Disruption of the *Trichoderma reesei cbh2* gene coding for cellobiohydrolase II leads to a delay in the triggering of cellulase formation by cellulose

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The role of the major conidial-bound cellulase – cellobiohydrolase II (CBH II) – in the triggering of cellulase formation in the fungus *Trichoderma reesei* was investigated by comparing the mutant strain QM 9414 with a recombinant strain unable to produce CBH II. For this purpose, the *cbh2* gene was isolated from a chromosomal gene bank of *T. reesei*, cloned into pGEM-7Zf(+), and disrupted by insertion of the homologous *pyr4* gene in its coding region to yield the plasmid vector pSB3. Transformation of the auxotrophic, *pyr4*-negative strain *T. reesei* TU-6 with pSB3 yielded 23 stable prototrophs, of which three were unable to produce CBH II – assessed by means of a monoclonal antibody – during growth on lactose or in the presence of sophorose. However, they formed cellobiohydrolase I (CBH I) at a rate comparable to strain QM 9414 under these conditions. Southern analysis of DNA of some CBH II⁻ and CBH II⁺ transformants confirmed that pSB3 had integrated at the *cbh2* locus in the CBH II⁻ strains. The latter displayed normal growth on glucose or maltose as carbon source. They showed retarded growth on cellulose as sole carbon source, however, and exhibited a lag in the time course of CBH I and EG I formation, although producing roughly the same final cellulase activities. It is concluded from these results that CBH II is not essential for induction of cellulase formation by cellulose, but that it contributes significantly to the formation of lower molecular mass inducers in the early phase of growth of the fungus on cellulose.

Introduction

Cellulolytic enzymes are adaptively formed by fungi, but the respective mechanisms mediating this adaption are still only rudimentarily known. In the fungus *Trichoderma reesei*, the mechanism by which cellulose triggers cellulase formation has been demonstrated to involve constitutive, conidial-bound cellulases (Kubicek *et al.*, 1988; El-Gogary *et al.*, 1989). This apparently enables the fungus to form a limited amount of cellulose degradation products upon initial contact with cellulosic substrates, which may either themselves be or become converted to the inducer of cellulase formation. This idea is supported by the demonstration both of a constitutive β -glucosidase and a constitutive β -linked disaccharide permease in *T. reesei* (Umile & Kubicek, 1986; Kubicek, 1987; Fritscher *et al.*, 1990).

The enzyme spectrum of the cellulase complex in *T. reesei* conidia is different from the one secreted into the medium. Thus, the predominant conidial cellulase is not cellobiohydrolase (CBH) I – which accounts for more than 60% of total secreted protein (Enari & Niku-Paavola 1987; Kubicek, 1992) – but rather cellobiohydrolase II, which is only about 15% of total secreted protein (Messner *et al.*, 1991). A major involvement of CBH II in the triggering of cellulase formation by cellulose has been confirmed by amplification of the *cbh2* gene in *T. reesei*, which resulted in recombinant strains in which cellulase formation was more efficiently induced by cellulose (Kubicek-Pranz *et al.*, 1991; Messner *et al.*, 1991). This suggests a major role of CBH II in cellulase induction.

In order to further support this possibility, we have constructed recombinant strains of *T. reesei* that contain a disrupted copy of the *cbh2* gene, and have analysed inducibility of cellulase formation by cellulose in these strains.

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Abbreviations: CBH I, II, cellobiohydrolase I, II; EG I, endoglucanase I.

Methods

Organisms. *Trichoderma reesei* QM 9414 was used for the construction of the genomic library. *T. reesei* TU-6, a *pyr4* auxotrophic mutant strain (Gruber *et al.*, 1990a) derived from *T. reesei* QM 9414, served as transformation host. As stated previously (Gruber *et al.*, 1990b), the term *pyr4* rather than *pyrG* is preferred, since the gene contains no introns.

Media and conditions for cultivation. Conditions for growth of *T. reesei* strains in submerged culture on cellulose or other carbon sources have been described previously (Kubicek, 1982). For induction by sophorose in a replacement system, mycelia were pre-grown for 20 h with glycerol as a carbon source, and then transferred to medium free of carbon source as described by Sternberg & Mandels (1979), and cultivated therein for a further 24 h. Conidia for inoculations were obtained by growing *T. reesei* strains on malt agar plates for 14 d at 28 °C.

