3 Results

The broad aim of the current work was to explore protein quality control in the filamentous fungus *T. reesei* and more specifically the role of the proteasome in this process. Towards this end, different mutant versions of the main secreted protein cellobiohydrolase I (CBHI) were expressed in *T. reesei*. The mutations introduced in the CBHI molecule were predicted to prevent formation of selected disulfide bridges, which would lead to misfolding of the protein, its accumulation in the ER, induction of the UPR pathway and possibly the ERAD pathway. The consequences of expression and secretion of three different mutated CBHIs on the UPR and ERAD pathways and hyphal physiology were investigated.

3.1 Proteomic characterisation of the proteasomes from *Trichoderma reesei*

Since there were no previous reports on isolation and characterisation of proteasomes from filamentous fungi, the first step in the current work was to purify the *T. reesei* proteasome for proteomic analysis. Extraction and purification of the fungal proteasome was performed by two methods. The first was a conventional method used for purification of the yeast proteasome. A resulting publication (Grinyer *et al*., 2007, Appendix 1) was the first to report purification and identification of the 20S proteasome from a filamentous fungus. The second purification method described and developed in this work was successful in purifying the entire 26S proteasome particle (Kautto *et al*., 2009 Appendix 2) also described in section 2.1.

3.1.1 Purification of the proteasomes

Cultivation of *T. reesei* and sample preparation for the chromatography steps were similar in both methods except that the lysis of the hyphae was carried out by sonication in the method by Grinyer *et al*. (2007) and by French press in the second purification method (Kautto *et al*., 2009). Further, the buffers used in the chromatography steps were different.

The presence of the 20S core particle in the eluted fractions was monitored during purification by measuring the chymotrypsin-like activity. The 20S Proteasome Assay Kit AK-740 (Biomol) used for chymotrypsin-like activity measurement detects the amount of fluorescence produced by cleavage of the fluorogenic substrate SUC-LLVY-AMC. Cleavage of the peptide LLVY from the 7-Amino-4-Methylcoumarin (AMC) will produce fluorescence which can be read using a 380 nm excitation and a 460 nm emission filter in a fluorometer (Stein *et al*., 1996).

Purification of the 20S proteasome

Purification of the 20S proteasome was performed by a three-step chromatography procedure. First, the fungal cell extract was fractionated on a DEAE-Affigel blue column. Fractions showing chymotrypsin-like activity were eluted at 270-312 mL with 100 mM NaCl. These eluates were pooled and then loaded onto Resource Q column and eluted at a salt concentration of approximately 300-340 mM NaCl. Eluted fractions were again pooled and concentrated into 1 mL for gel filtration by Superose 6 (Fig. 19). In the final step, proteasomes were eluted in fractions 23-40 in 100 mM NaCl; these fractions were pooled and applied for Western blotting to confirm purification of the 20S proteasome (see section 3.1.1.1, Fig. 21A). The purified 20S proteasome sample was further analysed by 2DE and mass spectrometry (section 3.1.3).

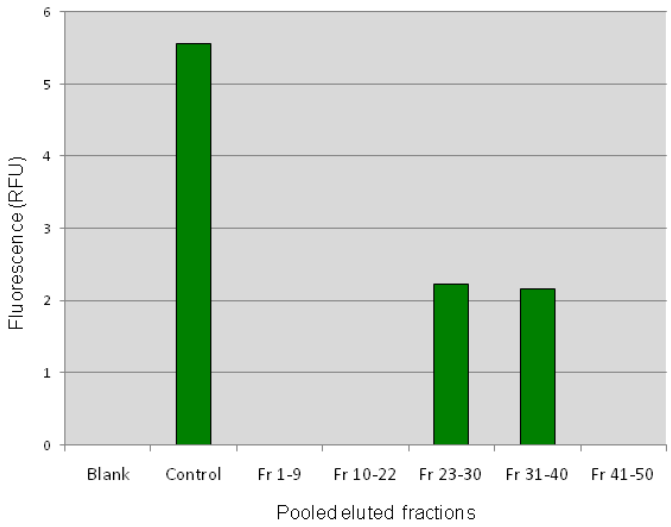

Figure 19. Pooled fractions 23-30 and 31-40 eluted from the Superose 6 column showing chymotrypsin-like activity. These fractions are results of three steps chromatography purification of fungal cell extract. First, the fungal cell extract with protein concentration of 134.4 mg/ml and chymotrypsin activity of 1.2 pmol/min was fractionated on a DEAE-Affigel blue column. Eluted fractions within 230-310 mL with 100 mM NaCl were further loaded onto Resource Q column and the proteasome was eluted at a salt concentration of approximately 300-340 mM NaCl. After fractions were concentrated down to 1 mL, they were loaded into Superose 6 column and the eluted fractions were pooled into five fractions as shown above: Fr 1-9, Fr 10-22, Fr 23-30, Fr 31-40 and Fr 41-50. Chymotrypsin activity was found in fractions 23 to 40 totalling 20.26 pmol/min. Control: human proteasome from the 20S Proteasome Assay Kit AK-740 (Biomol, USA).

Purification of the 26S proteasome

The fungal cell extract was applied onto a 1 mL POROS® HQ column (anion exchange, Applied Biosystems) at 5 mL min^{-1}, using an ÄKTA Explorer 10S FPLC system (GE Healthcare). The fractions showing chymotrypsin-like activity eluted at approximately 230□ 280 mM NaCl in buffer B (10 mM Tris - 10 mM NaH_2PO_4 (pH 7.5) - 1 mM ATP) (Fig. 20A) were pooled and concentrated down to 1 mL as mentioned in section 2.1.2. The concentrated sample was separated on a 150 mL Sephacryl S-500 HR (GE Healthcare Life Sciences, Uppsala, Sweden) size exclusion column at 0.5 mL min^{-1} with 1M NaCl in buffer B. Eluted fractions containing high chymotrypsin-like activity (Fig. 20B) were pooled and concentrated to 1 mL and stored at -80 ^{o}C until required. The total protein concentration of the purified proteasome sample was 2.7 mg mL^{-1}. A 133-fold purification yielded 1.7 mg of proteasome with chymotrypsin activity of 20.26 pmol/min. This is 16.8 times more than chymotrypsin activity in the crude extract, suggesting good purity of the 20S proteasome extract. Proteasome is a highly abundant protein, estimated at 0.5□1% of total soluble protein (Orlowski, 1990; Chen and Hochstrasser, 1995). Specific peptidase activity of the

final purified sample was more than 18-fold higher than that of the crude extract confirmed good purity of proteasome extracted from fungal cells.

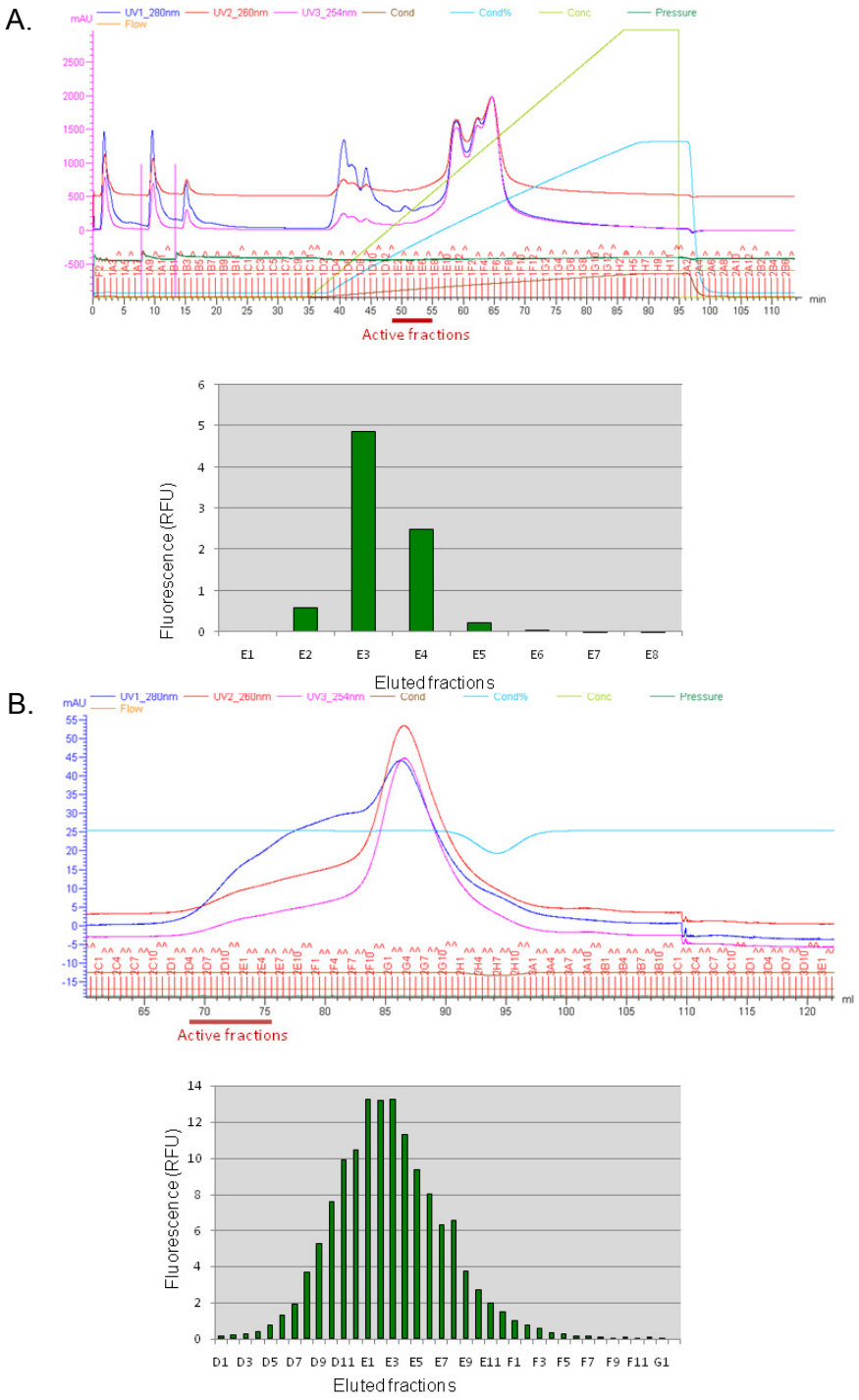

Figure 20. Chromatograms and graphs showing the fractions with chymotrypsin-like activity from the 26S proteasome purification. A. Chromatograph of POROS® HQ column and chymotrypsin-like active fractions were eluted at 230-280 mM NaCl; B. Chromatograph of the Sephacryl S-500 HR column and chymotrypsin-like activity of fractions (D1-F11) eluted from the Sephacryl S-500 HR column.

3.1.1.1 Western blotting of the purified 20S and 26S proteasomes

The purification of the proteasomes using the methods discussed above was confirmed by 1D SDS-PAGE and Western blotting (Fig. 21). A yeast polyclonal 20S antibody (PW9355, Biomol Int., USA) was used to detect the *T. reesei* 20S proteasome. The yeast 20S antibody plus a mixture of five 19S particle subunit antibodies (PW8315, PW8160, PW8250, PW8220, PW8215; Biomol Int., USA) were used to analyse the 26S proteasome sample (Fig. 21 B). Proteins from the 20S particle purified by the conventional method (Grinyer *et al*., 2007; Fig. 21A, lane 2) and proteins from the 26S (Fig. 21B, lane 3) proteasome purified using the method developed in this work (Kautto *et al*., 2009) exhibited a different protein profile on 1D SDS-PAGE with the 26S proteasome featuring a considerably larger number of proteins of higher molecular weights.

Lane 3 in the Western blot (Fig. 21A) shows the presence of the 20S proteasome particle with the antibody recognising proteins in the range of 20-35 kDa typical for the 20S core subunits. Western blot of the proteasome sample purified with the new method indicated the presence of 20S proteasome subunits and also the Rpt1, Rpt2, Rpt3, Rpt4 and Rpt6 subunits of the 19S particle (Fig. 21B, lane 4). Western blot analysis therefore provided evidence that the entire 26S proteasome had been purified with the new method. The antibodies detected protein bands with molecular weights corresponding to the known proteasome subunits in yeast (Glickman *et al*., 1998) and therefore provided further support to the notion that fungal proteasomes had been successfully purified.

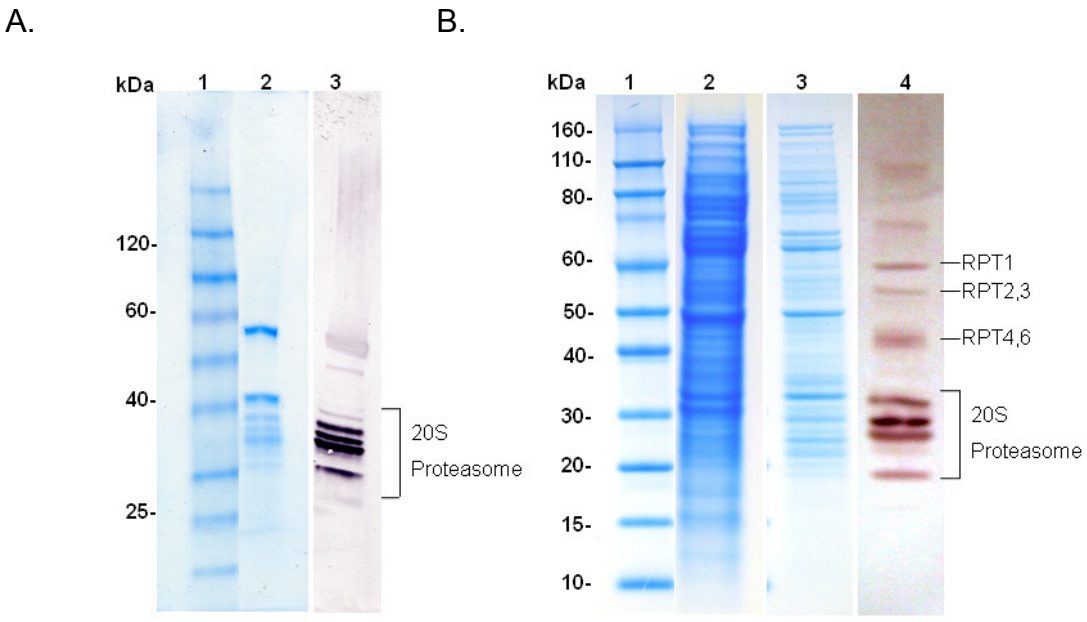

Figure 21. SDS-PAGE and Western blots of the *T. reesei* proteasomes purified by two different methods. A. 20S proteasome after purification using the three-step chromatography method. Lane 1: Molecular weight marker (Fermentas) Lane 2: 20 µg of purified 20S proteasome on SDS-PAGE gel stained with Colloidal Coomassie Blue. Lane 3: Purified 20S proteasome detected using a yeast 20S polyclonal antibody; B. 26S proteasome after purification using two-step chromatography method. Lane 1: Molecular weight marker (Invitrogen). Lane 2: Eluted and pooled fractions (E1-E7, 40 µg protein) from the POROS® HQ column stained with Colloidal Coomassie Blue. Lane 3: Eluted and pooled fractions (D1-F11, 25 µg protein) from the Sephacryl S-500 HR column on SDS-PAGE stained with Colloidal Coomassie Blue. Lane 4: Purified 26S proteasome detected by a mixture of yeast 20S polyclonal antibody and five polyclonal yeast anti-19S proteasome antibodies.

In summary, the two methods used for purification of the 20S proteasome particle and the 26S proteasome from *T. reesei* differed in terms of the number and type of columns and the buffers used to perform the chromatography. In the purification of the 20S proteasome, the three chromatography columns were affinity, ion exchange and size exclusion columns while the 26S proteasome was purified through two columns: an anion exchange and size exclusion column. The buffer for purification of the 20S proteasome was 50 mM Tris (pH 7.5) - 5 mM $MgCl_2$ - 10 % (v/v) glycerol - 5 mM ATP - 1 mM DTT - 1 mM PMSF - 0.1 % (v/v) fungal and yeast protease inhibitor cocktail (Sigma, P8215) whereas 10 mM Tris - 10 mM NaH_2PO_4 (pH 7.5) - 2 mM ATP was used for 26S purification. Application of the two-column method simplified the purification process and shortened purification time by 50 % compared to the conventional method. The most significant advancement achieved with the second purification method using the POROS® HQ column and Sephacryl S-500 HR was efficient purification of the entire 26S proteasome. Proteasome is a highly abundant protein

(0.5-1 % of total soluble protein; Orlowski, 1990; Chen and Hochstrasser, 1995). Specific chymotrypsin activity of the final purified sample was more than 16-fold higher than that of the crude extract which can be considered good. The patterns of subunits visualised on SDS-PAGE gels and by Western Blotting are very similar to those observed with highly purified 26S proteasomes from other organisms.

3.1.2 Proteome mapping of the *Trichoderma reesei* proteasome

The purpose of two-dimensional analysis of the *T. reesei* proteasome was to map out the subunits of the *T. reesei* proteasome for comparison with characterised proteasomes, and lay ground for studies into potential changes in proteasome expression upon production of various heterologous proteins in this fungal system.

Two-dimensional protein maps of the purified *T. reesei* proteasomes were produced after purification and desalting of the samples by protein precipitation. The 2D-gels contained 150 and 200 protein spots when separated across pI ranges of 3-10 or 4-7 as shown in Fig. 22 and 23 respectively. From these, 122 protein spots from the gel in Fig. 22 and 172 spots from the gel in Fig. 23 were excised and processed for mass spectrometry analysis and protein identification. For the 20S proteasome extract, protein spots for identifications were excised from the gel representing proteins separated across pI range 3-10. Note that Fig. 22 shows a cropped image of the pI range 3-7 for the simple reason that no protein spots existed beyond pI 7. An IPG strip with a pI range 4-7 was used to separate proteins in the 26S proteasome extract.

Protein spots cut from the above gels were prepared for MS/MS analysis as described in section 2.1.4. Peptide mass fingerprints of tryptic peptides were generated by matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in the MS mode. The instrument was then switched to MS/MS (TOF/TOF) mode where the eight strongest peptides from the MS scan were fragmented by collision-induced dissociation. Two types of databases were searched for protein identifications. The first search was against all known fungal proteins (cross species identification, CSI) and the second search

was against a custom-made, in-house *T. reesei* database developed by M. Traini (described in Grinyer *et al*., 2007).

All identified proteins are shown in Tables in Appendix 3 and 4, which also provide further information on each protein identified including the number of peptides matched, amino acid coverage, presence of conserved protein domains and relation of the identified protein to the proteasome. Thirty two unique proteins representing 50 different protein spots including one spot, which was a mixture of two different proteins (Fig. 22), were identified from the 20S proteasome extract using the CSI approach. Many of these proteins were identified from closely related fungal species such as *Gibberella zeae* and *Neurospora crassa*. In addition, a search of the custom-made *T. reesei* database featuring all species specific cDNAs translated into amino acid sequences, 45 unique proteins from 81 different spots were identified; this number included 11 spots which were a mixture of more than one protein. Each identified protein was submitted to a BLASTP search at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the name and function of the protein. Appendix 3 provides further information on each identified protein including the number of peptides matched, amino acid coverage and the presence of conserved protein domains found in proteasome subunits and proteasome-associated proteins.

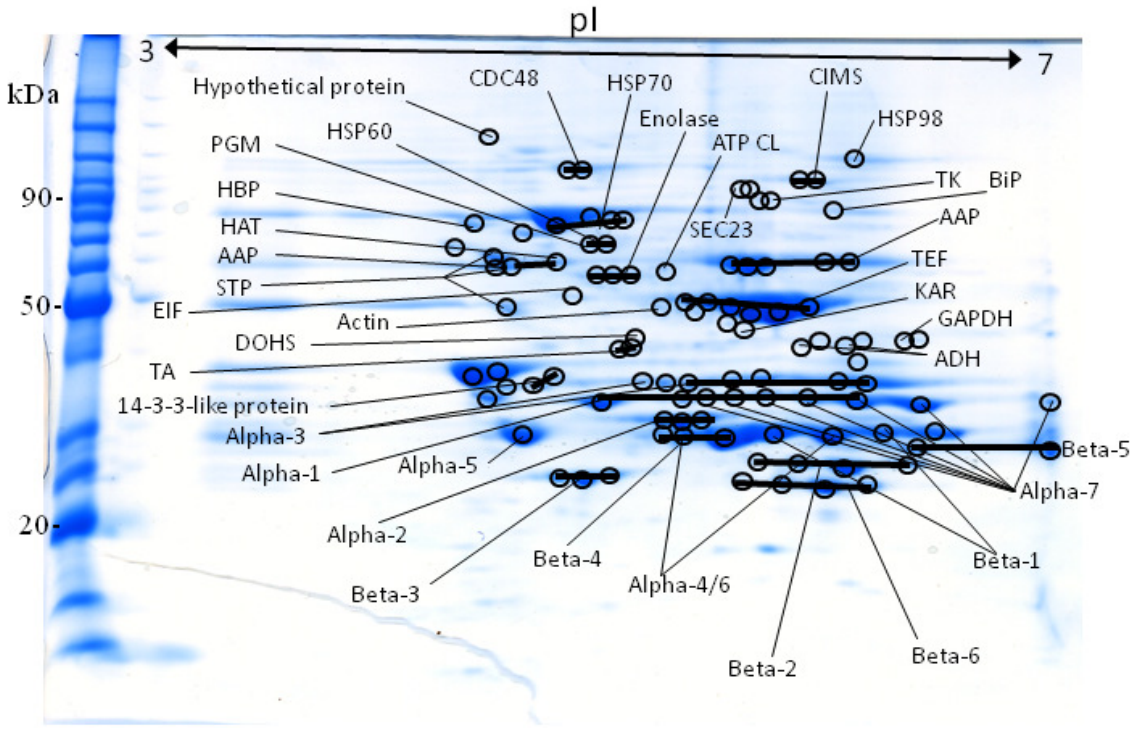

Figure 22. 2D-map of the purified *T. reesei* 20S proteasome. Protein identifications were made from MALDI and MS/MS analyses and the PMF data compared to all fungal proteins in the NCBI non-redundant protein database and an in-house *T. reesei* database. The proteins were focused across 11 cm 3-11 IPG strips in the first dimension and run on 4-12 % SDS-PAGE in the second dimension. Thereafter the gel was stained with Colloidal Coomassie Blue. The image has been cropped to show the pI range 3-7 only. Abbreviations used in the protein map include EIF, eukaryotic initiation factor; BIP, 78 kDa glucose-regulated protein; HSP, heat shock protein; EF, elongation factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KAR, ketol acid reductoisomerase; STP, serine/threonine phosphatase; ADH, alcohol dehydrogenase; DOHS, deoxyhypusine synthase; TA, transaldolase; TEF, translation initiation factor; ATP, CL ATP citrate lyase; AAP, aspartyl aminopeptidase; HAT, histone acetyltransferase; HBP, histone binding protein; PGM, phosphoglycerate mutase; Tk, transketolase; CIMS, cobalamin-independent methionine synthase; CDC, cell division control.

