regulation of different cellulase components of *T. reesei* and potentially lead to the development of cellulose-producing strains for...
specific industrial applications.

Materials and Methods

Strains, vectors and media

Plasmids, E. coli strains and filamentous fungi strains were listed in Table 1. Growth conditions, media, and genomic DNA isolation for filamentous fungi were as described in the literature [10].

General procedures

Recombinant DNA technology and double-stranded DNA sequencing were carried out by standard procedures [11].

Construction of recombinant plasmid pTRIL-eg3

The eg3 coding sequence was amplified by PCR from the constructed pAJ401-eg3 using primer A and B. Primer A is identical to nucleotide sequence at position 4–23 of the T. reesei eg3 cDNA, and primer B is complementary to the nucleotide sequence downstream of the eg3 gene terminator in the plasmid pAJ401-eg3.

Primer A: 5’-ACTA CTGCAGAACAAGTCCGTGGC-CCATT-3’ (PstI site underlined)
Primer B: 5’-GCGC TCT AGAATGACCATGATTACG-CCAAG-3’ (XbaI site underlined)

The amplified fragment was digested with PstI and XbaI, and cloned into PstI-XbaI sites of pTRIL to created plasmid pTRIL-eg3.

DNA sequencing

A 1.7 kb T. reesei eg3 cDNA fragment were sequenced using the dyeoxy sequencing technique [15] with primer 5’-GCTCTCCCCATCTCTCATC-3’ which is identical to the nucleotides from position –96 to –77 of the cbh1 promoter, and primer G (Table 2) which is identical to the nucleotides from position –301 to –281 of the cbh1 promoter, respectively. At the same time, a 416 bp fragment obtained by PCR with primer G and H (Table 2) was sequenced using the same method.

Transformation of Trichoderma reesei and isolation of the positive transformants

The Pcbh1-eg3-Tcbh1 cassette was amplified by PCR from the plasmid pTRIL-eg3 using oligonucleotide C and D as primers (Table 2), and co-transformed with plasmid pAN7-1 carrying a hygromycin resistance cassette into the recipient T. reesei strain Rut C30. The plates and media for T. reesei transformation and hygromycin B selection were the same as described by Penttilä et al. [10]. The hygromycin-resistant transformants were selected on minimal medium containing 100 µg/ml hygromycin B. Then, the positive transformants were

Table 1  The strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Genotype and characters</th>
<th>Origin and reference</th>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F-, SupE44, Δ(argF lacZya)U169, φ80lacZΔM15, hsdR17(r–, m+) recA1, endA1, gyrA96, thi-1, relA1, λ</td>
<td>Hanahan D [12]</td>
</tr>
<tr>
<td>T. reesei Rut C30</td>
<td>Mutant strain in which glucose repressor gene Cre1 was destroyed (Ilmen 1996)</td>
<td>Presented by Dr. Penttilä M, Biotechnology Institute, VTT, Finland</td>
</tr>
<tr>
<td>T. reesei Rut C30L13 and C30L29</td>
<td>The EGIII-overproducing and CBHI-negative strain</td>
<td>This study</td>
</tr>
<tr>
<td>pAJ401-eg3</td>
<td>The eg3 cDNA from T. reesei was inserted between the EcoRI and XhoI sites of plasmid pAJ401</td>
<td>Xiao ZZ et al. [13]</td>
</tr>
<tr>
<td>pAN7-1</td>
<td>The fungal plasmid with the hph gene under the control of A. nidulans gpd promoter and the trpA terminator</td>
<td>Punt PJ et al. [14]</td>
</tr>
<tr>
<td>pTRIL</td>
<td>4.7 kb plasmid in which the MCS with PstI, ApaLI, KpnI, SmaI, Sacl and XhoI sites was inserted between the 1355 bp T. reesei cbh1 promoter and the 595 bp terminator fragments</td>
<td>Wang TH et al. [9]</td>
</tr>
<tr>
<td>pTRIL-eg3</td>
<td>T. reesei eg3 was inserted into the PstI and XhoI sites of pTRIL under the control of the cbh1 promoter. MW 6.4 kb</td>
<td>This study</td>
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analyzed by PCR with primer E and F (Table 2) specifically amplified cbh1 to select the strains in which the cbh1 gene was disrupted. Further PCR was carried out by with primer G and H (Table 2) to specifically amplify Pcbh1-eg3-Tcbh1 cassette to select the strains in which the cassette was inserted into the correct chromosomal position.

Southern dot blot hybridization

Genomic DNA was extracted as described by Raeder and Brode [16]. Southern dot blot analysis was performed with an ECL direct nucleic acid labeling and detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer’s instructions.