Cloning of *cbh2* and gene disruption. Chromosomal DNA from *T. reesei*, grown in minimal medium, was isolated as described previously (Gruber *et al.*, 1990a). The DNA was partially digested with *Mbo*I and the resulting fragments of 15–22 kb were ligated into lambda EMBL3, previously cut with *Bam*HI. DNA was packaged into lambda particles *in vitro* and transfected into *E. coli* NM 539. Plaques of the *Trichoderma* gene bank were transferred onto nitrocellulose membrane filters (BA85, Schleicher and Schuell) and hybridized with a ³²P-labelled nick-translated *Dra*III fragment of the *T. reesei cbh2* gene from *T. reesei* L27, kindly supplied by Dr M. Ward (Genencor Int., South San Francisco, CA, USA). Hybridization was carried out at 66 °C for 18 h. The filters were then washed twice with 5 × SSC, additionally containing 0.1% (w/v) SDS and 0.1% (w/v) sodium pyrophosphate (30 min, 66 °C), followed by two further washings with 0.3 × SSC (containing the same additives as above; 30 min, 60 °C). The filters were exposed on Kodak X-OMAT AR film at –70 °C using intensifying screens. From 19000 plaques screened, 40 gave a positive hybridization signal; these were purified and their DNA was subsequently isolated. A 4.2 kb *Eco*RI fragment containing the *cbh2* gene and its flanking regions was isolated from one of them, and cloned into the *Eco*RI site of the polylinker in the *lacZ* gene in pGEM-7Zf(+) to yield pSB1.

The plasmid pSB3 (see Fig. 1) was used to replace a portion of the host *cbh2* gene with the homologous *pyr4* gene. To construct this vector a 2.7 kb *Sal*I fragment of pFG1 (Gruber *et al.*, 1990b), containing the *pyr4* gene and flanking regions, was cloned into the unique *Sal*I site of pSB1 containing *cbh2*.

To disrupt the *T. reesei* TU-6 *cbh2* gene, it was transformed with pSB3 essentially as described previously (Gruber *et al.*, 1990a).

Enzyme assays. Cellulase activity was assayed by measuring 'carboxymethylcellulase' (CMC-ase) activity as described previously (Kubicek *et al.*, 1988).

Quantification of CBH I and II. CBH I and II were quantified by a dot-blot immunotechnique using the monoclonal antibodies CH-5 and CE-16 (Mischak *et al.*, 1989).

Electrophoretic separation of cellulases and immunodetection. Culture supernatants were mixed with 0.5 vol. of 3 × electrophoresis sample buffer (Laemmli, 1970), boiled for 5 min, and centrifuged in an Eppendorf centrifuge (5 min, full speed). A sample (15 µl) of this supernatant was then loaded on 7.5% (w/v, polyacrylamide) gels (Laemmli, 1970) and subjected to SDS-PAGE. The separated proteins were then blotted to nitrocellulose (Burnette, 1981), followed by staining with monoclonal antibodies as described by Mischak *et al.* (1989). The monoclonal antibodies for detection of CBH I, CBH II and EG I were CH-5, CE-16 and EG-3, respectively (Mischak *et al.*, 1989; Luderer *et al.*, 1991). Freshly prepared substrate solutions were used for

each staining experiment. If the intensity of staining was to be compared, samples were loaded on the same gel. If two different antibodies were used to stain proteins on one single blot ('double-immunostaining'), this was done in a sequential order, starting with CH-5.

Results

Construction of *T. reesei* recombinant strains lacking CBH II formation

To generate strains of *T. reesei* that were specifically deficient in the production of CBH II, we employed a gene replacement strategy similar to that described by Miller *et al.* (1985). First (Fig. 1), we constructed a gene replacement vector, called pSB3; this contains a selectable *pyr4* gene from *T. reesei*, which is inserted into the coding region of the *cbh2* genomic clone. This vector was used to transform a *pyr4* auxotroph of *T. reesei* (strain TU-6).

A total of 23 *pyr4*⁺ transformants were screened for CBH II expression (by SDS-PAGE/Western blotting/ 'double immunostaining') during growth on lactose as a carbon source. The rationale behind this was that lactose promotes cellulase formation by *T. reesei* (Mandels, 1982), while growth on lactose is independent of the amount of cellulases secreted and hence not influenced by the possible lack of CBH II in some transformants. This eliminated the possibility of not finding putative CBH II[–] strains, due to their expected poor growth on cellulose. Three CBH II[–] candidates (*T. reesei* transformant strains SB 6, SB 11 and SB 23) were isolated (Fig. 2). Confirmation that a gene disruption event had

Fig. 1. Physical map of plasmid pSB3 used for interrupting the *cbh2* gene of *T. reesei* in this study. Open box, *pyr4* of *T. reesei* (the arrow below shows the 5' orientation, and the coding regions drawn thicker than the rest); shaded region, *cbh2* from *T. reesei* (arrow shows 5' orientation, with the coding region shaded).