Protein identifications from the 26S proteasome sample were conducted similarly to the identifications for the 20S proteasome. Forty five unique protein identifications were obtained from the in-house *T. reesei* database and six additional unique proteins were identified by CSI from related fungal species such as *N. crassa* and *G. zeae*. Combined protein identification results from the 26S proteasome sample are presented in Fig. 23.

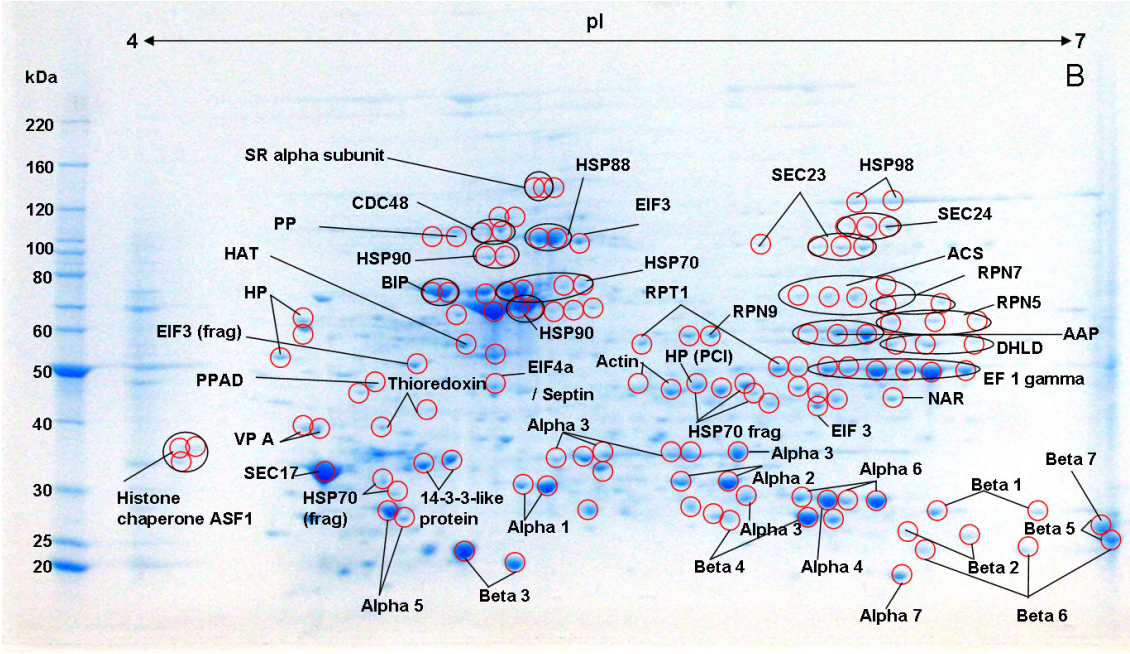

Figure 23. 2D-map of a purified *T. reesei* 26S proteasome with protein identifications. The proteins were focused across 11 cm 4-7 IPG strips in the first dimension and run on 4-12% SDS-PAGE in the second dimension. Thereafter the gel was stained with Colloidal Coomassie Blue. Protein identifications were made from MALDI and MS/MS analyses and the PMF data compared to all fungal proteins on NCBI non-redundant protein database and an in-house *T. reesei* database. Abbreviations used on protein maps include EIF, eukaryotic initiation factor; BIP, 78 kDa glucose-regulated protein; HSP, heat shock protein; EF, elongation factor; HAT, histone acetyltransferase; CDC, cell division control; HP, hypothetical protein; PPAD, porhyromonas-type peptidyl-arginine deiminase; PP, predicted protein; SR, sulfide reductase; VP, vacuolar protease; ACS, acetyl coA synthetase; DHLD, dihydrolipolyl dehydrogenase; NAR, norsolorinic acid reductase; RPT, regulatory subunit, ATP-ase; RPN, non-ATP-ase regulatory subunit.

Seven α (Alpha 1-7) and six β (Beta 1-6) subunits of the 20S proteasome particle were identified using the conventional purification method. However, it was not efficient in revealing the β7 subunit. The 26S proteasome 2D-map contained all 14 α and β subunits from the 20S particle and four subunits from the 19S particle. Table 7 shows the comparison of protein identifications achieved from the two purification methods.

Table 7. Comparison of protein identifications from the 20S proteome using a conventional purification method and the 26S proteome using the new purification method developed in this work.

	Conventional method (20S purification)	***New method (26S purification)***
Total number of protein spots analysed	122	176
Identifications by CSI	32	51
Identifications from the in-house *T. reesei* database	36	45
20S particle subunits	13	14
19S particle subunits	-	4
PIPs (proteasome interacting proteins)	8	9
Chaperones	4	7
No association to the proteasome	10	13

Several proteasome interacting proteins (PIPs; Verma *et al*., 2000; Guerrero *et al*., 2006) were co-purified along with the fungal proteasome, although their number and nature varied between 20S and 26S purification methods. Known PIPs, such as ER-resident chaperones BiP, stress-inducible 70 kDa heat shock protein Hsp70 and two stress response proteins, Hsp90 and Hsp98 were co-purified with the proteasome through both purification methods. The conventional method also resulted in co-purification of the mitochondrial chaperone Hsp60, which is known to be responsible for the transport and refolding of proteins from the cytoplasm into the mitochondrial matrix (Koll *et al*., 1992). A member of the 70 kDa heat shock family, chaperone Hsp88 only co-purified with the 26S proteasome. Hsp88 interacts with at least two other heat shock proteins known as PIPs, Hsp70 and Hsp30 and thus may also have a role as a PIP (Pleofsky-Vig and Brambl, 1998).

Six known PIPs were identified with both methods; these include 14-3-3-like protein, glyceraldehyde-3-phosphate dehydrogenase, translation elongation factor, actin, translation initation factor 1 and ATPase in ER (CDC48) which is required for the degradation of substrates by a ubiquitin-mediated pathway in yeast (Ghislain *et al*., 1996). The eukaryotic initation factor 4 α was co-purified with the proteasome by both methods indicating that the proteasome was also functioning during transcription in fungal cells (Lee *et al*., 2005). Known PIPs co-purified with either the 20S or 26S included UPR negative regulator serine/threonine phosphatase (Welkinda *et al*., 1998), eukaryotic initation factor 3, transaldolase, enolase, alcohol dehydrogenase and thioredoxin (Verma *et al*., 2000).

Several proteins not yet classified as PIP but shown to associate with the proteasome (Chung *et al*., 2001; Hedge and Upadhya, 2006; Fu and Sztul, 2003) were identified in this study. Examples include a histone acetyltransferase type B subunit, histone binding protein, histone chaperone ASF1, SEC23, SEC24 and septin.

Interestingly, chromatography purifications also resulted in the co-purification of other proteins that have not been described to associate with the proteasome so far. These include ketol acid reductoisomerase, deoxyhypusine synthase, ATP citrate lyase, phosphoglycerate mutase, transketolase, cobalamin-independent methionine synthase vesicular fusion protein Sec17, vacuolar protease A, norsolnic acid reductase, porphyromonas-type peptidyl arginedeiminase, dihydrolipyoldehydrogenase and aspartyl aminopeptidase, acetyl-CoA synthase, sulphite reductase alpha, hypothetical protein with PCI domain and hypothetical proteins. These proteins were consistently purified with either 20S or 26S proteasomes across triplicate purifications and are therefore likely to have some association to the proteasome. However, their role as PiPs needs to be confirmed by further studies, for example, by using quantitative analysis of tandem affinity purified *in vivo*-cross linked protein complexes (QTAX; Guerrero *et al*., 2006).

In summary, the two chromatography methods used to purify the *T. reesei* proteasomes resulted in identification of all the 20S proteasome and four 19S proteasome subunits. Homologues to several UPR related proteins and PIPs previously found to interact with the yeast proteasome were also identified from *T. reesei*. Some proteins that have not previously been known to interact with the proteasome were constantly co-purified throughout this work indicating that they had some association with either the 20S core particle or the 19S regulatory particle. The time required for proteasome purification could be shortened from five to two and a half days with the new method developed in this work. These approaches yielded proteasome samples with high purity and chymotrypsin activity. The new method also proved efficient in purification of the entire 26S proteasome (Kautto *et al*., 2009).

3.1.3 Visualisation of the proteasome by transmission electron microscopy (TEM)

To evaluate the integrity of the purified proteasomes, extracted from the *T. reesei* Rut-C30 mycelia grown in a minimal medium containing 2 % (v/v) glycerol and purified by the two step chromatography method were analysed using TEM. After buffer exchanges to reduce salt concentration from the eluted and pooled proteasome samples, they were loaded onto copper grids and negatively stained with 4 % (w/v) uranyl acetate to examine their structure by transmission electron microscopy. Image analysis revealed a structure similar to the yeast proteasome featuring a 19S particle and a 20S particle consisting of four stacked rings. Images taken from the purified preparations showed that the majority of particles featured the 20S particle alone (Fig. 24A and B). In Figure 24C, the 20S particle is capped at one end by a 19S particle.

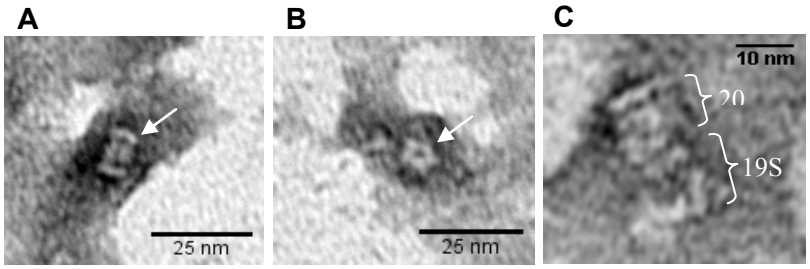

Figure 24. Transmission electron micrographs of the *T. reesei* 20S proteasome. A. The 20S particle; typical four-stacked rings are visible in the side-on views. B. The 20S particle; the central channel is visible in the end-on view. C. The 20S particle is capped on one end with the 19S particle. Samples were negatively stained with uranyl acetate. Bars: 25 nm and 10 nm.

Dissociation of the 20S particle from 19S may be due to the dilution of the sample to a level suitable for microscopy as also observed by Cascio *et al*. (2002) and Majetshak and Sorell (2008). While the assembly of the 20S particle is known to be assisted by chaperones, the nature and dynamics of association of the 19S with the 20S particle is still unknown. It is possible that the lid and base sub complexes of the 19S particle associate after the subunit Rpn10 has recognised and bound to the substrate as suggested by Rosenzweig and Glickman (2008) and Le Tallec, *et al*. (2009). Thereafter, the 19S complex will associate with the 20S particle by an ATP-dependent manner, triggering the next ATP-dependent reaction, opening of the gate in the 20S particle allowing the substrate to enter the degradative chamber. Although the 20S subunits and their role in substrate degradation are well defined, the nature of the interaction between the 20S and 19S subunits, and the exact function of the 19S lid subunits in this interaction is still unknown.

In summary, the *T. reesei* 26S proteasome purified in this work and visualised using TEM appeared very similar to the yeast (Groll, *et al*., 1997), *Drosophila melanogaster* (Waltz *et al*., 1998) and bovine red cell proteasomes (Adams *et al*., 1997) featuring four alpha and two beta rings forming the barrel-shaped 20S particle. Images showing the 19S regulatory particle attached to the 20S core particle confirmed that the method developed in this work was able to purify both the 19S and 20S proteasome particles together.

3.2 Generation of mutant (misfolded) cellobiohydrolase I

Mutations in the *cbh1* core gene were made by site-directed mutagenesis to examine secretion of the misfolded proteins and the effect of expression of the mutant proteins on protein quality control. The overall cellular response was explored using microscopy and microarrays. As discussed below, three different mutant *cbh1* genes were designed and expressed in *T. reesei*. The mutations were chosen to disturb formation of disulfide bridges in the CBHI protein leading to protein misfolding and induction of UPR and ERAD pathways.

3.2.1 Site-directed mutagenesis

Mutations to the *cbh1* gene were introduced by site-directed mutagenesis using a set of primers in which the cysteine residues had been replaced with proline-coding codons. To amplify a mutant *cbh1* with two cysteines replaced with proline at positions 4 and 25, three separate DNA fragments were amplified in the first round PCR (Fig. 14). Similarly four and five separate DNA fragments were respectively amplified to achieve substitution of four (Cys4, Cys25, Cys172, and Cys176) and five cysteine residues (Cys261 in addition to the previous four).

Seven different fragments amplified in the first round PCR had the size of approximately 313 bp (I), 86 bp (II), 506 bp (III), 282 bp (IV), 616 bp (V), 874 bp (VI) and 1388 bp (VII). After purification, the correct size of each fragment was confirmed by running the DNA on a 1 % (w/v) agarose gel along with the molecular weight marker. Different combinations of

the fragments were then used as a template for the second round PCR to construct three different mutant *cbh1* genes. All three mutated *cbh1* gene fragments were then introduced to the pCVt expression vector (section 2.2.1). Recombinant plasmids with introduced *cbh1* mutations inserts were partially sequenced in order to confirm that all intended mutations had been achieved. After transformation of the expression cassettes into *T. reesei*, the genomic DNA was extracted from selected transformants and sequencing was tried, however, without any success.

Two plasmids, pCVtΔ2 and pCVtΔ4 seemed to have the expected mutations, however the plasmid pCVtΔ5 had one extra point mutation in addition to the five designed mutations. The serine at position 211 had been changed to proline due to replacement of a thymidine (T) by cytosine (C).

3.2.2 Generation and screening of transformants

The expression cassettes were designed to facilitate homologous integration of the mutated *cbh1* genes into the endogenous *cbh1* locus by containing extended 5□and 3□sequences homologous to the targeted gene locus. Each expression cassette was excised from the pUC19 plasmid by digestion with *Sph*I, then purified prior to transformation into *T. reesei*.

Linear expression cassettes derived from the plasmids pCVt, pCVtΔ2 pCVtΔ4 and pCVtΔ5 (section 2.2.2.1) were introduced into *T. reesei* by protoplast transformation (section 2.2.3.1). Table 8 summarises the number of transformants obtained from each expression DNA following transformations and a second round selection on hygromycin-containing PDA plates. Same transformants were further screened using PCR and Southern blotting.

Table 8. Number of transformants obtained with plasmids harboring different *cbh1* mutant genes.

Strain	***CVt***	***CVtΔ2***	***CVtΔ4***	***CVtΔ5***
1st hygromycin selection	122	112	94	97
2nd hygromycin selection	45	59	49	72
Transformants showing homologous integration into the endogenous *cbh1* locus	11	5	8	2
Transformants carrying one gene copy of the *cbh1* gene (expression cassette) in the endogenous *cbh1* locus	1	2	4	1

Genomic DNA was extracted after the second hygromycin selection and the transformants were analysed by PCR to identify where homologous integration of the transforming DNA into the *cbh1* locus had occurred (section 2.2.4.2; Fig.15). Eleven CVt transformants, five CVtΔ2 transformants, eight CVtΔ4 transformants and two CVtΔ5 transformants were positive for homologous integration as assessed by PCR. All these transformants were further analysed by Southern blotting to determine the number of copies of each mutant *cbh1* gene in the *T. reesei* genome (Fig. 25). Genomic DNA was digested with the restriction enzymes *Bam*HI and *Eco*RV and hybridised with a *hph* probe attaching to the end of the hygromycin gene or a *venus* probe attaching to the venus gene (section 2.2.4.2; Fig. 15). A single band of 3.7 kb hybridising with the hygromycin gene region (shown for CVtΔ4 in Fig. 25 lane 6) when the DNA was digested *Bam*HI strongly suggested that the expression DNA and thus the *cbh1* gene was present as a single copy in the *T. reesei* genome and at the *cbh1* locus (Fig.15). A similar Southern analysis was performed for each of the other CBHI mutant strains (not shown).

When the digestion was carried out using *Eco*RV, a single copy of the gene cassette in the *cbh1* locus would produce a 4.7 kb band when probed with *venus* (data not shown).

When the genomic DNA was digested with *Bam*HI four different sized bands were detected in one of the pCVtΔ4 transformants (line 1 in Fig. 25). This means that the expression cassette had been integrated into the genome in four different places randomly, and one of them was in the *cbh1* locus as indicated by the 3.7 kb band in the blot.

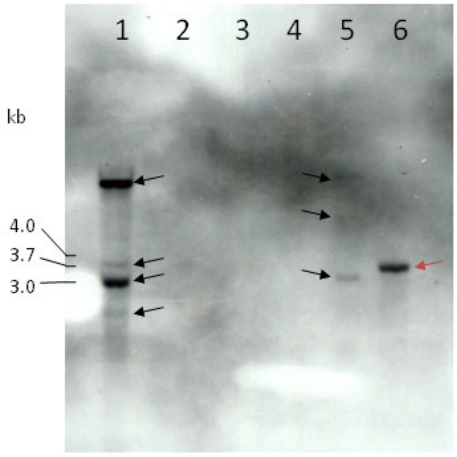

Figure 25. Number of copies of the expression cassette containing the mutated *cbh1* gene in the genome of the *T. reesei* pCVtΔ4 transformants. Lane 1, at least four copies; Lanes 2-4, zero copies; Lane 5, at least three copies; Lane 6, one copy of the *cbh1* gene (expression cassette) replacing the endogenous *cbh1*. The genomic DNA was digested by *Bam*HI enzyme.

In the CVt strain, the endogenous *cbh1* gene was replaced by a fusion construct carrying a non-mutant *cbh1* gene. Transformant strains with one copy of the three *cbh1* mutant genes in the genomic *cbh1* locus were chosen for further studies. These strains were named as CVtΔ2, CVtΔ4 and CVtΔ5. The transformant non-mutant strain was named CVt.

In summary, site-directed mutagenesis was used to replace the selected cysteines with proline in the *cbh1* core gene to promote conformational changes to the 3-dimensional structure of the CBHI core protein and therefore produce misfolded proteins. Three transformant (mutant) strains carrying a single copy of a particular mutant *cbh1* core gene in the endogenous *cbh1* locus were generated. All mutations were constructed using a previously constructed plasmid (pCVt) in which the *cbh1* core gene had been fused with the gene encoding the fluorescent protein Venus. Therefore, all mutant strains were also carrying the fluorescent reporter gene. The expression DNA cassettes prepared from the non-mutant pCVt, pCVtΔ2, pCVtΔ4 and pCVtΔ5 were transformed into *T. reesei* and strains carrying one copy of each type of expression DNA were chosen for further analysis. From now on, transformant strains carrying a mutant *cbh1* core gene may also be called "mutant CBHI strains" depending on the context. Similarly, the "*cbh1* core" gene may also be referred as *cbh1* gene.

3.3 Molecular characterisation of the mutant CBHI strains

The mRNA expression level of the mutant *cbh1* genes in the transformants, non-mutant CVt as well as the non-transformant Rut-C30 was determined by Northern analysis. A global view of the effects of expression of the mutant CBHI forms was pursued through microarrays discussed in Chapter 4.5.

In order to compare potential differences in the transcription of the mutant *cbh1* gene between the different transformant strains, Northern analysis was performed at an early time point, of 12 h and thereafter, at the onset of secretion during 24 h and at 48 h (Fig. 31).

The non-transformant RutC-30 expressing the native CBHI protein was used as a control strain for the expression analysis. The transformant strain CVt has a non-mutant *cbh1* gene fused to the DNA sequence encoding the fluorescent protein Venus. The three mutant strains generated from CVt were CVtΔ2, which has two cysteines replaced with proline, CVtΔ4 which has two additional substitutes to those in CVtΔ2 and CVtΔ5 which has one more cysteine replaced with proline in position 261 in addition to the mutations in CVtΔ4 (Fig. 12). Total RNA was extracted from cultures grown in the CLS-medium and blotted onto a nylon membrane. Northern blots were hybridised with dig-labeled *cbh1* (506 kb) and *gapdh* (528 kb) probes (Fig. 26).