Western hybridization analysis

The Trichoderma reesei culture was grown in minimal medium for 3 d. The mycelia were harvested and then inoculated into filter paper liquid medium and cultivated for 3 d. The mycelia were removed by centrifugation at 10,000 g at 4 °C for 10 min. The supernatants were ultrafiltered, and then the concentrated liquid was analyzed by the Western hybridization analysis using CBHI-antibodies. The remaining samples were stored at –20 °C for further analysis.

Enzymatic assays

The enzymatic activity of exocellulase in the culture supernatants was measured as the release of reducing sugars from filter paper according to the method reported by Mandels et al. [17]. The endoglucanase activity was measured using sodium carboxymethyl cellulose as a substrate (CMCase activity) as described by Wood et al. [18]. Protein concentrations were determined from the trichloroacetic acid-precipitated T. reesei culture media using the method of Lowry et al. [19], with bovine serum albumin as the standard. One unit of enzyme activity corresponds to 1 µmol of β-1,4-glycosidic bonds of substrate hydrolyzed in one minute during the initial period of hydrolysis. All measurements were performed with a spectrophotometer (Thermo Spectronic Helios β).

Results and Discussion

Isolation of eg3 gene and construction of plasmid pTRIL-eg3

A 1.7 kb fragment that included the 1.5 kb coding region of eg3 gene and 0.2 kb of the 3’ flanking vector sequence was amplified as expected. This fragment was inserted into the PstI-XbaI sites of the pTRIL, producing the recombinant plasmid pTRIL-eg3 (Fig. 1). Plasmid pTRIL-eg3 contained the 1.5 kb eg3 gene cDNA under the control of the 1350 bp cbh1 promoter from T. reesei (Fig. 2). The eg3 fragment encoding 397 amino acids was correctly fused into pTRIL after the ATG start codon as confirmed by sequencing.

Trichoderma reesei co-transformation and isolation of the transformants lacking the cbh1 gene

P_{cbh1}-eg3-T_{cbh1} cassette amplified from the pTRIL-eg3 was co-transformed with plasmid pAN7-1 into T. reesei

![Gel electrophoresis of PCR to amplify eg3 gene](image)
strain Rut C30 as described above. 112 hygromycin-resistant transformants were obtained and cultivated in shaker flasks on cellulase-inducing medium. Chromosomal DNAs were extracted from all these transformants. PCR amplification identified two that were lack of cbh1 among these transformants, and designated as T. reesei Rut C30L13 and L29 respectively (Fig. 3).

PCR result indicated that the Pcbh1-eg3-Tcbh1 cassette had been inserted into the chromosomal DNA of both L13 strain (Fig. 4, lane 1) and L29 strain (Fig. 4, lane 4). The 416 bp fragment sequencing results indicated that the strains contained partial sequence of cbh1 promoter and eg3 structure gene.

Dot blot hybridization and Western blot analysis

Southern dot blot hybridization and Western blot analysis demonstrated that in T. reesei strain Rut C30L13 (Fig. 5, dot 6) and L29 (Fig. 5, dot 2) the cbh1 gene was absent, and neither strain secreted CBHI as expected (L13, Fig. 6 lane 1; L29, Fig. 6 lane 2). The results suggested that the Pcbh1-eg3-Tcbh1 cassette replaced the cbh1 gene by homologous recombination. While in Rut C30L23, a heterogenous recombination but not homologous recombination happened, as the cbh1 gene still existed in this strain (Fig. 5, dot 4).

Enzyme production by the transformant strain Rut C30L29

The filter paper activity and endoglucanase activity (measured against CMC) of the culture supernatant from transformant L29 were measured (Fig. 7, Table 3). The results indicated that the filter paper-hydrolyzing activity of the strain Rut C30L29 was reduced to 60% of that of the parent strain Rut C30, while the CMCase activity was increased by 30% than that of parent strain. These results were consistent with that of replacement of the CBH1 gene via homologous recombination with the Pcbh1-eg3-Tcbh1.
Karhunen [20] reported that expression of one copy of the eg1 cDNA under the control of the cbh1 promoter at the cbh1 locus produced less EGI compared with CBHI produced by the wild-type cbh1 locus. In our experiment, the expression of one copy of eg3 cDNA under the control of the cbh1 promoter resulted in a 30% increase CMCase activity compared with the parent strain. This result was similar to that reported by Karhunen [20].

In this study the production of endoglucanase enzymes was improved in the biotechnically important filamentous fungus T. reesei. Expressing of one copy of eg3 cDNA under the control of the strong cbh1 promoter in T. reesei, CBHI-negative strains with high endoglucanase activity were constructed. The T. reesei strain Rut C30L29 constructed in this study is beneficial to molecular biological research and potentially has industrial applications. The enzyme with novel cellulase profiles derived from the transformants L13 and L29 could possibly be used in the textile industry, such as cotton finishing, improving the stonewashing effect. The new strains may also provide a more economical way to produce cellulase for feed modification.

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