Fig. 2. Screening of *cbh2* gene disruption in strains of *T. reesei* grown on lactose as carbon source. Strains were grown for 4 d, and then 20 μ l aliquots of the culture supernatant were subjected to SDS-PAGE and Western blotting. The blots were subsequently stained with monoclonal antibodies against CBH I and CBH II; positions of the bands are indicated by arrows. Individual stable transformants are indicated by their SB number over each track.

Fig. 3. Comparison by Southern blot analysis of *T. reesei* TU-6 (*pyr4*-negative) and some of the transformants. Genomic DNA (1–2 μ g) was digested with *Eco*RI, fractionated by electrophoresis on a 0.8% gel, blotted to nylon membrane (Hybond-N; Amersham), and probed with a 2.15 kb *Sal*I/*Bgl*I fragment of the *T. reesei cbh2* gene. Molecular mass markers (in kb) are indicated on the left. Strains and their phenotypes (in parentheses) are indicated over each track.

occurred was provided by Southern blotting of genomic DNA that had been digested with *Eco*RI. In *T. reesei* TU-6, *cbh2* was detected on a single 4.2 kb fragment. The predicted increase in size of the *Eco*RI fragment of the *cbh2* gene from 4.2 to 6.9 kb was observed in all transformants. However, in the strains that lacked the CBH II protein band in immunostaining of SDS-PAGE/Western blots, the 6.9 kb fragment was the only one, indicating integration at the *cbh2* locus (Fig. 3).

Induction of cellulase formation in the CBH II⁻ *T. reesei* strain

The final titre of CBH I, formed by all the various transformant strains of *T. reesei* during growth on lactose, was roughly the same (data not given), which suggested that their capacity for cellulase secretion was not altered by transformation and the subsequent screening procedure. In order to confirm that their inducibility of cellulase formation was indeed comparable, three randomly chosen CBH II⁺ positive (SB 5, SB 17 and SB 20) and three CBH II⁻ transformants, as well as the original strain QM 9414, were pre-grown on glycerol as carbon source, and then transferred to a replacement medium containing sophorose to induce cellulase formation. The results obtained were comparable within each group of transformants, and are shown for QM 9414 (CBH II⁺) and SB 11 (CBH II⁻) in Fig. 4:

Fig. 4. Induction of CBH I and II formation by sophorose in *T. reesei* QM 9414 and in transformant SB-11. Strains were induced by 4 mM-sophorose, and samples of the culture supernatant were withdrawn after the times indicated and subjected to SDS-PAGE and Western blotting. Staining with monoclonal antibodies against CBH I and II was carried out. The relative positions of molecular mass marker proteins and the positions of CBH I and II are indicated.

Fig. 5. Formation of cellulase activity (a) and CBH I and EG I (b) by *T. reesei* QM 9414 (■) and by transformant SB 11 (□) during growth on Avicel cellulose. Samples were withdrawn after the times indicated and the supernatant subjected to activity assay or to SDS-PAGE/Western blotting. Immunostaining was carried out subsequently, using monoclonal antibodies CH-6 (Mischak *et al.*, 1989) and EG-3 (Luderer *et al.*, 1991) to detect CBH I and EG I, respectively. The relative positions of molecular mass marker proteins and of CBH I and EG I are indicated.

irrespective of the presence of an intact *cbh2* gene, both strains started to produce CBH I immediately after transfer to the sophorose-containing medium, showing maximal titres after about 20–24 h. Quantification by the dot-blot immunotechnique indicated roughly equal levels of secreted CBH I (73 and 68 µg ml⁻¹, respectively).

Growth and cellulase formation of CBH II⁻ strains on cellulose as a carbon source

In our initial experiments, when CBH II⁺ and CBH II⁻ transformants were grown on cellulose as carbon source, no specific differences in the rate of cellulase formation was observed between them (data not shown). Since the cellulase-production medium of *T. reesei* contains peptone, which is used as a carbon source prior to cellulose (unpublished results), we suspected that this may mask a possible difference between the strains. These experiments were therefore repeated with media from which peptone had been omitted. The results (shown for QM 9414 and SB 11, respectively; Fig. 5) indicated that the CBH II⁻ strain required about 18–24 h longer to induce cellulase formation on cellulose. Its growth was correspondingly delayed. Thereafter, however, the rate of enzyme formation was comparable to that of the CBH II⁺ control. Quantification of the amount of CBH I secreted into the medium by dot-blot scanning (John *et al.*, 1984) yielded consistent results.