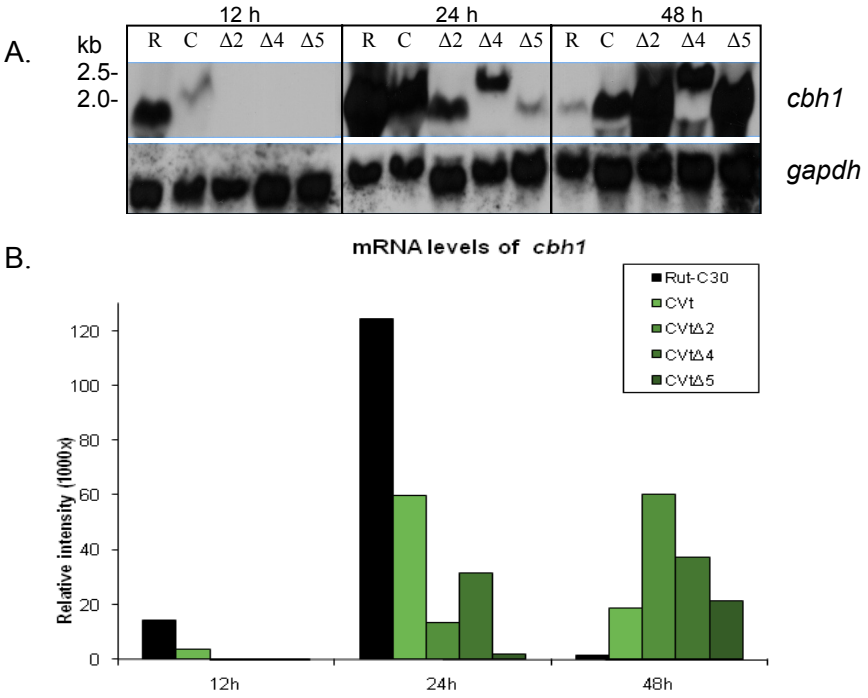

Figure 26. Northern blots of (A) Rut-C30 (R), CVt (C), CVtΔ2 (Δ2), CVtΔ4 (Δ4) and CVtΔ5(Δ5) after 12 h, 24 h and 48 h growth in the CLS medium inducing *cbh1* expression. (B) Intensity of the hybridising band reflecting mRNA levels of *cbh1*. The intensity of the bands has been normalised against *gapdh.*

The expected transcript size for the native *cbh1* gene in the non-transformant Rut-C30 strain was 1.9 kb, which was seen at 12 h, 24 h and 48 h time points in Fig. 26A. The approximately 1.7 kb transcript of the *cbh1* core gene was seen in CVtΔ2 and CVtΔ5 at 24h and 48 h timepoints. However, in CVtΔ4, a larger transcript of about 2.4 kb was obtained in CVt and CVtΔ4 (Fig. 26A). This 2.4 kb band corresponded to the expected sized transcript originating from the *cbh1* core-*venus* gene fusion, because the same sized band was observed in strains CVt and CVtΔ4 when hybridised with a *venus* probe (data not shown). The lack of the 2.4 kb mRNA transcript in CVtΔ2 and CVtΔ5 and the fact that no Venus fluorescence was detected in the hyphae of these strains, suggested that Venus transcript was not produced in these strains. Initially, Southern blotting showed integration of the entire expression cassette into the *cbh1* locus of each transformant (section 3.2.2). However, when Northern blotting of these same strains was carried two years later, a different view appeared (Fig. 26). Therefore, it could be possible that part of the expression cassette, e.g. *venus* gene had been lost in the strains CVtΔ2 and CVtΔ5 over time; these strains were maintained on PDA plates without hygromycin. Northern analysis has been performed three times with the *cbh1* probe and twice with *venus* probe to confirm the results.

The differences in the transcript levels of the expression cassette integrated in the *cbh1* locus, hybridised with a *cbh1* probe, were determined within the first 48 h of culturing from each of the five *T. reesei* strains of (Fig. 26B). In Rut-C30, *cbh1* was highly transcribed within the first 24 h before a down-regulatory effect observed after 48 h that could be due to the pulsing nature of *cbh1* expression observed earlier and discussed in section 3.4.2. Transcription of *cbh1* in the CVt strain was low at the 12 h time point, increased at 24 h and began to decrease again at 48 h showing a cycling effect similar, but slower to that in Rut-C30. In contrast, *cbh1* transcripts were only detected after 24 h and 48 h time points in all CBHI mutant strains. When the CBHI mutant strains were compared with each other, it seemed that the rate of transcription was affected by the increasing number of mutations, with CVtΔ5 having the slowest rate (Fig. 26).

3.4 Biochemical characterisation of the mutant CBHI strains secreting the Venus protein

Transformant strains harboring a mutant *cbh1* gene (mutant CBHI strains) and the transformant CVt carrying a non-mutant form of the *cbh1* gene were cultured along with the non-transformant Rut-C30 strain to study the effects of the different mutations on secretion of the CBHI and Venus proteins. Shake flask cultivations were carried out in a medium that induced the *cbh1* promoter and samples were removed daily up to day 7 (section 2.2.5.1).

3.4.1 Microscopic examination of the CBHI mutant strains

The expression of Venus protein in *T. reesei* was determined under fluorescence microscopy from samples removed after 36 h and 4 d cultivation in the CLS medium. Fluorescence emission at 515 nm corresponding to Venus fluorescence was not detected in the two CBHI mutant strains CVtΔ2 and CVtΔ5, either at 36 h or 4 d (Fig. 27A and B). In comparison, CVt and CVtΔ4 exhibited green fluorescence at both time points. These observations are in agreement with the Northern analysis (Fig. 26), which showed incorrect

transcript processing. In the strain CVt, fluorescence was scattered throughout the hyphae at 36 h and during the 4 d time point it seemed to localise at the cell envelope. Localisation of the fluorescence in the cell envelope may indicate that the protein is in transit to the external cultivation medium. Intracellular fluorescence in CVtΔ4 was weaker than in CVt and mostly localised in the cytoplasm at both time points.

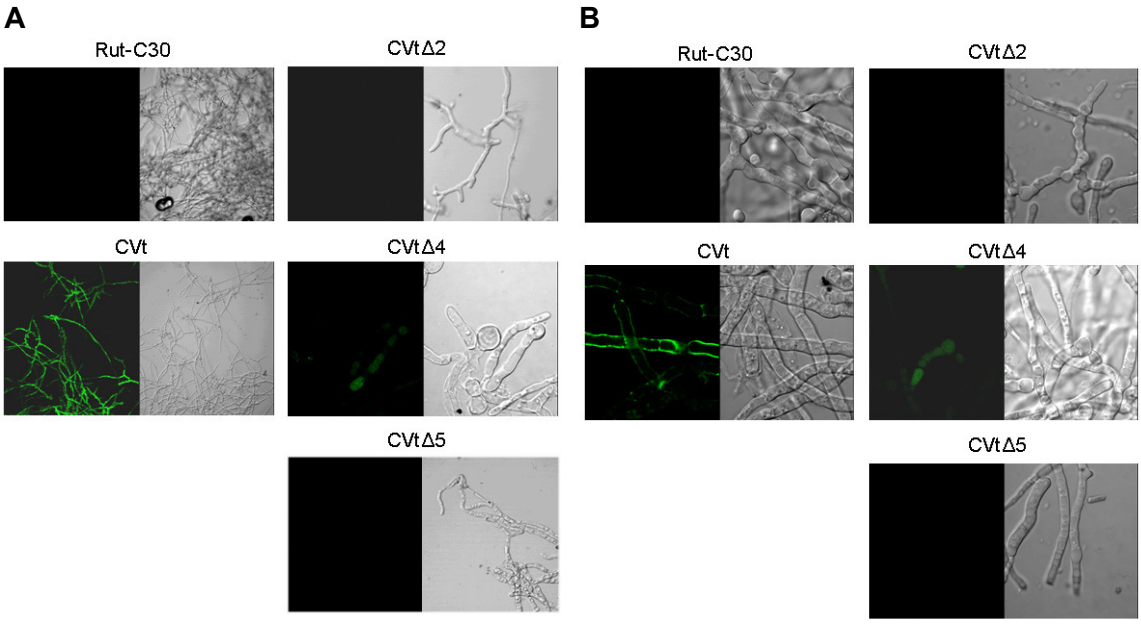

Figure 27. Fluorescence of Venus in the *T. reesei* hyphae. A. 36 h cultivation; B. 4 d cultivation in the CLS medium. Note that magnification of Rut-C30, CVt, CVtΔ2 and CVtΔ5 was x 40 and x 100 in CVtΔ4. In B, magnification was x 100 for all strains.

Hyphae from the 12 h - 7 d cultures, observed under a light microscope, showed considerable morphological changes. A remarkable decrease in the hyphal diameter can be seen in the *T. reesei* strain CVtΔ4 compared to the other *cbh1* mutant strains, the non-mutant CVt and the non-transformant Rut-C30 (Fig. 28A) indicating that this strain was under considerable physiological stress. An increased amount of vacuoles present in the CVtΔ5 hyphae suggested a similar effect but through a different response. During nutrient starvation in *Aspergillus*, hyphal growth was reduced by 50-70 % during the period where there was no glucose, vacuoles were appearing in the hyphae, and there was a ≤ 50 % decrease in the hyphal diameter (Pollack and Marten, 2008).

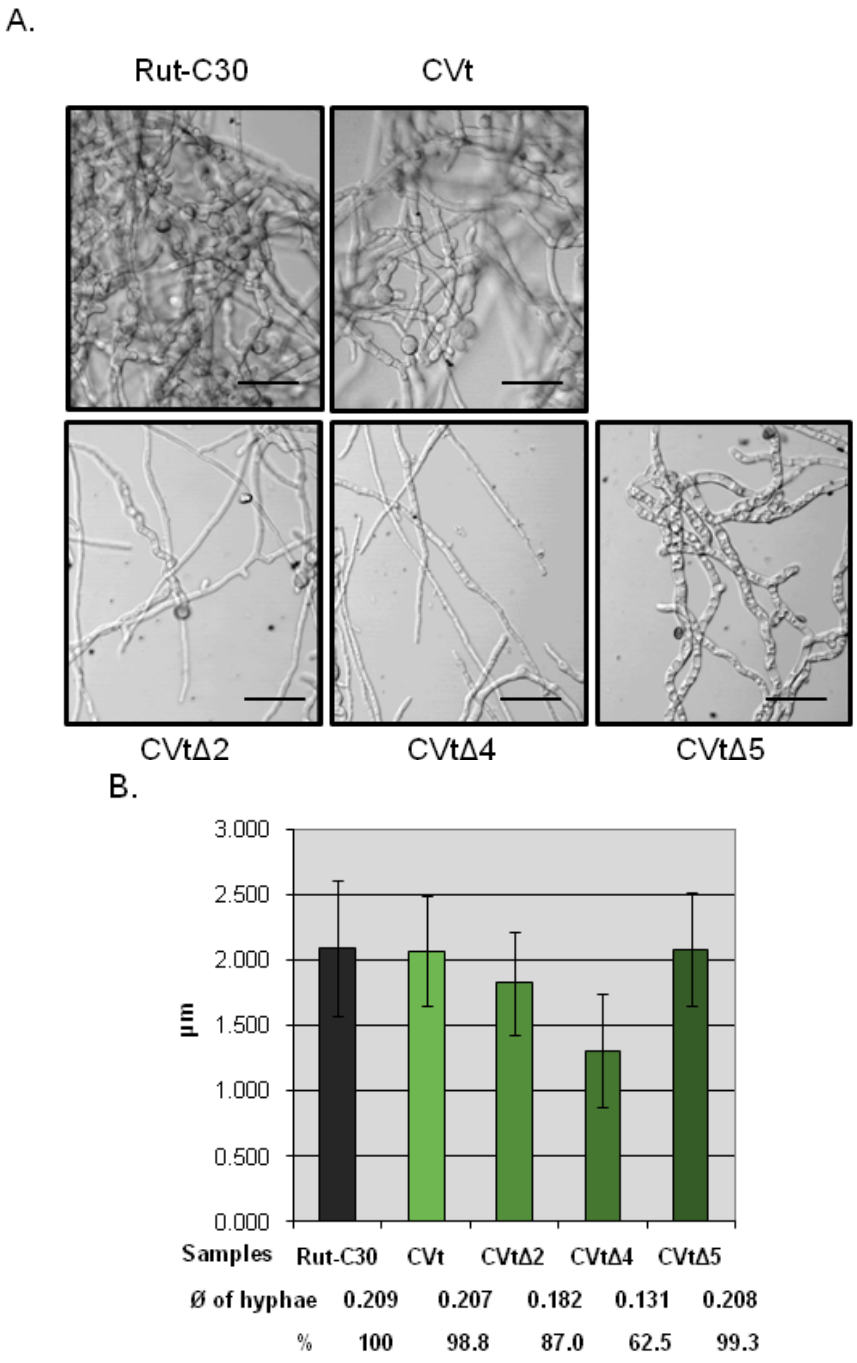

Figure 28. Physiological changes in the hyphae of different transformants and Rut-C30. A. Hyphae under the light microscope at 36 h cultivation in the CLS medium. Rut-C30 and CVt showed healthy growth, while CVtΔ2 and CVtΔ4 showed considerably thinner hyphae. In CVtΔ5, an increased amount of vacuoles can be observed in the hyphae. Bar 10µm. B. Diameter of the hyphae after 36 h growth in the CLS medium. Values represent the mean of 50 measurements; error bars show two standard deviations.

Comparison of the diameters of hyphae in the CBHI mutant strains and CVt to the non□ transformant Rut-C30 is shown in Figure 28B. The hyphal diameter of CVtΔ4 was only 62.5 % of the diameter of Rut-C30 in 36 h old hyphae, while only slight differences could

be observed within strains CVtΔ2 (88.7 %) and CVtΔ5 (99.1 %). Not only the diameter of hyphae but also a lot of vacuoles inside hyphae have been shown to reflect to the ongoing stress in fungal cells, as seen strain CVtΔ5 (Matsuura and Takagi, 2005). There were also differences in the colony phenotype (Fig. 29). For example, CVtΔ4 did conidiate later than the other strains and CVtΔ5 had less compact colony formation.

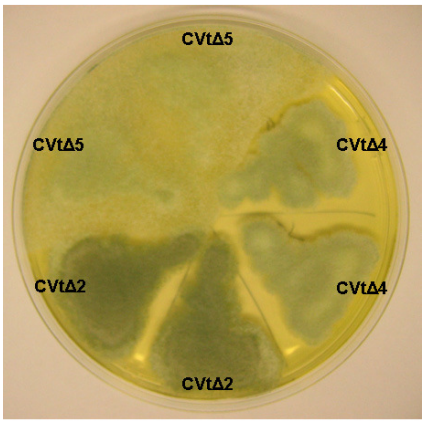

Figure 29. *T. reesei* transformants grown on a PDA plate for five days showing different colony phenotypes.

These morphological differences suggested that the *T. reesei* strains carrying mutations in the *cbh1* gene were experiencing some form of stress.

3.4.2 The effect of mutations on protein secretion

The main protease produced by *T. reesei* is an acidic protease "*Trichoderma*-pepsin" (Eneyskaya, *et al*., 1999). This and many other proteases are typically secreted into the cultivation medium and are active at a low pH. It has also been established that heterologous proteins produced and secreted by *T. reesei*, such as Venus, maybe degraded by these proteases (Haab *et al*., 1990; Marcolles-Clark *et al*., 1996).

The pH of the culture supernatant was measured over 7 days and appeared to follow a similar pattern in all the transformants (Fig. 30). Generally, the pH dropped as the biomass increased in the early growth phase, but returned close to pH 6 (starting pH was 6.5) in the late phase. In the non-transformant Rut-C30, the pH was lowest at day three (pH 3.5) but then increased to the same level as in the transformants (pH 5.7). This phenomenon has also been observed in earlier cultivation studies (personal information, J. Te□o, Macquarie University, Sydney, Australia). In the transformants CVtΔ2 and CVtΔ5, the initial decrease

of pH was not as significant compared to Rut-C30. These strains reached their lowest pH on day 2 (pH 5) while with CVtΔ4, the pH of the culture supernatant decreased only by 0.5 units from the original pH (ph 6.5) during the entire cultivation.

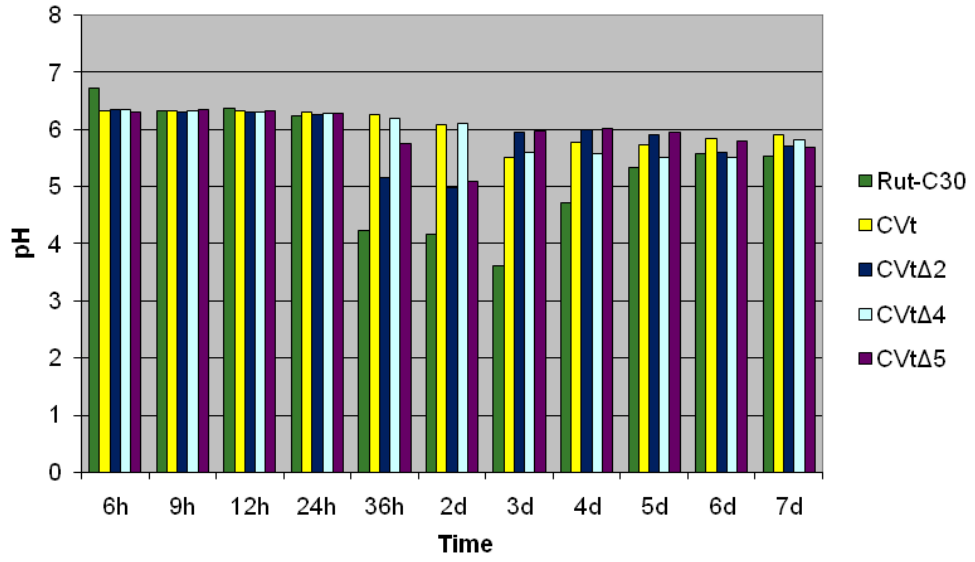

Figure 30. pH-profiles of the culture supernatants of all transformants and the non-transformant Rut-C30. All transformants exhibited a similar pH-profile.

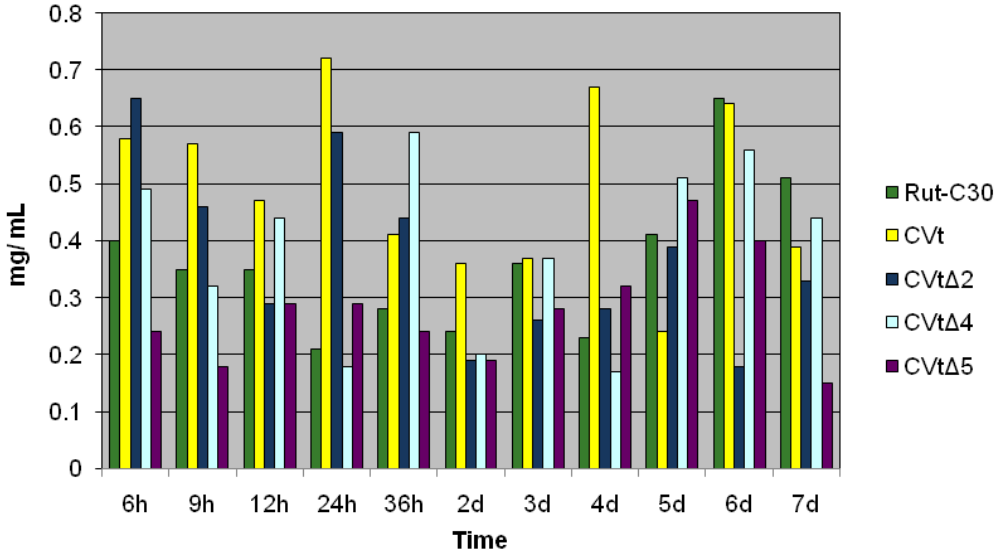

Figure 31. Total protein concentration in the supernatants of all transformants including CVt, and the non-transformant Rut-C30 measured over 6 h to 7 d in cultures grown on CLS medium. Note that the protein readings from the culture supernatants up to 36 h were affected by the soy protein which was an ingredient in the culture medium.

The amount of total secreted protein was measured from the same cultures (Fig. 31). It should be noted that at the early time points (6-12h) the total protein concentration in the

culture medium included the soy protein used as a nutrient. The effect of soy protein can also be seen in Fig. 32 displaying proteins stained with Coomassie Colloid Blue G250 on an SDS-PAGE gel. While the amount of secreted protein was different between all the strains, the profiles revealed an interesting cycle in protein secretion. The increase-decrease pattern seemed to be continuing at a 24 h cycle in all strains. The "pulsing" time in protein secretion seemed to be longer in the mutant strains. This pulsing behaviour has been also shown by Godlewski *et al*. (manuscript in preparation) in our laboratory in studies where secretion of the CBHI-Venus fusion protein in the hyphae of the *T. reesei* Rut-C30 and CVt strains was visualised by fluorescence microscopy. The studies indicated that the secretion of CBHI was highest at 21 h and started to pulse in a 22-24 h cycle on a *cbh1*- inducing medium similar to the medium used here.

In general, the protein profiles exhibited a similar overall pattern in all strains. The strain CVt, carrying a non-mutated *cbh1* gene fused with the *venus* fluorescent reporter gene, showed the highest total secreted protein concentration (about 0.67 g/L) at day 4 on the CLS medium (Fig. 31).

3.4.3 Protein profiles in the mutant CBHI strains

To obtain a profile of the total secreted proteins, 40 µL of the supernatant from each strain collected at 12 h, 24 h, 36 h, 2 d, 3 d and 5 d was run on SDS-PAGE (Fig. 32). The expected molecular weight for an intact CBHI-Venus fusion protein is about 78 kDa, and the CBHI core is about 51-65 kDa depending on the amount of glycosylation (Penttilä *et al*., 1998). The expected molecular weight of Venus is 27 kDa.

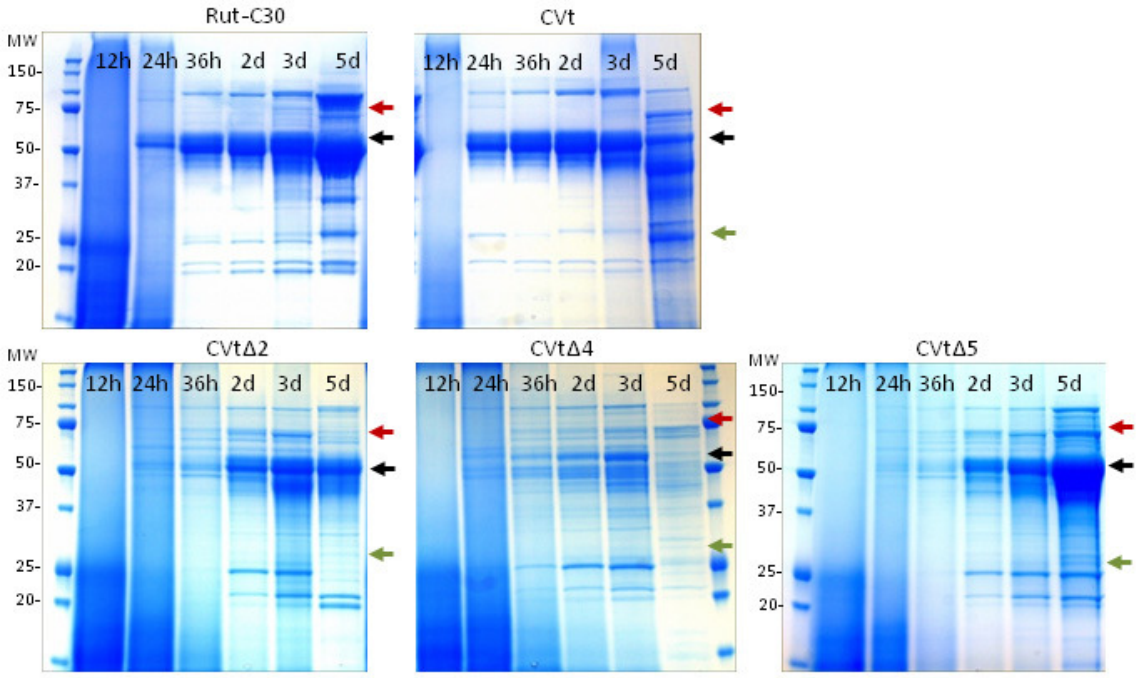

Figure 32. SDS-PAGE separation of total secreted proteins stained with Coomassie Colloidal Blue G250 from Rut-C30, CVt, CVtΔ2, CVtΔ4 and CVtΔ5. Black arrows point to the CBHI band (50-65 kDa). Green arrows indicate the expected location of a 27 kDa band that would correspond to the size of the Venus protein. Red arrows indicate the location of a protein exhibiting a molecular weight of 78 kDa. Each well was loaded with 40 µL of culture supernatant containing secreted protein.

Profiles of the secreted proteins in the mutant strains were slightly different. However, it seemed that none of the strains secreted an intact fusion protein as indicated by Western blots in Fig. 33A and B, and the CBHI and Venus were seen as separated in the culture medium. A strong signal was obtained from a band of about 50-65 kDa corresponding to the CBHI core (with glycosylation) in all strains (Fig. 33A). Faint signals received from higher molecular weight bands most probably reflect non-specific hybridisation since these bands were also visible in the non-transformant Rut-C30. Hybridisation with a polyclonal anti-GFP antibody supported the previous results from fluorescence microscopy of the fungal hyphae (Fig. 27) and Northern blotting (Fig. 26) indicating that the Venus protein was not produced in CVtΔ2 and CVtΔ5 (Fig. 33B).

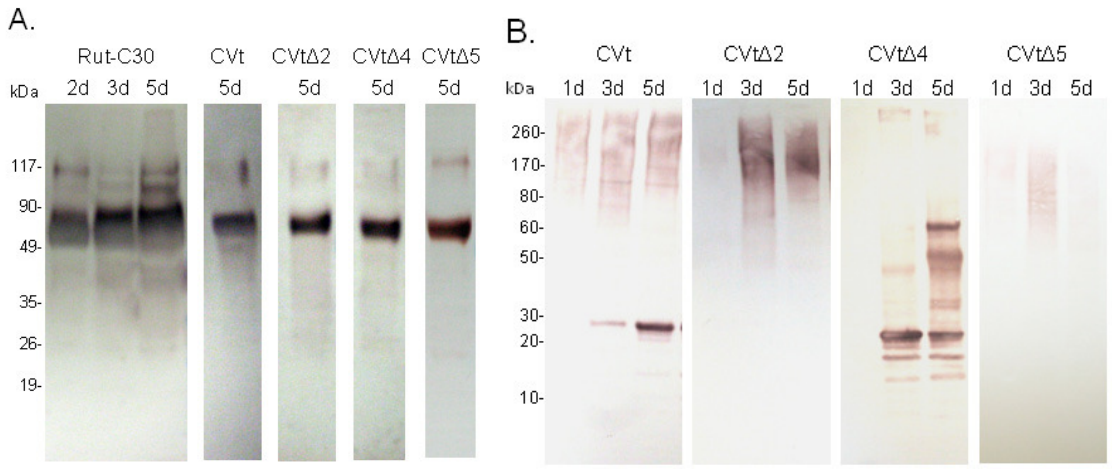

Figure 33. Western blot analysis of the total secreted proteins in the *T. reesei* culture supernatants. A. Blots hybridised with a monoclonal CBHI antibody; B. Blots hybridised with polyclonal GFP antibody. Culture supernatants were collected after 1 d, 2 d, 3 d and 5 d. CBHI (65 kDa) was secreted by Rut-C30 already after 2 d. In all transformants carrying a mutated *cbh1* gene, CBHI only appeared in the culture medium at a somewhat later stage. Venus (27 kDa) was secreted by CVt and CVtΔ4 after 3 d. No Venus secretion was observed from transformants CVtΔ2 and CVtΔ5.

In Rut-C30, CBHI was secreted into the culture medium at 24 h and the secretion continued strongly until day 5 (Fig. 33). It appeared that CBHI secretion outside the hyphae was delayed in all transformants. This delay may have been caused either by the Venus tag or mutant CBHI. The delay was less prominent in CVt which expressed the non-mutant CBHI. Mutations in the CBHI protein may have contributed to the secretion delay and multiple mutations may have caused a longer delay in the maturation and secretion of the CBHI protein. These findings are in agreement with the Northern blotting analysis (section 3.3. Fig. 26). Karakura *et al*. (1999) have demonstrated that a single amino acid substitution can increase or reduce the secretion of a particular protein when the substituted residue is crucial for protein structure. A yeast strain carrying a mutant bovine β-lactoglobulin (Trp19Tyr) secreted six times more β-lactoglobulin compared to a non-mutant β lactoglobulin strain, whereas the mutant strains with Phe or Ala at position 19 were found to have low secretion levels of β-lactoglobulin (Karakura *et al*., 1999). In addition, the secretion kinetics studies revealed that maturation of β-lactoglobulin was slower in the Phe mutant strain and faster in the Tyr mutant strain, when compared to the non-mutant β lactoglobulin strain.

In conclusion, there seemed to be two types of transformant strains, those expressing the Venus part of the fusion protein in addition to CBHI (CVt and CVtΔ4) and those

expressing CBHI only (CVtΔ2 and CVtΔ5). Separation of the CBHI part from Venus should not pose a problem for the experimental goals addressing the effects of the expression of mutant proteins on protein quality control since it was shown that production of Venus did not trigger UPR or ERAD pathways (section 3.5.1) which were of specific interest in this study. Therefore, any changes recorded would be the result of the production of the mutant CBHI molecules.

3.4.4 Cellulobiohydrolase activity in culture supernatants

Cellobiohydrolase activity was determined by hydrolysis of the fluorogenic substrate 4□ nitrophenyl β-D-lactopyranoside by the culture supernatants at 37 °C for 1 h (Henriksson *et al.*, 2000). Figure 34 shows the differences in cellobiohydrolase activity found in the culture supernatant of the CBHI mutant strains compared to the non-mutant strain CVt cultured in the CLS-medium for five days.

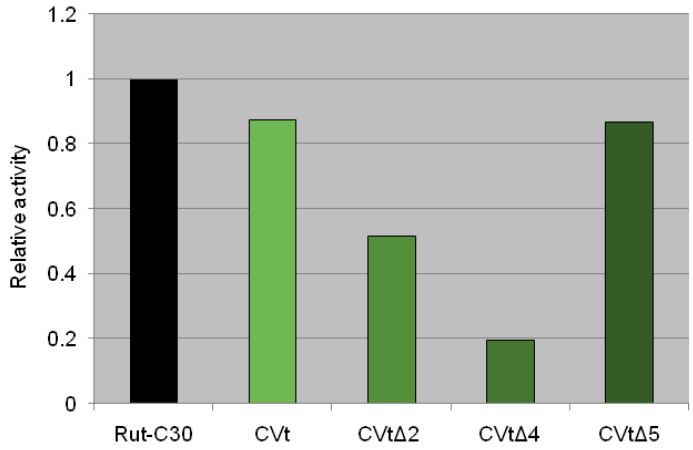

Figure 34. Cellobiohydrolase activity of the culture supernatants from the non-mutant CVt and the different CBHI mutant strains compared to the non-transformant Rut-C30 after 5 d cultivation in the CLS medium. Rut-C30 has been given the value 1.

The cellobiohydrolase activity measured from the culture supernatants of the CBHI mutant strains CVtΔ2 and CVtΔ4 was notably lower when compared to the non-mutant strain CVt after 5 d cultivation. CVtΔ2 carried two mutations at the C-terminal end of the CBHI enzyme and in addition, two additional mutations were introduced more close to the active site of CBHI in strain CVtΔ4. Conformational changes in the CBHI protein, caused by replacement of cysteine with proline, were probably sufficient to change the substrate binding site in both CVtΔ2 and CVtΔ4. The active site of CBHI is at the end of a tunnel in

a sandwich-shaped structure formed by two sheet structures (Fig. 12). In the strain CVtΔ4, two replacements were nearer to the active site at cysteine pairs C172-C210 and C176-C209. Three acidic residues, Glu212, Asp214 and Glu217 are known to be the catalytically important residues (Divne *et al*., 1998). Destroying the formation of disulfide bridges by mutations in one cysteine in a pair of cysteines forming a disulfide bridge close to the active site may have changed the conformation so that the substrate might not be able to bind to the active site. Interestingly, the strain CVtΔ5, which carried an additional mutation to the *cbh1* showed higher cellulase activity than CVtΔ4. In the CVtΔ5 strain, the last cysteine replacement with proline might have changed the structure closer back to the original 3D structure, and therefore the enzyme would bind better to the active site. Another possible explanation for the above could be that CVtΔ5 may have lost the mutant CBHI gene, for which however, the evidence is somewhat contradictory.

In conclusion, physiological changes similar to the changes previously seen in the hyphae of *Aspergillus* under starvation were observed in all CBHI mutant strains whereas the non-mutant (CVt) and non-transformant (Rut-C30) strains seemed healthy under similar cultivation conditions. The pulsing mode of protein secretion was observed in all strains progressing in approximately 22-24 h cycles. Inclusion of the Venus tag and/or mutations in the CBHI protein seemed to have delayed the secretion of CBHI. CBHI and Venus were seen as separate components in the culture medium in CVt and CVtΔ4. Finally, cellulase activity was lower in the CBHI mutant strains when compared to the non-transformant Rut-C30 producing an intact CBHI enzyme.

3.5 Microarray profiling of gene expression in the mutant CBHI strains

Expression and secretion of the mutant CBHI forms were likely to have broader effects on gene expression in *Trichoderma reesei*. These were explored by genome-wide microarrays. Special emphasis was on the effects of the expression and secretion of mutant CBHI forms on the UPR and ERAD pathways.

Genome wide expression profiling was carried out to monitor cellular responses caused by the expression of the mutated CBHI proteins in *T. reesei*. Three time points were chosen based on preliminary work using Northern blotting which showed that *cbh1* expression started after 9 h cultivation in the CLS medium (data not shown). CVtΔ2, CVtΔ4 and CVtΔ5 mutant strains along with the non-mutant CVt strain and the non-transformant Rut C30 were cultivated in the CLS medium for 12 h, 24 h and 48 h. Total RNA was extracted and reverse-transcribed to cDNA which was labelled with CY3 and CY5 dyes, mixed and placed onto microarray slides. CustomArray^{TM} 12K slides (Combimatrix, USA) contained in total 12 000 of 30-40 mer oligoprobes, generated from 9129 different *T. reesei* cDNAs, based on the second round of genome sequencing (http://genome.jgipsf.org/Trire2/Trire2.home.html). These 9129 probes corresponded to each gene ORF of *T. reesei*. Since there was a special interest in exploring the ERAD and UPR pathways, four to eight replicate probes for the chosen 64 genes related to UPR and ERAD, and two housekeeping genes were spotted on the slide; this set of 66 genes was called Set1 and analysed separately to Set2. Set2 included the rest of the 9129 genes (9129-66 genes) spotted once on the same Custom Array slides. The dye swap replicates were applied through out the whole experiment. Non-mutant transformant strain CVt was used as an internal control. Genes were considered to have an expression difference if the expression was at least 1.5 x up-regulated or 1.5 x down-regulated across replicate slides (dye swap). The 1.5 fold cut off was chosen because it has been commonly used in published fungal microarray studies (e.g. Guillemette *et al*., 2007).

Statistically relevant data would require a number of biological replicates to determine the reproducibility. This study was lacking biological relicates, but gene expression was analysed in technical replicates in Set 1 (4 to 8 replicates) and also dye swap replicates. Multiple spots of the same gene provided a quality control and inceased confidence. Also the four replicates of housekeeping genes were showing constant expression in all conditions, and therefore performed as a quality control as did the constant readings from the spike control. The Set2 was analysed based on expression changes in one or two technical replicates and dye swap replicates and therefore expression changes can only be considered as a preliminary guide.

3.5.1 Global view of differentially expressed genes

Overall view into gene expression

The data from all 9129 genes which were expressed under the chosen experimental conditions in the three different mutant strains at three time points (12, 24 and 48 h) were further filtered on the basis of fold change. A total of 701 genes were considered differentially expressed (DE) during cultivation of the *T. reesei* transformant strains expressing mutant CBHIs when compared to the strain CVt expressing a non-mutant CBHI. Further down the terms "up-regulated" and "down-regulated" refer to gene expression related to the CVt strain.

The Venn diagram in Figure 35 shows the numbers of unique and common genes which were differentially expressed in each sample after 12 h, 24 h and 48 h of *cbh1* induction. Overall, the biggest changes in gene expression were observed after 12 h cultivation (Fig. 35A), when 574 genes were differentially expressed; of these, 40 genes were common to all strains. At 24 h, only 160 genes changed expression and no common genes were identified (Fig. 35B). At 48 h, 255 genes were expressed differentially and from these, eight genes were common to all strains (Fig. 35C).

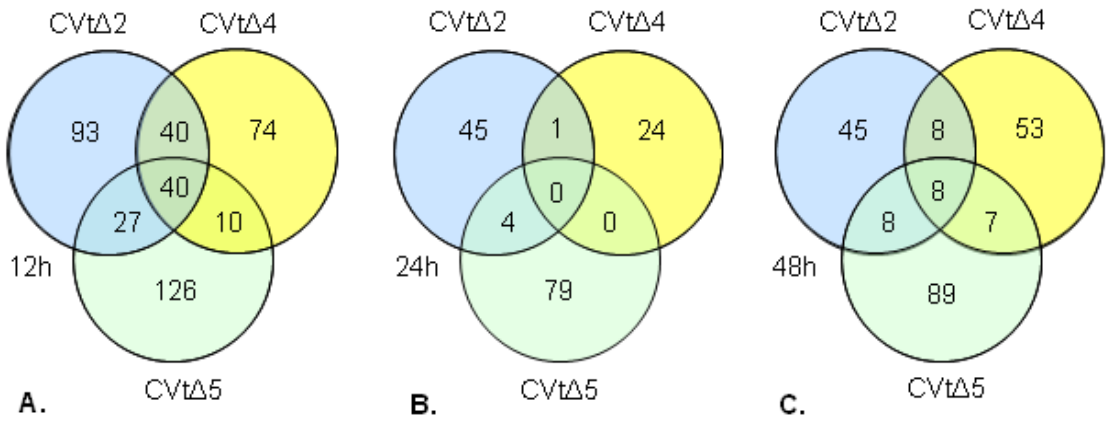

Figure 35. The numbers of up-regulated (≥ 1.5 fold) or down-regulated (≤ 1.5 fold) genes from the entire *T. reesei* genome analysed by microarrays. Three CBHI mutant strains were compared to a non-mutant strain in the same culture conditions at three time points: A. 12 h; B. 24 h and C. 48 h. The colours indicate the three different mutant CBHI strains: CVtΔ2 blue, CVtΔ4 yellow and CVtΔ5 green.

To gain more information on the global perspective and potentially identify general shifts in gene expression during production of the different mutant CBHIs, all differentially expressed genes were categorised by their gene ontology (GO) terms using Blast2GO

program (http://www.blast2go.de). Overall the fungal genes showing an expression difference were not limited to ERAD and UPR related genes. Putative functions were assigned to 67.3 % of the transcripts. Of the genes that were differentially expressed, 32.7 % showed no significant similarity to any other sequence in the current genome or protein databases and thus their function could not be determined. Some of these genes might be specific to filamentous fungi or more specifically, *T. reesei*. This group, called "unclassified" also included some genes encoding proteins of which the function or name is currently unknown. The others were classified as being involved in "cell growth, cell cycle, development and proliferation" (4.5 %); "transcription and DNA repair" (4.9 %); "translation" (1.5 %); "ribosome structure and biosynthesis" (4.3 %), "metabolic pathways" (35.8 %); "protein folding/UPR" (5.6 %); "protein elimination/ ERAD" (1.9 %); "protein trafficking" (1.5 %), and "transport" (8.1 %) in the cell. The "metabolic pathway" class included genes representing amino acid metabolism (9.7 %); fatty acid and lipid metabolism (2.8 %); carbohydrate metabolism (23.6 %); hydrolases (6.0 %); dehydrogenases (5.1 %); oxidoreductases (18.2 %); peptidases and proteases (6.3 %); reductases (2.8 %) and transferases (13.9 %). It was not surprising that most of the differentially expressed genes belonged to the class "metabolic pathways" which are associated with the general developmental events in the cell.

The non-mutant transformant strain CVt was used as an internal control for the entire microarray experiment. It contained the same expression cassette as the mutant strains, but the *cbh1* gene was intact. The non-transformant Rut-C30 strain was included in the microarray experiment for comparison of the changes to the internal control, the non-transformant strain CVt. We were also interested to see if the inclusion of the fluorescent tag - heterologous Venus - had any effects on expression of the genes involved in UPR and ERAD pathways.

Differentially expressed genes in Rut-C30

T. reesei Rut-C30 strain carries the native non-mutated *cbh1* gene, but not the heterologous Venus tag. When the overall gene expression profile in the non-transformant Rut-C30 was analysed, changes were recorded in 128 genes. Twenty one genes were differentially expressed at 12 h, 37 genes at 24 h and 70 genes at 48 h. Most of the changes were in the expression of genes involved in "cell growth, cell cycle, development and proliferation"

(15) "metabolic pathways" (48) and "unclassified" function (39), and were summarised in Table 9. Some genes encoding proteins involved in "protein folding and the UPR pathway" as well as "protein elimination and ERAD pathway" were up-regulated at 12 h and 24 h and down-regulated at 48 h in the Rut-C30 strain. These genes encoded proteins such as Hsp101, mitochondrial chaperone bcs1 and ubiquitin, 26S proteasome associated ubiquitin N-terminal protein and an F-box protein involved in the ubiquitin cycle. These proteins are believed to have multiple tasks in the cell and could be involved in ERADication of short lived regulatory proteins.

Table 9. Functional groups of protein-coding genes which were differentially expressed in Rut-C30 at 12, 24 and 48 h, cultured in the CLS medium. Transformant CVt carrying a non-mutant *cbh1* gene tagged with the *venus* gene was used for comparison to Rut-C30. Numbers in red represent amount of up-regulated genes and numbers in green represent amount of down-regulated genes.

Functional class	***12 h***	***24 h***	***48 h***
Cell growth, cell cycle, development and proliferation	0 / 1	4 / 5	2 / 3
Transcription	0 / 1	0 / 3	2 / 1
Translation	0 / 0	0 / 0	0 / 0
Ribosomal proteins and biosynthesis	0 / 0	0 / 0	0 / 0
Metabolic pathways	2 / 10	4 / 6	18 / 8
Protein folding / UPR	0 / 1	0 / 0	1 / 0
Protein elimination / ERAD	0 / 1	0 / 1	3 / 0
Trafficking	0 / 0	0 / 0	0 / 0
Transporters	1 / 1	2 / 1	6 / 1
Unclassified	0 / 3	4 / 7	19 / 6

There were no expression changes in Rut-C30 when compared to the internal control CVt in genes coding for proteins such as HAC1, ER-resident chaperones or proteasome related proteins, which indicate secretion stress. Therefore it can be concluded that expression of the fusion protein, non-mutated CBHI tagged with the fluorescent protein Venus, did not seem to induce stress on the protein secretion pathway in CVt. Expression of genes encoding "transporters" was down-regulated in Rut-C30 most probably because of the difference in the secretion rate between these strains. The same observations can be made based on analysis of genes involved in "metabolic pathways". All differentially expressed genes in strain Rut-C30 were assigned to their functional classes at each time point and are presented in the supplementary material, Table 1.