The delayed growth of the CBH II⁻ transformants was specifically observed on cellulose as carbon source, and not on maltose, glycerol and lactose (data not shown).

Discussion

The present paper is the first to describe the construction and properties of a CBH II⁻ recombinant strain of *T.*

reesei, although *cbh2*-gene disruption has been also carried out by others (Suominen *et al.*, unpublished data; cited in Uusitalo *et al.*, 1991; A. Harkki, personal communication). Besides the usefulness of this phenotype in studies on the regulation of cellulase formation – as described here – such strains may serve various attractive purposes: they may serve as recipient strains in studies where the expression of a manipulated copy of *cbh2* in a homologous system is desirable (e.g. site-directed mutagenesis of *cbh2* or studies on the function of its promoter). It may also be worth considering the use of the *cbh2* structural gene as a reporter gene in the study of other *Trichoderma* promoters, since CBH II is secreted into the medium and both fluorogenic substrates (Van Tilbeurgh & Claeysens, 1985) as well as monoclonal antibodies (Mischak *et al.*, 1989) are available for detection of CBH II with high sensitivity. This is particularly worthwhile, since conventionally used reporter genes (*lacZ*, *uidA*; cf. Van Gorcom & Van den Hondel, 1988; Roberts *et al.*, 1989) are not secreted and the gene products are prone to proteolysis.

An additional application of a CBH II⁻ strain is that it may serve as a source for purifying endoglucanase (EG) I that is essentially free of CBH II. Due to the very similar physico-chemical properties of EG I and CBH II (Enari & Niku-Paavola, 1987; Kubicek, 1992), and the appearance of enzyme–enzyme complexes (Tomme *et al.*, 1990), purification of either enzyme from culture filtrates of *T. reesei* is difficult, and cross-contaminated ‘purified’ preparations actually caused some confusion about the substrate specificities of these two enzymes, and their contribution to the synergistic breakdown of cellulose (Kyriacou *et al.*, 1987). Penttilä *et al.* (1987) solved this problem by expressing individual cellulase cDNAs in *Saccharomyces cerevisiae*, to obtain single enzymes only. This strategy, however, suffers from the drawback that the cellulases secreted by yeast are overglycosylated

(Penttilä *et al.*, 1987; Van Arsdell *et al.*, 1987), which may affect some enzymic properties. We thus recommend the use of a CBH II⁻ *T. reesei* strain for purifying EG I.

We used CBH II⁻ strains of *T. reesei* to elucidate the role of CBH II in triggering cellulase formation. The results showed that strains lacking CBH II exhibit a considerable lag in growth on cellulose and corresponding cellulase formation. This finding is in accordance with the postulation of a major role of CBH II in cellulase induction by cellulose (Messner *et al.*, 1991), although it clearly shows that CBH II is not essential for this process. Apparently, in the absence of CBH II, CBH I also can initiate the attack on cellulose, albeit at a lower rate. On the other hand, similar growth and cellulase formation rates were observed with CBH II⁻ and CBH II⁺ strains when peptone was present in the cellulose-containing medium. In this case, the conidia germinate while using peptone as a carbon source and the mycelium starts to secrete cellulases into the medium which then attack cellulose. Since the hyphae secrete EG I, it is possible that the synergism between CBH I and EG I is sufficient for an optimal rate of hydrolysis, and thus CBH II is dispensable. Such an assumption also appears to be substantiated by the findings that growth of CBH II⁻ strains on cellulose, after overcoming the lag, took place at a rate comparable to the CBH II⁺ strains. Thus the lag during the phase of initial attack by the conidial-bound cellulases is most probably the result of the fact that they lack EG I (Messner *et al.*, 1991), and the CBH II⁻ strains therefore contain only a single cellulase. The data are therefore consistent with the assumption that a synergism of at least two cellulolytic enzymes is needed for an optimal rate of cellulolysis (Henrissat *et al.*, 1985).

If these interpretations are correct, then we are faced with the fact that *T. reesei* conidia use 'exo-exo'-synergism, whereas *T. reesei* mycelia may use both 'exo-endo' as well as 'exo-exo' synergism to degrade cellulose. It is tempting to speculate on the physiological advantages of these two different mechanisms.

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