Expression differences in strains carrying different mutant cbh1 genes

All three mutant strains showed different expression profiles at each time point. Overall, the largest effect was observed in strain CVtΔ5 with 398 differentially expressed genes, whereas CVtΔ2 had 319 genes and CVtΔ4 265 genes that were differentially expressed (Table 10). At the 24 h time point the gene expression seemed to be lowest in each strain, which could be explained by the fact that protein expression also seemed to be lowest in all mutant strains when compared to the control strain CVt (Fig.30).

Table 10. Differentially expressed genes of CVtΔ2, CVtΔ4 and CVtΔ5 after 12 h, 24 h and 48 h cultivation in the CLS medium.

		12 h	***%***	***24 h***	***%***	***48 h***	***%***	***Total***	***%***
	All differentially expressed genes	574		160		255		989	
CVtΔ2	Total	200		50		69		319	100.0
	Down-regulated	148	74.0	28	56.0	37	53.6	213	66.8
	Up-regulated	52	26.0	22	44.0	32	46.4	108	33.9
CVtΔ4	Total	164		25		76		265	100.0
	Down-regulated	86	52.4	14	56.0	20	26.3	121	45.7
	Up-regulated	78	47.6	11	44.0	56	73.7	144	54.3
CVtΔ5	Total	203		83		112		398	100.0
	Down-regulated	66	32.5	54	65.1	10	8.9	129	32.4
	Up-regulated	137	67.5	29	34.9	102	91.1	271	68.1

The response of the strain CVtΔ2 with two mutations was predominantly down-regulation of gene expression across the entire genome (66.7 %), whereas in strain CVtΔ4 with four mutations, the gene expression was varying with 45.7 % of genes down-regulated and 54.3 % up-regulated. Interestingly, the effect of the five mutations in strain CVtΔ5 on global gene regulation was the opposite to strain CVtΔ2 with two mutations: two thirds of genes in CVtΔ5 were up-regulated (67.7 %). It seemed that having multiple mutations introduced into the *cbh1* gene exerted an increased effect on total gene expression.

3.5.2 Time scale view into overall changes in gene expression in the mutant strains

The varying growth rate also seen at the transcriptional level and in protein production in the CBHI mutant strains did not allow direct comparison of gene expression between the strains, therefore the expression analysis was conducted across the different time points in

each individual strain. These changes were compared against the changes in gene expression in the non-mutant transformant CVt.

Gene expression in CVtΔ2

Analysis of the strain carrying two mutations in the *cbh1* gene revealed that the expression levels of 200 genes had changed at 12 h, 50 genes at 24 h and 69 genes at 48 h of cultivation in the *cbh1* inducing medium (Fig. 36).

The majority of down-regulated genes belonged to the class "metabolic pathways". Also genes involved in the "cell growth, cell cycle, development and proliferation", "transcription and DNA repair", "translation", "ribosome structure and biosynthesis", "stress response and UPR", and "transport" were down-regulated at the 12 h time point. It is known that the maturation rate of the mutant proteins is slower than that of the non-mutant proteins, which is reflected by a general down-regulation response at this time point (Fig. 36). Here again, most of this may be explained by the fact that these genes were related to the developmental events active in the hyphae in the CVtΔ2 mutant which appeared to be under physiological stress (Fig. 28) and growing slower than the hyphae of the comparison strain CVt.

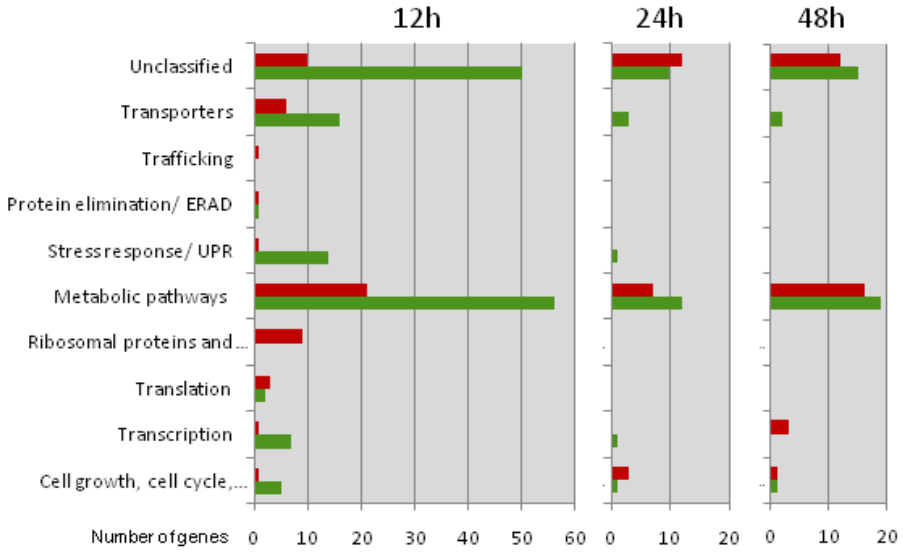

Figure 36. Up-regulated and down-regulated genes assigned to their functional classes in strain CVtΔ2 at 12 h, 24 h and 48 h. Up-regulated ■; down-regulated ■.

In summary, expression of the CBHI protein with two mutations did not trigger a significant stress response in the fungal cells. Protein phosphatase 2c, which is a stress inducible serine/threonine protein phosphatase (Scheen, 1998), was upregulated at a later time point of 48 h. All differentially expressed genes in strain CVtΔ2 were assigned to their functional classes at each time point and are presented in the supplementary material, Table 2.

Gene expression in CVtΔ4

In the strain CVtΔ4, the total number of differentially expressed genes was 265, which was less than the number of differentially expressed genes in the two other CBHI mutant strains. More than one third of genes in CVtΔ4 changed expression at 12 h, however, the function of a large portion of these genes remains unknown. In CVtΔ4, expression of the *cbh1* gene with four mutations induced considerable changes not only in the expression of genes related to "protein folding and UPR pathway", and "protein elimination and ERAD pathway" but also of the genes in other functional classes, e.g. "transporters", "ribosome structure and biosynthesis" at the early time point of 12 h (Fig. 37). The genes related to "metabolic pathways" were the up-regulated significantly at 12 h. Many genes related to the functional classes "ribosome structure and biogenesis" as well "transporters" were induced at 12 h while there were not many gene expression changes at the later stages compared to the non-mutated CVt strain. Expression of genes related to the functional class "intracellular trafficking" was slightly increased as a function of time. All differentially expressed genes in strain CVtΔ4 were assigned to their functional classes at each time point and are presented in the supplementary material, Table 3.

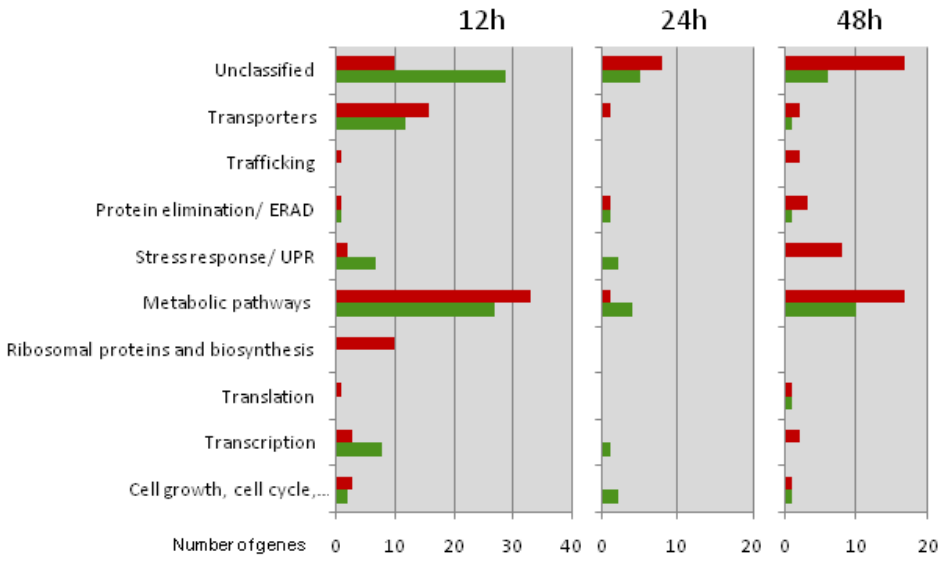

Figure 37. Up-regulated and down-regulated genes assigned to their functional classes in strain CVtΔ4 at 12 h, 24 h and 48 h. Up-regulated ■; down-regulated ■.

The general gene expression profile in strain CVtΔ4 (assessed at 12 h, 24 h and 48 h) was different to that of strain CVtΔ2, in that there were more up-regulated genes at 12 h and 48 h. Overall, gene expression was generally down-regulated at the 24 h time in all CBHI mutant strains (Table 10). This provides further evidence for the earlier finding that gene transcription and protein secretion occurs in pulses in the *T. reesei* hyphae (section 3.3 and 3.4.2 and Godlewski *et al*., manuscript in preparation).

The strain CVtΔ4 with four mutations in the *cbh1* gene showed a significant stress response at 48 h (Fig 37). This is indicated by the up-regulation of genes in the functional class protein elimination/ERAD and stress response/UPR. Also the transcription related genes along with metabolic pathways genes were up-regulated at 48 h again reflecting the delay in both gene transcription and protein secretion in the CBHI mutant strains.

Gene expression in CVtΔ5

Changes in the gene regulation in strain CVtΔ5 carrying five mutations in the *cbh1* gene featured more up-regulation of genes than the other two strains. All the differentially expressed genes in strain CVtΔ5 were assigned to their functional classes at each time point and are presented in the supplementary material, Table 4.

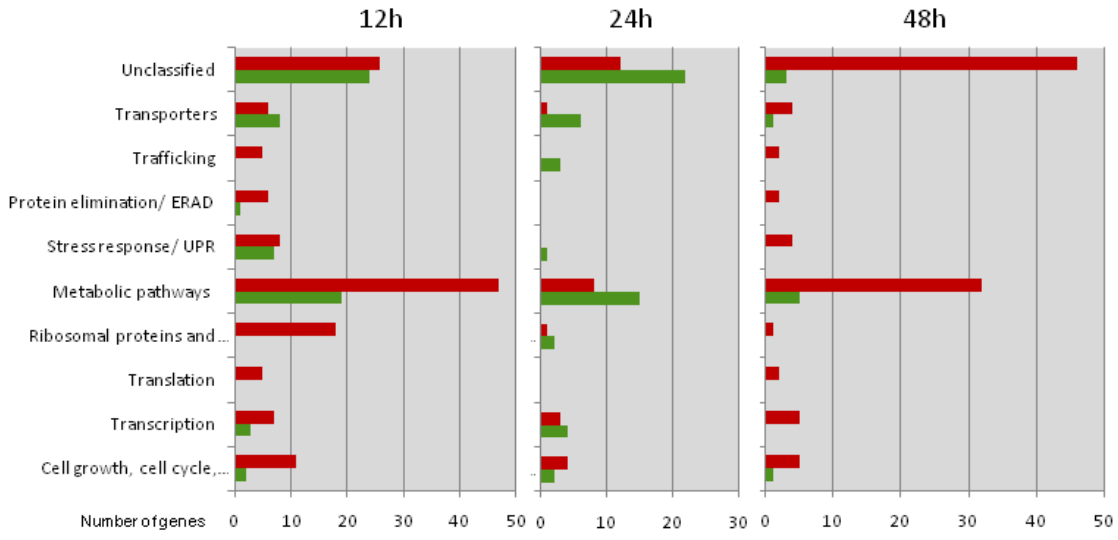

Figure 38. Up-regulated and down-regulated genes assigned to their functional classes in strain CVtΔ5 at 12 h, 24 h and 48 h. Up-regulated ■; down-regulated ■ .

In addition to the up-regulation of genes related to "metabolic pathways", genes involved in "ribosome structure and biogenesis" were significantly induced at 12 h in strain CVtΔ5 (Fig 38). This strain had the largest number of up-regulated genes belonging to the class "unclassified" at the 48 h time point. This strain was also showing up-regulation in genes involved in "stress response and UPR" and "protein elimination and ERAD" already at 12 h and then again at 48 h.

In summary, amongst the three CBHI mutant strains examined, CVtΔ2 did not show major expression changes in the genes involved in the UPR and ERAD pathways, while CVtΔ4 was showing a stress response at 48 h and CVtΔ5 was already showing signs of stress at 12 h. All the mutant strains exhibited stress related physiological changes such as low biomass synthesis and thinner hyphae during cultivation when compared to the non-mutant CVt (section 3.4.1). In strains CVtΔ2 and CVtΔ4, gene expression in all functional classes seemed to gradually shift from down-regulation to slight up-regulation as a function of time. In strain CVtΔ2, the genes related to 'metabolic pathways' were down-regulated significantly at 12 h, but in strains CVtΔ4 and CVtΔ5, genes related to 'metabolic pathways' were mainly up-regulated. Generally, in all strains, the differentially expressed genes in the different functional classes were different at each time point. Only 2.5 to 6.3 % of the differentially expressed genes were common in each strain either at two time points

or all three time points, probably reflecting the different time frames of growth of each of the mutant strains.

3.5.3 Set1: Differentially expressed genes involved in protein folding / UPR and protein elimination / ERAD

Set1 included probes for 66 selected genes related to the UPR and ERAD pathways and two housekeeping genes. Our primary interest was to explore expression changes in these pathways when transformants carrying mutated *cbh1* genes were cultivated in the *cbh1* inducing medium, CLS. The UPR and ERAD genes were chosen based on literature searches and were present as four to eight replicates on the microarray slides. In the case of using eight replicates for a particular gene, four of them were oligonucleotide probes binding to a different part of the same gene. The complete gene list is shown in section 2.3.1. Differential expression of Set1 genes is shown in Table 11.

Table 11. Differentially expressed genes when compared to CVt strain in Set1 encoding proteins involved in UPR and ERAD. jgi Trire, gene number from the second round sequencing of *T. reesei* genome; fold changes for down-regulation shown in green; fold changes for up-regulation shown in red; multiple numbers indicate replicates and numbers in brackets indicate dye swap replicates.

			RutC30	CVtΔ2	CVtΔ4	CVtΔ5	RutC30	CVtΔ2	CVtΔ4	CVtΔ5	RutC30	CVtΔ2	CVtΔ4	CVtΔ5
TR2 code	Gene	Function	12h	12h	12h	12h	24h	24h	24h	24h	48h	48h	48h	48h
46902	HAC	UPR		↓(3.303, 0.591) ↓(3.397, 0.619) ↓(2.946, 0.645) ↓(2.811, 0.572) ↓(2.695, 0.652)										
119890	TIGA	UPR											↑(0.614, 7.382)	
122920	BIP	UPR		↓(3.669, 0.543) ↓(3.565, 0.509) ↓(4.4, 0.505) ↓(3.968, 0.46)	↓(3.667, 0.644) ↓(4.54, 0.621) ↓(4.257, 0.664)	↓(0.596, 4.462) ↓(0.652, 2.931) ↓(0.613, 2.302) ↓(0.574, 2.814)							↑(0.451, 12.325) ↑(0.368, 7.251) ↑(0.386, 9.595) ↑(0.407, 8.18)	
122415	PDI	UPR		↓(5.237, 0.395)									↑(0.427, 26.836)	
52050	CPR6 (PPI)	UPR		↓(0.683, 0.644) ↓(1.594, 0.603)	↓(1.775, 0.635)									
119903	DER1	UPR											↑(0.619, 2.347)	
35465	LHS1(CER1, Hsp-like)	UPR						↓(0.638, 1.545)	↓(0.659, 2.053)				↑(0.655, 1.814) ↑(0.609, 2.544)	
28928	PRPA	UPR		↓(1.634, 0.497)	↓(1.764, 0.642) ↓(2.301, 0.62)									

			RutC30	CVtΔ2	CVtΔ4	CVtΔ5	RutC30	CVtΔ2	CVtΔ4	CVtΔ5	RutC30	CVtΔ2	CVtΔ4	CVtΔ5
TR2 code	Gene	Function	12h	12h	12h	12h	24h	24h	24h	24h	48h	48h	48h	48h
73678	CAL	UPR		↓(2.022, 0.619) ↓(1.603, 0.631) ↓(2.066, 0.669) ↓(1.704, 0.644)		↓(0.612, 1.998)							↑(0.65, 7.332)	↑(0.541, 3.923)
82512	19S RPN4	ERAD			↓(11.622, 0.445)	↓(6.388, 0.359)								
68304	19S RPN5	ERAD				↑(0.655, 2.512) ↑(0.667, 2.081)								
80843	19S RPN8	ERAD				↑(0.606, 2.393)							↓(1.816, 0.665)	
48366	19S RPN12	ERAD				↑(0.569, 1.876)								
76010	20S ALFA7	ERAD				↑(0.602, 2.412) ↑(0.619, 2.232)								
66707	20S BETA6	ERAD												↑(0.578, 1.539)
22994	CDC48	ERAD												↑(0.592, 4.836) ↑(0.638, 4.995)
121397	SEC61	ERAD		↓(3.315, 0.506) ↓(3.52, 0.571) ↓(3.792, 0.533)		↑(0.664, 1.904)							↑(0.612, 5.107) ↑(0.628, 4.888) ↑(0.651, 4.992)	
72606	UBA1	ERAD											↑(0.577, 2.689)	
80400	UCH1	ERAD	↑(0.528, 1.656)				↑(0.64, 1.538)				↓(0.53, 1.969)			
21246	UFD1	ERAD			↓(1.768, 0.6)									

Studies into ER quality control in yeast have shown that when misfolded or incorrectly assembled proteins accumulate in the ER, where they will cause an increase in the expression of several genes encoding ER-resident foldases and chaperones. These chaperones include calnexin, calreticulin, Grp94, PDI, heat shock proteins Hsp70 and Hsp40, as well as proteins involved in functions associated with the secretory processes such as vesicular transport proteins, e.g. COPII (Nishikawa *et al*., 2005; Kohno *et al*., 2008). In the current study, 19 genes represented by more than four replicates on the microarray slides were differentially expressed amongst Set1 genes. There were a number of cases where only one or two of the four replicates showed differential expression. This was considered as a significant change in the cases where the difference to the comparison strain was in the range of 2-26 (shown in Table 11).

ER- resident chaperones

The *bip, pdi, cpr6 (ppi), prpA, TigA* and *cal* genes encoding ER-resident chaperones were down-regulated at 12 h in all CBHI mutant strains when compared to non-mutant strain CVt. This may indicate that *bip* gene was already up-regulated in CVt and therefore showing low expression in all mutant strains (Table 11). The binding protein, BiP is believed to be one of the stress sensors in the ER. Accumulation of misfolded proteins triggers the release of BiP from the lumenal domain of the regulators of UPR. In addition to this crucial sensor role in UPR, BiP has multiple tasks in the ER (Gűlow *et al*., 2002). BiP binds to unfolded proteins to prevent their aggregation and also participates in the translocation of misfolded proteins for their degradation by ERAD. The translocon channel is believed to be masked by BiP when not in use. The *bip* gene was strongly down-regulated at 12 h (three from four spots) in all mutant *cbh1* strains which may also be a result from the delay in their growth and general development when compared to CVt. Up-regulation of *bip* was apparent in CVtΔ4 at 48 h in all four spots on the microarray slide reflecting the ER stress this particular strain was under.

Protein disulfide isomerase (PDI) catalyses the formation, reduction or isomerisation of disulfide bonds in the newly synthetised proteins in ER. The strain CVtΔ4 carrying four mutations in the *cbh1* gene was the only one in which *pdi* expression was highly up-regulated; at 48 h with value 26.836. At 12 h time point PDI was down-regulated in one of the four spots (5.237). TigA is also a member of the PDI-family and was showing similar

expression response as *pdi*. Peptidyl-prolyl-isomerase, PPI is known to improve the efficiency of protein disulfide isomerase. The *ppi* gene was partially down-regulated at 12 h along with *pdi*. Calnexin is an ER resident chaperone, which promotes the proper folding of glycoproteins together with PDI-family proteins; the *cal1* gene encoding calnexin was partially down-regulated at 12 h and up-regulated at 48 h in the strains CVtΔ4 and CVtΔ5 carrying four or five mutations in the *cbh1* gene. Misfolded proteins are translocated from the ER by the hydrophobic protein Der1, and the gene encoding this protein was also up-regulated at 48 h. The heat shock protein LHS1, of which the function is essential for protein translocation into the endoplasmic reticulum (Tyson and Stirling, 2000) was the only gene which changed expression at 24 h; it was up-regulated in one of four spots in strain CVtΔ2 and CVtΔ4.

Overall, the strongest induction of ER chaperones was observed at 48 h in strain CVtΔ4. Most of these ER-resident folding chaperones were co-regulated along with the strongly up-regulated *bip* at 48 h indicating that misfolded CBHI in CVtΔ4 induced the UPR pathway.

Proteasome genes

The genes from Set1 that are known to be involved in ER-associated degradation and protein elimination were mainly up-regulated. Some of the genes encoding proteasome subunits were up-regulated in strains CVtΔ5 and CVtΔ4 at 12 h and 48 h, respectively. Two 20S subunit and three 19S subunit-encoding genes showed expression changes in CVtΔ5; β6 was up-regulated at 48 h and α7, Rpn5, Rpn8 and Rpn12 were all up-regulated at 12 h. The β6 subunit of the 20S proteasome particle is thought to be the subunit which binds to the nuclear matrix and anchors the proteasome to the nucleus (Tokumoto *et al*., 2000). The α7 subunit, a subunit of the outer rings of the 20S proteasome complex, is known to stabilise the 26S complex (Bose *et al*., 2004).

The gene coding for Rpn8 was down-regulated at 48 h in strain CVtΔ4. Rpn8 is an essential non-ATPase regulatory subunit of the 26S proteasome. Several domains and motifs such as the JAB domain (Jun activation-domain binding protein domain), originally described as a regulator of transcription, and the C-terminal KEKE-motif, a putative site of

protein-protein interaction, have been identified in the Rpn8 protein. Rpn8p that forms complexes with Rpn13p and Doa1p has also been shown to interact with tRNA and its mitochondrial targeting factor (pre-Msk1p) in *S. cerevisiae*. These findings suggest that Rpn8 has an additional role in transcription as well as in protein degradation (Tian *et al*., 2008; Brandina *et al*., 2007).

In addition to *rpn8,* another gene (*rpn5*) encoding a lid subunit of the 26S proteasome, was up-regulated at 12 h in strain CVtΔ5. This non-ATPase subunit of the 19S regulatory particle is a proteasome regulator and plays a role in mediating proper proteasome assembly (Yen *et al*., 2003). *Rpn12,* the gene encoding a subunit of the 19S particle was up-regulated in this study. Rpn12p, also known as Nin1p, associates with Nob1p that has been shown to be essential for the function of the proteasome in growing yeast cells. Nob1p is necessary for activation of Cdc28 kinase, which is essential for the normal cell cycle and is also involved in ribosome synthesis (Kominami *et al*., 1995; Tone *et al*., 2000; Tone and Toh-e, 2009). In our study, RNA-binding protein Pno1p, which is known to form a complex with the 19S proteasome together with Nob1p (Fatica *et al*., 2004), was up-regulated in all strains at 12 h in the Set2 gene expression studies (see p.134).

The observation that five genes encoding proteasome subunits were up-regulated together with genes coding for proteasome assembly proteins (Table 11), supported the idea that at least part of the mutant CBHI in CVtΔ5 was degraded by the proteasome. The gene encoding ubiquitin-selective AAA-ATPase, Cdc48, was also up-regulated at 48 h in CVtΔ5; this gene is involved in ubiquitin-mediated protein degradation as well as in a variety of important cellular processes including membrane fusion, as a complex together with Np14/Ufd2 (Schuberth *et al*., 2004). These findings provided support that expression of the mutant CBHI in CVtΔ5 induced the ERAD pathway.

The gene encoding an ubiquitin activating enzyme E1, was up-regulated in strain CVtΔ4 only. E1 enzyme activates and transfers ubiquitin to ubiquitin-conjugating enzymes as the first step of ubiquitination of a substrate for proteasomal degradation (Attaix *et al*., 2002).

ERAD-related genes

PrpA is a protein disulfide isomerase-related protein A, which has shown to have disulfide isomerase activity (Haiping *et al*., 2004). In our study, the PrpA-encoding gene was down-regulated at 12 h in strains CVtΔ2 (one of four spots) and CVtΔ4 (two of four spots). Transcriptional regulation of *sec61* at 12 h was showing down-regulation in three replicate spots in strain CVtΔ2 and up-regulation at 48 h only in strain CVtΔ4 in three replicate spots. Sec61p is the major component of a channel-forming translocon complex and mediates protein translocation across ER including co- and post-translational ER import (Nakatsukasa and Brodsky, 2008). Down-regulation of *sec61* at the 12 h time point may have been caused by a delay in the expression of mutant proteins compared to the non-mutant CVt strain.

In summary, genes in Set1 showed a very different response in relation to protein folding and protein elimination in the three strains expressing different forms of misfolded CBHI. The strain CVtΔ2 carrying two mutations in the *cbh1* gene did not show any stress response in the secretion pathway while CVtΔ4 carrying four mutations in the *cbh1* gene showed up-regulation of many genes related to UPR at 48 h. Interestingly, the additional mutation in the *cbh1* gene in strain CVtΔ5 seemed to induce up-regulation of genes involved in ER-associated degradation even at 12 h.

3.5.4 Set2: Global profiling of expression changes

Set2 included 9063 gene probes corresponding to genes in the *T. reesei* genome. This set did not include the 66 Set 1 probes and was used for initial profiling of genome-wide gene expression in the CBHI mutant strains and to provide additional information to that obtained with Set1 concentrating on gene expression related to cellular stress. A probe for each gene was spotted at least once on the microarray slides. Gene expression was analysed in technical replicates, dye swap replicates, therefore expression changes can only be considered as a preliminary guide. The four replicates of the housekeeping genes showed constant expression in all conditions, and therefore performed as a quality control and increased precision to the experiment. Constant results of spike control provided an indication of a good quality of the experiment.

There were some interesting changes in the gene expression which could be counted as significant if there were high fold changes related to the control (CVt) and if the change was seen in both dye swap replicates. A high portion of differentially expressed genes from Set2 had an assigned function in protein folding and stress response based on the Blast2Go search. Expression changes were observed in a total of 37 spots on the microarray slides corresponding to 21 genes. The majority of the differentially expressed genes were observed in strains CVtΔ2 at 12 h and in strain CVtΔ5 at 12 h and 48 h.

Differentially expressed genes involved in protein folding / UPR and protein elimination / ERAD

Cells adapt to ER-stress by arresting global protein synthesis while simultaneously activating specific transcription factors and their downstream targets. These processes are mediated in part by serine/threonine-protein phosphatase PP1, which dephosphorylates the translation initiation factor eIF2α leading to subsequent general inhibition of protein synthesis (Drexler, 2009). In the current study, protein phosphatase 2c, an evolutionarily conserved serine/threonine protein phosphatase, was up-regulated only in strain CVtΔ2 at 48 h (Table 3 on the attached DVD; fold change is indicated as the ratio of median, 2.021/0.66).

A large number of ER-resident chaperones and other foldases were partially down-regulated at the 12 h time point and up-regulated at 48 h in strain CVtΔ4 only. Transcriptional up-regulation was partly changed at 12 h in the genes encoding three chaperones belonging to the family of peptidyl-prolyl-isomerases (CVtΔ2, 29.127/0.152; CVtΔ4, 28.062/0.189; CVtΔ5, 26.671/0.14), which are known to assist the PDI chaperones in the formation of disulfide bridges in the nascent polypeptide. Prefoldin (PFD), a molecular chaperone has been shown to stabilise and deliver unfolded proteins to a chaperone to facilitate in folding in *S. cerevisiae* (Vainberg *et al*., 1998). Prefoldin subunit 6 - encoding gene as well as the T complex protein, which is a molecular chaperone and belongs to the TCP-1 chaperonin family showed up-regulation at 12 h in strain CVtΔ5 (2.874/0.572; 3.615/0.658). TCP-1 chaperones are known to assist in the folding of proteins upon ATP hydrolysis and play a role in the folding of actin and tubulin *in vitro* (Altschuler *et al*., 2009). In yeast, prohibitins have shown to function as chaperones that regulate the turnover of membrane proteins by mitochondrial ATP-dependent protease (Steglich *et al*.,

1999). In this study, prohibitin 1 (PHB1) and prohibitin 2 (PHB2) encoding genes showed up-regulation at 12 h in strain CVtΔ5 (1.596/0.6; and 2.143/0.602 respectively). Hsp70 family protein and Hsp90 binding co-chaperone Sba1-coding genes were also up-regulated in strain CVtΔ5 at 12 h (3.06/0.506).

There seemed to be a clear need for the proteasome function already after 12 h of growth as shown by Set1. This was indicated by up-regulation of the gene encoding Pno1p (CVtΔ2, 2.848/0.653), which is an uncharacterised nuclear protein known to form a complex with Nob1p, another nuclear chaperone. Nob1p, in a complex with the 19S regulatory particle facilitates the maturation of the 26S proteasome (Tone and Toh-E, 2002). This need was further indicated by up-regulation of the *Ump1* gene involved in proteasome maturation in CVtΔ5 (5.015/0.668) at 48 h and the gene encoding the ubiquitin ligase complex F-box protein GRR1 in strain CVtΔ4 (1.894/0.647) at 24 h.

A number of genes related to the UPR and ERAD pathways among the Set2 genes were down-regulated. For example, the gene encoding the translocon protein Sec66 was down-regulated in CVtΔ4 at the 24 h time point. Sec66 is a component in a complex of a translocation apparatus and specific for the signal polypeptide (Feldheim *et al*., 1993). Some heat shock protein-coding genes were also down-regulated in all strains at 12 h. These included three genes encoding small heat shock proteins, sHSPs, which are thought to contribute to the development of thermotolerance (a form of stress) in eukaryotic organisms (Osteryoung and Vierling, 1994). A high number of genes encoding the Hsp70□ family proteins were amongst the other HSP genes that were down-regulated. Hsp78□ encoding genes were partly down-regulated at 12 h in all CBHI mutant strains. Hsp78 is a calpain (ATPase chaperone) homologue in mitochondria, which can act as a chaperone and called mt-hsp70. When this chaperone binds to a misfolded protein, the protein escapes degradation (Schmitt *et al*., 1995).

Overall it can be concluded that four mutations in the *cbh1* gene in CVtΔ4 caused the strongest UPR activation and up-regulation of BiP, a known UPR signal protein at 48 h. In addition, five chaperone-related genes were up-regulated at the 48 h time point in this strain. Strain CVtΔ5 carrying five mutations in the *cbh1* gene showed the strongest

response to the protein elimination pathway by up-regulation of ERAD related genes, which featured many proteasome-encoding genes (12 h and 48 h) and translocon-related genes at the 48 h time point. In addition, some heat shock protein related genes and genes encoding folding chaperones showed up-regulation in strain CVtΔ5 at 12 h indicating that the cell had recognised mutant CBHI proteins and tried to refold them. There were no significant signals of UPR stress in strain CVtΔ2 other than activation of the serine threonine signalling pathway at 48 h. Probably the two mutations in the *cbh1* core gene were either not enough to trigger this response or the mutations have been corrected, which was indicated by up-regulation of the gene encoding DNA damage responsible protein (1.704/0.656) at 48 h.

Differentially expressed genes in cell growth, cell cycle, development and proliferation

The genes assigned in the classes cell growth, cell cycle, development and proliferation were predominantly up-regulated in all CBHI mutant strains at 12 h and 48 h. Down-regulated genes at the early time point included genes encoding proteins related to cell cycle control, conidiation and sexual development. The strongest transcriptional response (24 genes in total) was observed in strain CVtΔ5 at the 12 h time point, and may have partially been caused by a delay in cell growth compared to the other mutant strains and the non-mutant strain CVt. CVtΔ5 was growing slower than the two other mutant strains and biomass formation was clearly weaker compared to the other CBHI mutant strains and the non-mutant strain at an early time point (personal observations). Strains CVtΔ5 and CVtΔ2 started to conidiate at an early stage in a liquid culture, which is one way of survival in non-optimal conditions.

Expression of genes encoding hydrophobin 1 and cell division control protein 2 was down-regulated at later time points (24 h and 48 h) and hydrophobin II (HFBI) expression was induced in strains CVtΔ2 (24 h: 29.251/0.275; 48 h: 15.485/0.414) and CVtΔ5 (24 h: 35.255/0.464; 48 h: 42.234/0.344). Hydrophobin II (HFBII) is found on the conidial cell wall of *T. reesei* and is secreted into the medium at high concentrations (Linder *et al*., 2005). Hydrophobin I has been shown to have a role in hyphal development while hydrophobin II is involved in sporulation (Askolin *et al*., 2005). Both hydrophobins have been connected to stress response upon nitrogen or carbon starvation in *Magnaporthe*

grisea (Kim *et al*., 2001) and *Metarhizium anisopliae* (St Leger *et al*., 1992). No changes in the expression of the hydrophobin genes or signs for early conidiation were detected in CVtΔ4 indicating that this strain may have responded to stress in a different manner.

Differentially expressed genes in transcription

The majority of the genes classified to the "transcription and DNA repair" class were up-regulated in CVtΔ5 at 12 h and 48 h. The *ace1* gene encoding ACEI which is known to act as a repressor of cellulase and xylanase expression was up-regulated at 48 h in CVtΔ4 (7.223/0.639) only. The *ace2* gene coding for ACEII, a transcriptional activator of cellulase and xylanase genes in *T. reesei* (Aro *et al*., 2001, Aro *et al*., 2003), was down-regulated only in CVtΔ4 (1.692/0.597) at 12 h. This is in agreement with earlier findings that under stressed conditions the cell is regulating expression of the main cellulases by reducing the expression of the activator *ace2* and inducing the expression of the repressor *ace1* translating to a RESS response.

Differentially expressed genes in translation

When the cell is subjected to ER stress, immediate changes occur to inhibit translation initiation in order to protect the cell from further accumulation of unfolded proteins, as well as to preserve nutrients and energy. This is usually mediated by down-regulation of the relevant genes (Sheikh and Fornase, 1999; Drexler, 2009). In contrast to the above, the corresponding genes in this study were mainly up-regulated. For example, genes coding for eIF-2α and eIF4E which are the two major factors controlling translation initiation in mammalian cells (Hinnebush, 1997; Kaufman, 1999) were up-regulated in some CBHI mutant strains. The observed up-regulation may be due to the mode the stress was applied in this study, i.e. by expression of a native misfolded protein instead of drug treatment.

Differentially expressed genes in ribosome structure and biogenesis

Cellular proteins are synthesised at the ribosomes in the cytoplasm and therefore, maintaining an appropriate amount of small and large ribosomal subunits is critical for translation and cell growth. A linear relationship between growth rates and cellular concentrations of ribosomes in exponentially growing cultures of enterobacteria has been

shown by Schmidt *et al*. (1985). *S. cerevisiae* cells generate new ribosomes at a rate of 2,000/min accounting for 50 % of the total cellular transcription (Warner *et al*., 2001). This also consumes an enormous fraction of the resources of a rapidly growing cell.

In this work, 37 genes encoding proteins in "ribosome structure and biosynthesis" were predominantly up-regulated amongst all strains at 12 h (CVtΔ2 9 genes; CVtΔ4 10 genes; CVtΔ5 18 genes, Attachment 3, 4 and 5). These included genes for small and large ribosomal proteins and small nuclear ribonucleoproteins. Up-regulation of genes encoding several mitochondrial ribosomal proteins and ribosome associated chaperones was also noted. Only two ribosomal genes in CVtΔ5 showed a decrease in expression.

Over 200 genes encoding ribosomal proteins (RPs) are regulated by RNA polymerase II in yeast (Warner, 1999). Ribosome biogenesis genes have shown to be markedly and co-ordinately down-regulated in response to a number of environmental stress conditions (Schawalder *et al*., 2004). Defects in the yeast secretory pathway were shown to cause transcriptional repression of genes coding for ribosomal proteins. In the current study, genes in the class "ribosomal structure and biogenesis" were up-regulated in all CBHI mutant strains at the 12 h time point. This is different to previous findings (Sims *et al*., 2005) where the UPR response was triggered by chemical means e.g. by using DTT or A23187. In our case, the effects were caused by expression of a homologous mutant protein which may have lead to a different regulatory feedback further elaborated in the Discussion (p.169-171).

Genes regulating the synthesis of small and large ribosomal subunits are known to be regulated independently in *S. cerevisiae* (Mitzuta *et al*., 1994; Zhao *et al*., 2003). However, in this study, both small and large ribosome subunit encoding genes were up-regulated indicating the requirement for expression of both classes of genes under the growth conditions used.

Differentially expressed genes in metabolic pathways

A large portion of Set2 genes belonging to the "metabolic pathways" class were affected at all time points in all CBHI mutant strains. Strain CVtΔ2 had the strongest response with expression of 131 genes altered, while CVtΔ4 strain had the weakest response showing changes within expression of 95 genes in the class "metabolic pathway genes". In comparison, CVtΔ5 had significant expression changes in 106 genes. The "metabolic pathway" genes were further assigned to subclasses, which helped to tease out particular trends in gene expression within these subclasses (Table 12).

Table 12. Changes in gene expression in metabolic pathway subclasses in each mutant strain at three time points (↓ down-regulated, ↑ up-regulated).

Metabolic pathway	*12h*						*24h*						*48h*						
	CVtΔ2		*CVtΔ4*		*CVtΔ5*		*CVtΔ2*		*CVtΔ4*		*CVtΔ5*		*CVtΔ2*		*CVtΔ4*		*CVtΔ5*		
	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	Total
Unclassified	5	2	1	6	1	8	0	0	0	1	5	1	3	2	0	4	1	1	41
Amino acids	11	4	3	2	0	7	0	0	0	0	1	0	0	0	0	1	0	5	34
Fatty acids, lipids	1	1	2	1	1	1	0	1	1	0	1	0	0	0	0	0	0	0	10
Carbohydrates	10	1	7	1	7	4	8	4	2	0	3	1	4	10	2	8	1	10	83
Hydrolases	4	0	4	0	3	2	1	0	0	0	0	2	1	0	0	2	0	2	21
Dehydrogenases	2	2	1	3	0	5	0	1	0	0	0	1	0	1	1	0	0	1	18
Oxidoreductases	5	9	4	11	2	7	2	1	1	0	2	2	4	2	3	3	2	4	64
Peptidases, proteases	4	0	2	0	1	2	2	0	0	0	0	1	2	1	2	1	1	3	22
Reductases	1	0	0	3	0	4	0	0	0	0	1	0	0	0	0	0	0	1	10
Transferases	12	2	3	6	4	7	0	0	0	0	2	0	5	0	2	1	0	5	49
Up and down reg. all together	76		60		66		20		5		23		35		30		37		352

Carbohydrate metabolism

The total number of differentially expressed genes in the "carbohydrate metabolism" subclass was 83 of which 44 genes were up-regulated and 39 down-regulated. The majority of genes were down-regulated in each strain at the early time points (12 – 24 h) and up-regulated after 48 h of cultivation. The majority of the down-regulated genes belonged to a group of genes coding for enzymes involved in lignocellulose degradation, such as endoglucanases (17 spots on the microarray corresponding to 12 genes), exoglucanase, endoxylanases and cellulose hydrolases. Up-regulated genes featured, for example genes encoding endo- and exoglucanases (CBHI, Cel61b, endo-1,3-beta glucanase, endoglucanase, glucan 1,3-beta glucosidase, CiP; cellobiose inducing protein) and xylanase III.

The transcriptional down-regulation of the genes encoding main secreted proteins under stress conditions is called RESS (**re**pression under **s**ecretion **s**tress) and has been observed previously in *T. reesei* (Pakula *et al*., 2003) and *A. thaliana* (Martinez and Chrispeels, 2003). All strains expressing mutant CBHI showed the RESS response in production at the 12 h and 24 h time points, while at 48 h, the transcriptional response was mainly up-regulated for the genes involved in carbohydrate metabolism.

Oxidoreductases

Oxidoreductases are enzymes that catalyse oxidation/ reduction reactions in the cell by transferring an electron from one molecule to another during oxidation (Mathews and van Holde, 1990). A large subclass of genes encoding oxidoreductases was showing both up- and down-regulation in each strain expressing a mutant CBHI. Regulation differences between the CBHI mutant strains and the non-mutant CVt in genes encoding oxidoreductases might reflect the delay observed in the cell growth and differences in proliferation in the CBHI mutants.

Amino acid metabolism

In addition to up-regulation of the accustomary UPR target genes, ER stress has also been shown to activate genes involved in amino acid metabolism (Okada *et al*., 2002). The majority of amino acid metabolism related genes were up-regulated in CVtΔ5 at 12 h and 48 h and down-regulated at 12 h in CVtΔ2 and CVtΔ4. This may also be an indication of the different growth rates of the mutant strains.

Peptidases, proteases and hydrolases

Proteases play various roles in cellular metabolism, nutrition and morphogenesis (Archer and Peberdy, 1997). They have been shown to be responsible for post-translational processing of extracellular hydrolytic enzymes in *T. reesei* (Chen *et al*., 1993). In earlier work with *Aspergillus oryzae*, it was found that transcription of genes encoding secreted proteases was induced when the pH in the culture medium changed from neutral to acidic, suggesting that expression of these genes was regulated by pH (Biesebeke *et al*., 2005). In the current study, there were only a few changes in the expression of protease, peptidase

and hydrolase subclass genes and the majority of the changes were observed in strain CVtΔ5 at 48 h.

The serine endopeptidase gene was down-regulated in all CBHI mutant strains at 12 h. The chymotrypsin-like and trypsin-like proteases that belong to serine proteases are responsible for catalysing the hydrolysis of peptide bonds. In contrast, genes encoding the proteasome β2-subunit, which has trypsin-like (TL) and β5-subunit chymotrypsin-like (ChTL) activities did not show regulation changes at any time point (Wolf and Hilt, 2004). Therefore, the major expression changes in encoding peptidases, proteases and hydrolases may occur later than 48 h when the pH of the culture medium dropped down to 3.62 on day 3 (section 3.3.1).

Transporters

Transporters are transmembrane proteins, which have a function in substrate specific and active transport across the membrane. Transporters use the energy of ATP to force the ions or small molecules through the membrane participating in multiple pathways e.g. glycan biosynthesis and metabolism, and signal transduction (Parales *et al*., 2008). The most significant response in gene regulation of transporters was observed at 12 h in CVtΔ4 with 26 genes. Fewer changes were observed at 24 h and 48 h time points. The majority of the differentially expressed genes were found to code for MFS transporters, ABC transporters and amino acid transporters. Regulation changes at the 12 h time point in genes encoding different transporters could be explained by the fact that fungal hyphae were still in a fast growth phase, when an active transport system was required.

Differentially expressed genes in "trafficking"

As expected, very few expression changes were shown in genes related to "intracellular trafficking" at the time points used in this experiment. Many of these genes are believed to be expressed at later stages, during active protein secretion, which appears to occur after three days of cultivation in the CLS medium in *T.reesei* (section 3.3.1).

The majority of up-regulation changes in genes involved in intracellular trafficking were observed in strain CVtΔ5 with five mutations in the *cbh1* gene. This may indicate a need

for proteins active in trafficking, including proteins mediating ubiquitination of misfolded proteins and directing them for degradation by the proteasome. In support to this notion, genes coding for proteins such as a coatomer subunit, protein transport protein SEC6 (1.64/0.638) and vacuolar protein sorting-associated protein 27 (6.557/0.617) were all up-regulated in strain CVtΔ5, mainly at 48 h. Also, the gene encoding the vacuolar sorting protein 29 was up-regulated in CVtΔ4 (3.644/0.644) at 48 h.

There were also genes in this class that were down-regulated. For example, the gene coding for coatomer subunit II was down-regulated in CVtΔ5 at the earlier 24 h time point indicating that at this stage, the secreted proteins were not directed into the secretory vesicles. The finding that misfolded proteins are excluded from the COPII vesicles may explain the observed down-regulation (Pagant *et al*., 2007).

Summary of gene expression in Set1 and Set2 arrays

Even though the results from Set2 gene expression can only be considered as a preliminary guide, there were high values in expression changes which further supported findings from Set1 which featured multiple replicates of selected genes related to UPR and ERAD. While indications of stress were not obvious in Set1 with strain CVtΔ2 at any of the time points in the genes spotted on the microarray slides, there were some signs of stress in CVtΔ2 at 48 h as indicated by the expression of genes other than those in Set1. These included the up-regulation of serine-threonine phosphatase, which could be considered as the first signal of ER-stress and induction of the gene coding for the proteasome assembly protein Pno1p, which signals an increased need for ERAD function. Induction of the *hfb II* gene at 24 h provided further support to the observation that strain CVtΔ2 was, indeed, under significant stress.

Indications of secretion stress were more obvious in strain CVtΔ4 expressing a CBHI protein with four mutations. In addition to up-regulation of the UPR pathway related genes seen in Set1, changes were also recorded in regulation of genes coding for the ACEI and ACEII proteins, which are regulators for the cellulase and xylanase biosynthesis. ACEI, the repressor of the main cellulases and xylanases was up-regulated and the activator ACEII of main cellulases and xylanases was down-regulated leading to RESS.

In the strain CVtΔ5 with five mutations in the *cbh1* gene, where RESS was also detected at the gene expression level, expression of the stress induced transcription factor eIF2α was up-regulated at 12 h along with a number of proteasome subunit genes. Also, along with many of the cell-growth related genes, expression of two stress related hydrophobin genes was up-regulated in CVtΔ5. Potential folding problems were indicated by up-regulation of many genes encoding chaperones and folding enzymes already at 12 h. In addition, activation of the ERAD function was not only shown at 12 h by up-regulation of genes encoding proteasome subunits but also in the up-regulation of genes coding for a proteasome maturation chaperone and a proteasome assembly factor. All these findings together would suggest that strain CVtΔ5 carrying five mutations in the *cbh1* gene was under a lot of stress related to secretion. It also seemed that the CVtΔ5 strain could not refold the misfolded protein leading to its translocation to the ERAD pathway.

RESS was observed in all CBHI mutant strains at 12 h and 24 h. Gene regulation leading to slowing down of the carbohydrate metabolism could be also explained by faster growth of the reference strain, CVt used in this experiment. As discussed earlier, different mutations in a protein may change the protein production. Interestingly, genes related to ribosome biogenesis and structure were induced in all mutant strains at an early time point in the current study, which was different to the earlier observations in studies addressing stress conditions (Sheikh and Fornase, 1999; Drexler, 2009).

3.5.5 Northern analysis to confirm the microarray results

Northern analysis was carried out for mRNAs for a selection of genes encoding proteins involved in the UPR and ERAD pathways. cDNAs were synthesised for the genes *bip* (Trire 246902), *cal* (Trire 273678), *rpn8* (Trire 80843), and *gapdh* (Trire 119735) and were used as probes. These particular genes were selected on the basis that they showed significant up- or down-regulation in the microarray experiment (Set1). Samples of total RNA were extracted from the fungal transformants CVt, CVtΔ2, CVtΔ4 and CVtΔ5 grown under cellulase-inducing conditions for 12 h at which point most genes where affected across the strains. The specific mRNA signals on the Northern blots were quantified using ImageJ program and their intensity normalised against the signal of *gapdh* (Fig. 39).

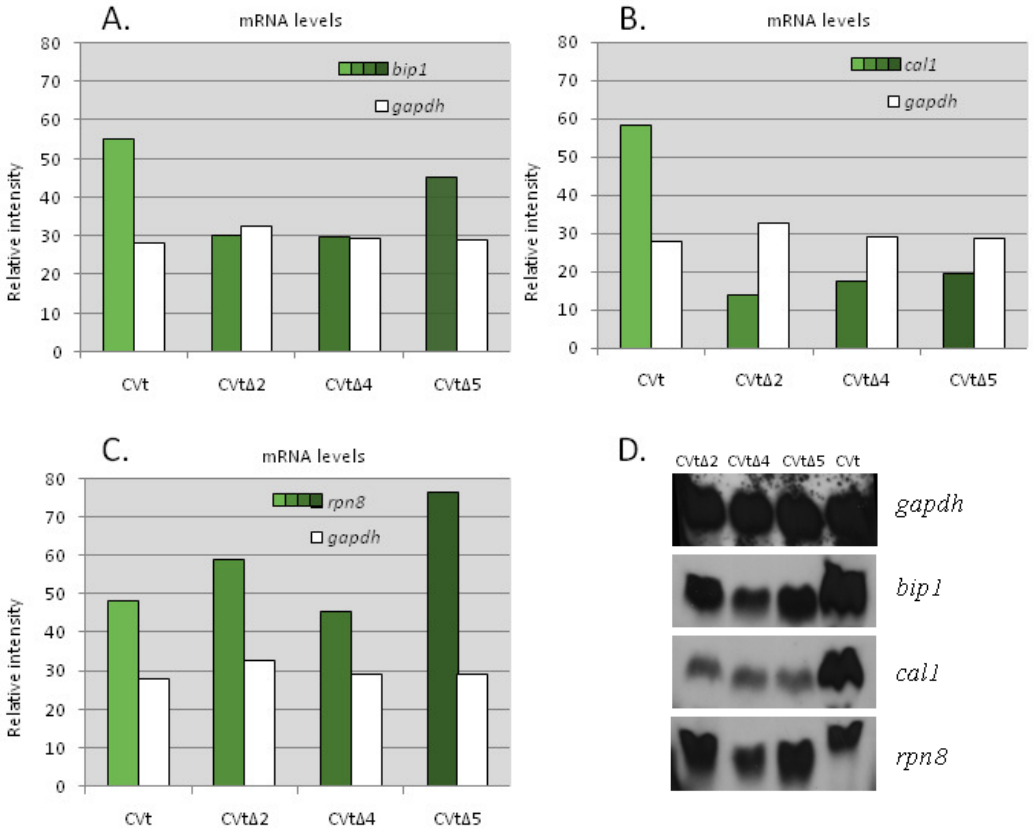

Figure 39. The mRNA levels of *bip1*, *cal1* and *rpn8* in the *T. reesei* non-mutant strain CVt and CBHI-mutant strains CVtΔ2, CVtΔ4 and CVtΔ5 after 12 h growth in the CLS medium. The graphs of quantified mRNA signals were normalised against the signal of *gapdh* and the results are shown as relative intensity of the signal. A. *bip1*; B. *cal1*; C. *rpn8*. D. The mRNA signals on Northern blot films.

The UPR related genes *bip1* and *cal1* showed higher expression levels in the non-mutant CVt than in any strains carrying a mutant *cbh1* gene as seen in Fig. 39A and B. The *bip1* mRNA level was significantly higher in CVtΔ5 compared to the levels in other mutant strains as expected (Supplementary data, Tables 1-3). Expression of the *rpn8* gene encoding the 19S proteasome non-ATPase subunit was up-regulated in strain CVtΔ5 (Fig. 39 C).

Expression of the UPR response indicator gene *bip1* was decreased in the microarray analysis (Set1) in strain CVtΔ2 (one of four spots), CVtΔ4 (three of four spots) and CVtΔ5 (four of four spots) after 12 h growth. Expression of the *cal1* gene was decreased in CVtΔ2 (four of four spots), CVtΔ4 (zero of four spots) and CVtΔ5 (one in four spots). *Rpn8* was up-regulated in one spot of four in the microarray at 12 h expression in strain CVtΔ5 only.

While the spot-to-spot comparison may not seem optimal, the overall trend as shown in Fig. 39 confirms the expression trend seen in the microarray analysis. The high amount of mRNA (20µg) used for the Northern blotting most probably increased the sensitivity of the detection method, which could explain the observed down-regulation of *bip1* and *cal1* in the strains CVtΔ4 and CVtΔ5 and up-regulation of *rpn8* in CVtΔ2 (Fig. 39), although these changes were not detected in the microarray analysis.

3.6 The effects of the expression of mutant CBHI under proteasome inhibiting conditions

The production of a mutant form of CBHI in strain CVtΔ4 showed some indications of secretion stress: first, CBHI expression and secretion was delayed by the *cbhI* gene mutation (as evidenced by Northern blotting and 1D SDS-PAGE) and secondly, the CVtΔ4 showed a reduction in the amount of biomass produced and in the hyphal diameter. The aim of this experiment was to investigate how *T. reesei* strain CVtΔ4 responded to the extra stress applied by inhibiting the proteasome compared to CVt and RutC-30. The particular interest was taken to see how the 26S proteasome, UPR and ERAD pathways were affected when the proteasome was inhibited by MG132.

Global profiling of gene expression under proteasome-inhibiting conditions was carried out using Set1 and Set2 microarrays. This experiment was carried out using strains CVtΔ4 with four mutations in the *cbh1* core gene, the non-mutant transformant CVt and the non transformant strain Rut-C30. Mutant strain CVtΔ4 was chosen for this particular experiment because it was showing significant secretion stress by activating the UPR genes at 48 h. Proteasome activity was inhibited by adding the inhibitor MG132 to the cultures after 46 h cultivation in the CLS medium and the samples were collected after a further 2 h of incubation.

Across the three fungal strains investigated, 187 spots on the microarray slide showed a significant up- or down-regulation upon addition of the proteasome inhibitor MG132 when compared to the controls (i.e. each strain without the addition of MG132). Differential gene

expression under proteasome inhibition conditions was not limited to the genes related to secretion stress and ERAD, but in addition, genes encoding proteins functioning in different pathways were also affected (Fig. 40). Unfortunately, 21-50 % of all differentially expressed genes did not find a match in genes or functional groups listed in various databases and were thus assigned as "unclassified".

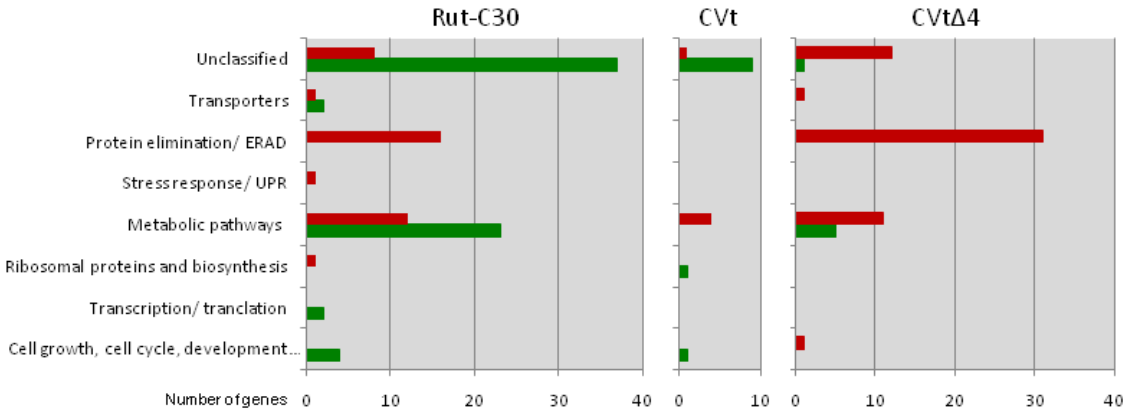

Figure 40. Up-and down-regulated genes assigned to their functional classes in strains Rut-C30, CVt and CVtΔ4 under proteasome inhibition conditions. Up-regulated ■ ; down-regulated ■ .

It appeared that inhibition of the proteasome activity had a different effect on each strain. A large portion of the differentially expressed genes in the functional class "metabolic pathways" was affected in the strains Rut-C30 and CVtΔ4 while only a few genes were up-regulated in CVt. The "carbohydrate metabolism" and "hydrolase" subclass genes were up-regulated in Rut-C30 and down-regulated in CVtΔ4. Several endoglucanase genes and xylanase III gene were up-regulated in CVtΔ4. No "carbohydrate metabolism" genes were affected in CVt. Different reactions of the strains to proteasome inhibition may relate to their different growth characteristics and timing which are reflected in gene expression.

The addition of proteasome inhibitor MG132 in the fungal cultures caused a widespread up-regulation in genes coding for proteasome subunits in Rut-C30 and CVtΔ4, while CVt did not show any expression changes in the proteasome genes. Five (CVtΔ4) or four (Rut-C30) of the seven genes encoding α-subunit proteins and four (CVtΔ4 and Rut-C30) of the seven genes encoding β-subunit proteins were up-regulated. Amongst these were genes encoding the subunits which are known to have peptidyl-glutamyl peptide hydrolysing activity (β1) and trypsin-like activity (β2) (Heinemayer *et al.*, 1997). Genes encoding subunits α1, α3, α5, α6, α7, β3 and β4 were strongly up-regulated in Rut-C30 and CVtΔ4

(3-7 spots from 4-8 replicates) which indicates a strong response to inhibition of the proteasome activity.

In addition to the effects on the expression of genes encoding 20S proteasome subunits, inhibition of the proteasome with MG132 also affected genes coding for 19S subunits including genes for Rpn3, Rpn8, Rpn9 and Rpt1. Rpn8 is thought to participate in deubiquination of the substrate, because it contains a highly conserved metalloisopeptidase domain (MPN) and this activity is necessary for deubiquination (Wei *et al*., 1998). Rpn9 is suggested to play a key role in facilitating the assembly of the 26S proteasome or in stabilising the structure of the 26S proteasome (Takeuchi *et al*., 1999b). Because of its location within the 19S lid particle and association with Rpt1 and Rpt6, Rpn3 is thought to have a role in modulating the activity of Rpt1 and Rpt6 subunits (Bailly and Reed, 1999).

In addition to proteolytic enzymes housed in the proteasome subunits, genes encoding other proteases and peptidases were up-regulated in Rut-C30 and CVtΔ4 including carboxypeptidase Y, dipeptidyl peptidase, vacuolar protease A and serine endopeptidase, but showed no response in strain CVt. This suggested an increased need for non proteasomal proteolytic activity especially in CVtΔ4, where three of the above protease genes were upregulated.

In previous work carried out with chicken erythroblast HD6 cells, chicken embryo fibroblasts (Kawazoe *et al*., 1998) and rat HepG2 cells (Liao *et al*., 2006), inhibition of the ubiquitin-proteasome pathway was shown to cause induction of the genes encoding major heat shock proteins including Hsp90, Hsp70A, Hsp70B, Hsp47, Hsp40 and Hsp25. The lack of induction of the Hsp-genes in this study was an unexpected result, which could be due to only one sampling time (46 h cultivation plus 2 h incubation after addition of the inhibitor). In previous studies with yeast (Lee and Goldberg, 1988), samples were taken at several time points up to two hours after addition of MG132 which facilitated the detection of up-regulation of the Hsp-genes. Different results between mammalian, yeast and *T. reesei* cells may be explained by the experimental set up or differences in the cell wall/envelope structure between yeast and fungal cells which may have affected penetration of MG132 and thereby timing of the event.

In summary, inhibition of the proteasome by MG132 had a different effect on the three strains tested. The number of affected genes varied from 16 in CVt to 109 in Rut-C30. The proteasome 19S subunit genes and 20S subunit genes showed large expression changes in Rut-C30 and CVtΔ4, while no effect in proteasomal gene expression was detected in CVt. There were also transcriptional changes in the expression of genes related to metabolic pathways in all strains. The previously found up-regulation of genes coding for heat shock proteins was only detected in strain Rut-C30.

3.6.1 Northern analysis to confirm the microarray results on proteasome inhibition

Northern blotting was utilised to test and confirm any expression changes within the group of genes encoding the 20S proteasome subunits in *T. reesei* grown with and without MG132 addition. Samples of total RNA from the CBHI mutant CVtΔ4 and the non-transformant Rut-C30 were isolated and subjected to Northern blotting (Fig. 41). cDNAs were synthetised for the genes *alpha 5* (*α5*; Trire 55644), *alpha 7* (*α7*; Trire 76010), *beta 1* (*β1*; Trire 78882), *beta 5* (*β5*; Trire 121009) and *gapdh* (Trire 119735) and were used as probes. These genes showed significant up-regulation in the microarray experiment (Set1). The specific mRNA signals were quantified using the ImageJ program and normalised against the signal of *gapdh*.

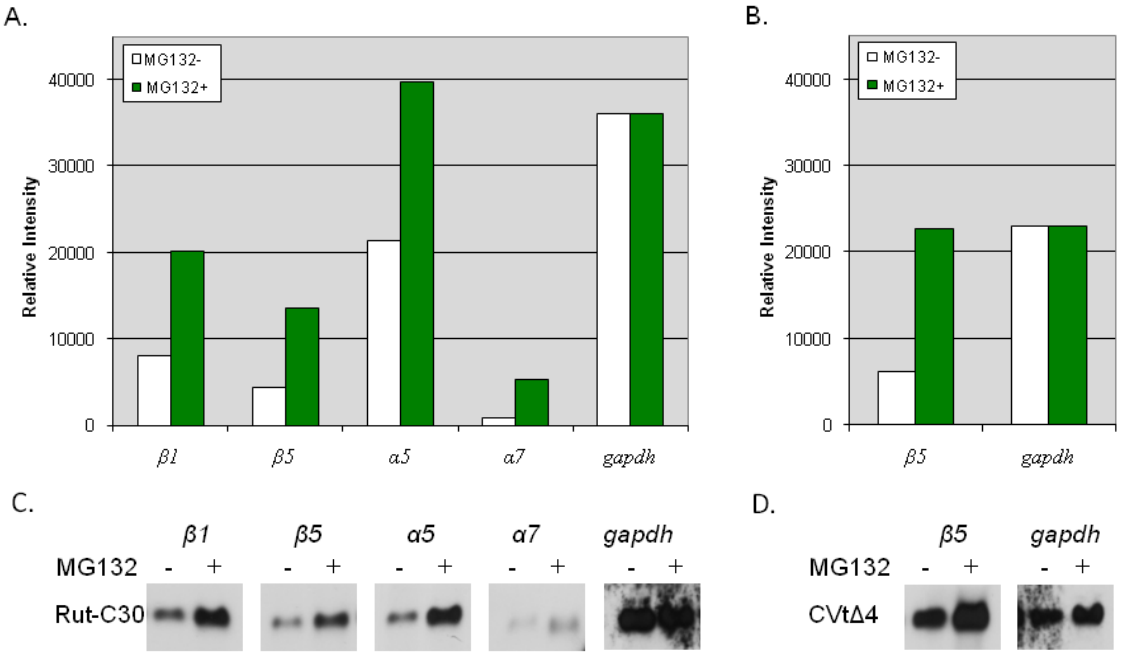

Figure 41. The mRNA levels of *α5, α7, β1* and *β5* genes in the *T. reesei* non-transformant strain Rut-C30 and the CBHI-mutant strain CVtΔ4 grown in with or without MG132. The graphs of quantified mRNA signals were normalised against the signal of *gapdh* and results are shown as relative intensity. A. Rut-C30; B. CVtΔ4; C. mRNA signals on Northern blot films from Rut-C30; D. mRNA signals on Northern blot films from CVtΔ4.

Expression of the proteasome genes *alpha 5, alpha 7, beta 1* and *beta 5* was up-regulated in the non-transformant Rut-C30 and the CBHI mutant CVtΔ4 treated with MG132. Similarly, Northern blots showed increased mRNA expression for the above genes in samples treated with MG132. This increase varied from 1.9-fold (*α5*) to 5.5-fold (*α7*) when compared to mRNA expression in untreated cultures in Rut-C30 (Fig. 41A) and in CVtΔ4 (Fig. 41B).

The results also showed that the extent of up-regulation of *β5,* the gene coding for the subunit exhibiting chymotrypsin-like activity, a signature activity of the proteasome, was higher in CVtΔ4 producing a mutant CBHI when compared to Rut-C30 producing a native CBHI.

In summary, Northern analysis of the mRNA transcribed from the selected proteasome encoding genes generally confirmed the results in the microarray analysis by showing up-regulation of all genes tested.

3.7 Visualisation of the *Trichoderma reesei* proteasome and interaction between the 20S proteasome and mutated (misfolded) CBHI in fungal hyphae

Degradation of the misfolded CBHI protein by the proteasome was investigated by fluorescence microscopy and by TEM. The possible interaction between CBHI and the 20S proteasome in *T. reesei* was determined using a dual immunolabelling procedure under fluorescent microscopy. A closer examination into the localisation of the 20S proteasome and the mutant CBHI protein in strain CVt4 was also carried out at the EM level. Immunolabelling for fluorescence microscopy was performed with five different strains: Rut-C30 with native CBHI, CVt transformant producing an intact CBHI fused to the Venus fluorescent protein and the three CBHI mutant strains CVtΔ2, CVtΔ4 and CVtΔ5. Immunolabelling for the TEM study was performed with one mutant strain, CVt4, along with CVt and Rut-C30.

3.7.1 Visualisation of the 20S proteasome in *Trichoderma reesei* hyphae

Mycelia from 19 h and 2 d old cultures of *T. reesei* Rut-C30 were immunolabelled with the polyclonal yeast anti-20S-ab (PW9355, Biomol) and anti-rabbit Alexa Fluor® antibody (Molecular Probes®). To-Pro3 (Molecular Probes®) was used to stain the nuclei. Proteasomes were localised in the cytoplasm and close to the nuclei in 2 d old immunolabelled hyphae (Fig. 42 A.). The proteasomes were mostly seen in the germinating conidia (Fig. 42 B.) and at the tip of the young hyphae (Fig. 42 C.) indicating an acute need for proteasomes during the active stage of growth. (See also supplementary data on DVD.)

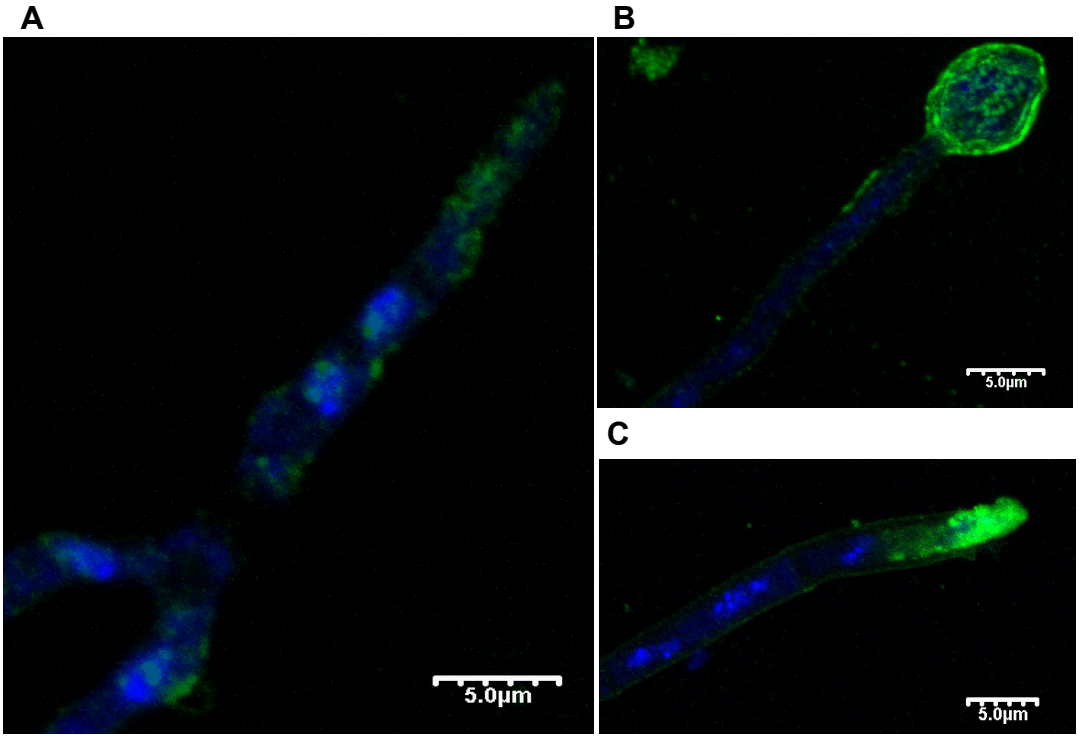

Figure 42. *T. reesei* Rut-C30 grown for 19 h and 2 d in the CLS medium and immunolabelled with a polyclonal anti-yeast 20S antibody which was detected by Alexa Fluor®488(green). ToPro3 stain was used for nuclear staining (blue). A. 2 d old mycelia showing the 20S proteasome located inside the hyphae. B. Proteasomes in the conidia of 19h old hyphae. C. Proteasomes at the actively growing hyphal tip.

3.7.2 Localisation of immunostained mutant CBHI and the 20S proteasome

To determine intracellular localisation of the secreted CBHI and the 20S proteasome in the *T. reesei* hyphae, ultrathin resin-embedded sections (0.7 µm) from hyphae cultured for two days were double-immunolabelled with the polyclonal anti-20S yeast proteasome antibody (PW9355, Biomol) and monoclonal CBHI-antibody (CI-261, Roal Ltd, Finland). The samples were later hybridised with the fluorescent secondary antibodies Alexa Fluor®488 and Alexa Fluor®594 (Molecular Probes®), respectively. Intracellular localisation of CBHI and the 20S proteasome was studied using a confocal laser scanning microscope (LSM310, Olympus, Japan) with an argon laser (488 nm).

Figure 43 shows the distribution of immunolabelled CBHI and 20S proteasome particles in the non-transformant *T. reesei* Rut-C30 producing native CBHI. The CBHI label (Fig. 43A) was distributed all over the cytoplasm and the proteasome label (Fig. 43B) was localised

mainly in the cell envelope. The merged picture (Fig. 43C) showed no obvious co□ localisation (yellow colour) of CBHI and the 20S proteasome.

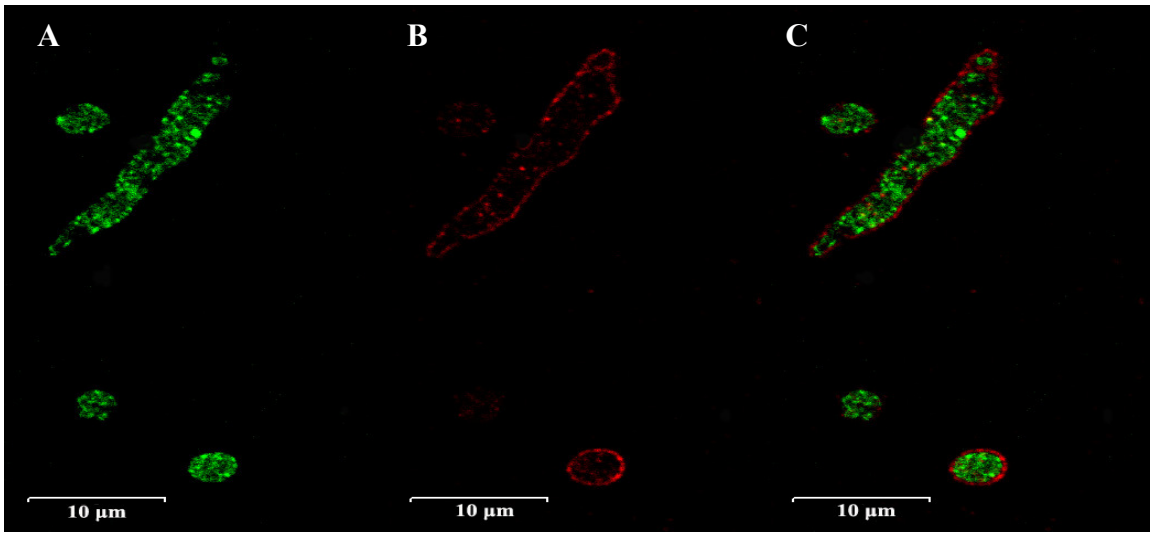

Figure 43. Indirect immunofluorescence microscopy of CBHI and 20S proteasome particles in *T. reesei* Rut□ C30. Sections of White resin embedded hyphae were treated with monoclonal anti-CBHI antiserum and polyclonal anti-20S proteasome antiserum. Antibody binding was detected with Alexa Fluor® 488 goat anti-rabbit IgG conjugate and Alexa Fluor® 594 goat anti-rabbit IgG conjugate, respectively. A. CBHI; B. 20S proteasome; C. Co-localisation of CBHI and 20S in the merged images.

Immunolabelling results of the transformant CVt producing a CBHI-Venus fusion protein are shown in Figure 44. In a longitudinal section of the strain CVt hyphae, the 20S proteasome particles and CBHI did not show co-localisation (Fig. 44C). As in strain Rut□ C30, CBHI was distributed all over the cytoplasm showing concentrated regions of label (Fig. 44A). The septum area of the hyphae contained no label (Fig. 44A, white arrow). Signals from proteasome particles (Fig. 44B) were mainly localised on the cell envelope, although there seemed to be some places of co-localisation in the cytoplasm with the CBHI label (Fig 44C, white circle).

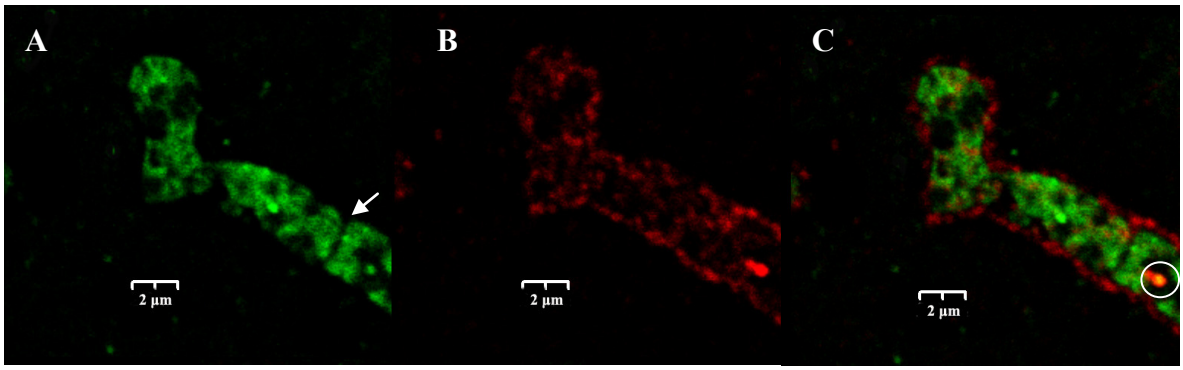

Figure 44. Indirect immunofluorescence microscopy of CBHI and 20S proteasome particles in *T. reesei* CVt. A. CBHI; B. 20S proteasome; C. Co-localisation shown in yellow in the merged images inside the white circle.

Similarly, indirect immunolabelling of the CBHI and 20S proteasome particles in strain CVtΔ2 with two mutations in the *cbh1* gene indicated that the CBHI signal was distributed in the cytoplasm forming dense, coloured regions (Fig. 45A). The proteasome signal was seen mainly in the cell envelope region (pointed white arrow in B). There was no obvious co-localisation in the merged picture (Fig. 45C).

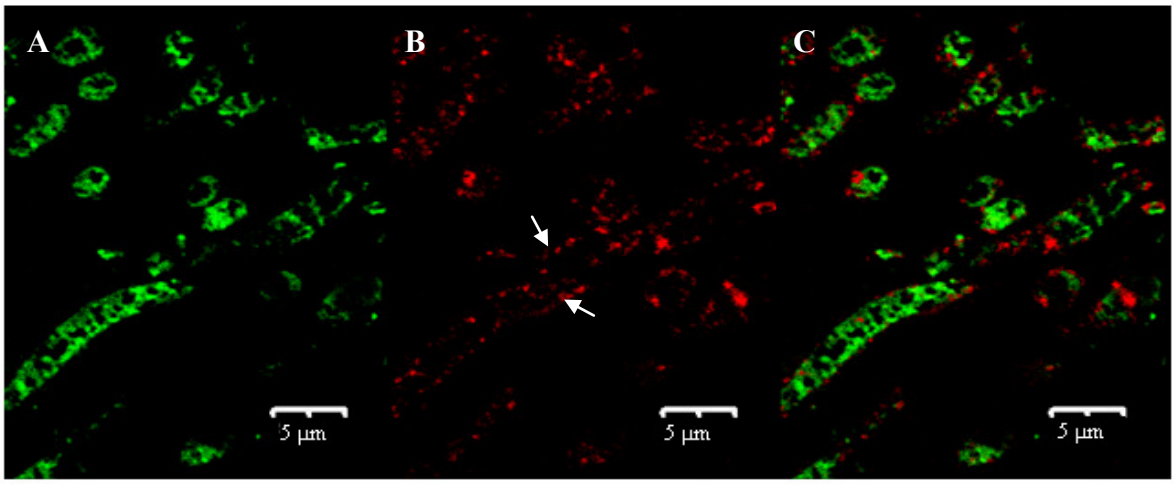

Figure 45. Indirect immunofluorescence microscopy of CBHI and 20S proteasome particles *T. reesei* CVtΔ2. A. CBHI; B. 20S proteasome; C. Co-localisation in the merged images.

In strain CVtΔ4 with four mutations in the *cbh1* gene (Fig. 46C, white arrows), the CBHI and 20S proteasome labels were showing a clear co-localisation. In addition, the mutant CBHI (Fig. 46A) appeared more aggregated in this strain compared to the other two strains producing mutant CBHI proteins (Fig. 45A and 47A). In comparison to Rut-C30 (Fig. 43A) and CVt (Fig. 44A) the proteasome label seemed to occur all over the cytoplasm appearing as bigger "blobs", instead of localising in the envelope region. Proteasomes are known to

have a tight interaction with the ER at the cytosolic side of the ER membrane (Rivett, 2002) and this may be the site of co-localisation of the 20S proteasome particles and the aggregated CBHI proteins in strain CVtΔ4 (Fig. 46C).

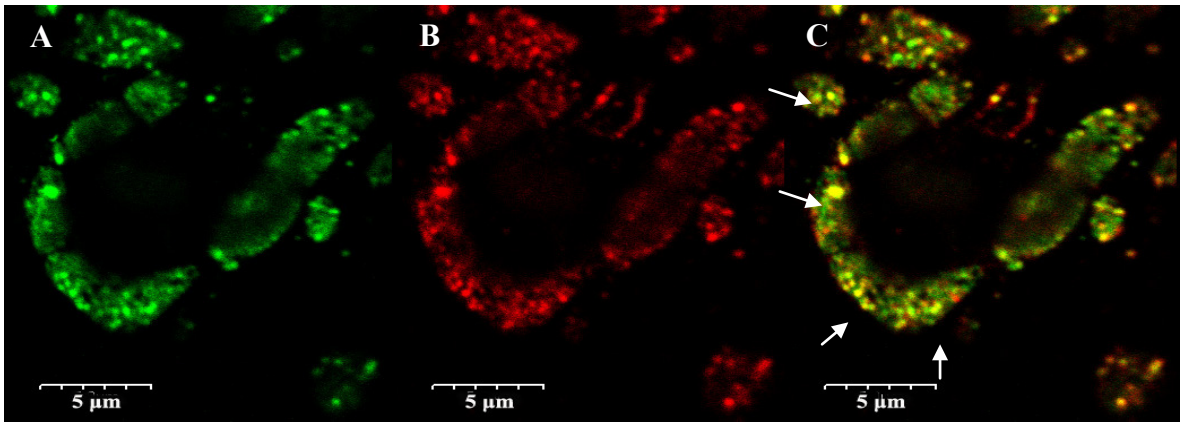

Figure 46. Immunocytochemical localisation of the mutant CBHI and 20S proteasome particles in *T. reesei* CVtΔ4 hyphae. A. CBHI; B. 20S proteasome particles; C. Co-localisation in the merged images (yellow spots pointed by white arrows).

One explanation why misfolded proteins are retained in the ER is that they are immobilised through an extensive interaction with foldases and thus unable to move on to ER exit sites (Lippincott-Schwartz *et al*., 2000). Such accumulation of misfolded protein in the ER can be seen in Fig. 46C. Also, studies by Godlewski *et al*. (unpublish data) have shown co□ localisation of the mutant CBHI and ER in strain CVtΔ4. Indications of accumulation of the mutant CBHI in strain CVtΔ4 were also suggested by the microarray results showing activation of the gene encoding Lhs1p, an Hsp family protein, which together with Bip, Sec63p and the nucleotide exchange factor Sil1p, play a key role in protein import into the ER and in proper protein folding in the ER lumen.

Shown in Figure 47 are the immunolabelled CBHI and 20S proteasome particles in strain CVtΔ5 expressing CBHI with five mutations. In this strain, CBHI was distributed all over the cytoplasm (Fig. 47A) and showed less intensive labelling compared to all other strains. The label also appeared to be more uniform throughout the cytoplasm and no obvious concentrations were apparent. The proteasome label was associated with the cell envelope and accumulation of the label can be seen at the apical regions especially in young hyphae (Fig. 47B, white arrows). Similarly to the mutant strain CVtΔ2, no obvious co-localisation of the CBHI and 20S proteasome labels was observed (Fig. 47C).

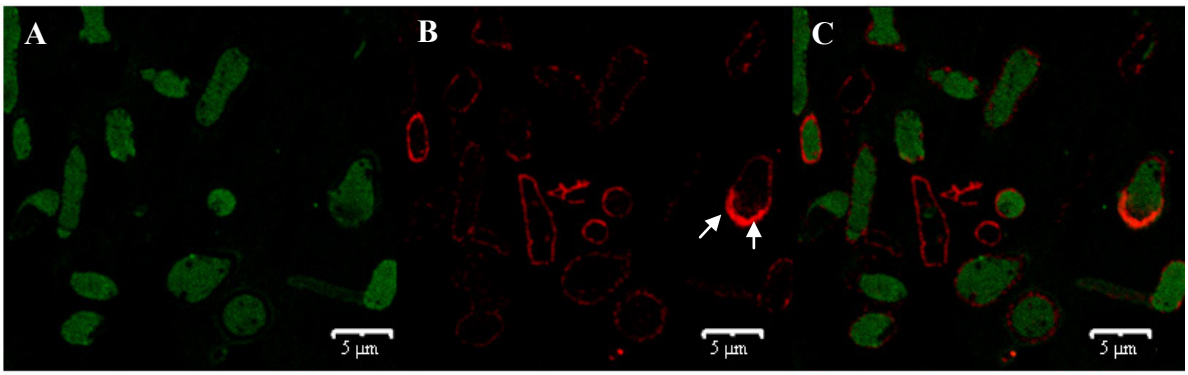

Figure 47. Immunocytochemical localisation of the mutant CBHI and 20S proteasome particles in *T. reesei* CVtΔ5 hyphae. A. CBHI; B. 20S proteasome particles; C. Co-localisation in the merged images

In conclusion, indirect immunolabelling showed that the co-localisation of mutant CBHI protein and 20S proteasome particles was most obvious in strain CVtΔ4, where they seemed to aggregate on the ER membrane. The microarray data supported this view showing up-regulation of genes encoding several ER-resident foldases and chaperones, e.g. Bip, PDI, PPI, calnexin and the Hsp-like protein LHS at 48 h, the same time point that at which the above observations were made. It seems probable that CBHI carrying four mutations was at least partially refolded in the ER, and progressed further in the secretory pathway as CBHI was seen secreted into the culture medium (Section 3.3.2). However, the aggregation may have affected the overall cellulase activity measured in CVtΔ4 culture supernatant which was five times lower than in CVtΔ5.

Strain CVtΔ2 with two mutations in CBHI showed rather similar distribution of both the CBHI and proteasome labels to CVt expressing a non-mutant CBHI and Rut-C30, a non□ transformant strain with an intact CBHI. There was no obvious co-localisation between the two labels in CVtΔ2 or CVtΔ5. CBHI from all mutant strains was secreted into the external growth medium later than in Rut-C30. Therefore it seems that progression of the mutant CBHI in the secretory pathway had been delayed and the enzyme may be trapped inside the cell for some time. Strain CVtΔ5 carrying five mutations in the *cbh1* gene did not show as dense and granular label of CBHI as the other four strains, probably because most of the mutant CBHI had already been degraded by the proteasome at 12 h, as indicated by the transcription analysis which showed the activation of the ERAD pathway (Section 3.5.2 and Discussion p. 168-169).

To confirm the co-localisation of the 20S proteasome with the mutant CBHI in the hyphae of CVtΔ4, immunolabelling of ultrathin sections cut from Rut-C30, CVt and CVtΔ4 hyphae was performed and samples were analysed by TEM.

3.7.3 Interaction and subcellular localisation of CBHI and 20S proteasome particles under transmission electron microscopy

A more detailed localisation of the 20S proteasome particles and the CBHI protein with four mutations was carried out by immunolabelling of ultrathin 0.5 µm sections cut out from hyphae of *T. reesei* Rut-C30, CVt and CVtΔ4 strains cultured in the CLS medium for two days. Double immunolabelling of the sections placed on Carbon-Formvar coated 200 mesh nickel grids was performed using monoclonal CBHI-antibody (CI-261, Roal Ltd, Finland) and polyclonal 20S proteasome antibody (PW9355, Biomol) as primary antibodies and IgG conjugated 10 nm (CBHI) and 5 nm (proteasome) gold particles (Molecular probes) as secondary antibodies. Gold-labelled sections were inspected and photographed under a transmission electron microscope (Hitachi -17000 EM; Fig. 48). The amount of gold particles reflecting the amount of the CBHI protein and 20S proteasome particles were calculated based on the size of the particles using the Cell^P program. The size variation in gold particles was set based on the manufacturer s instructions. The distribution of the gold particles in the hyphae was also determined as described in section 2.4.4.

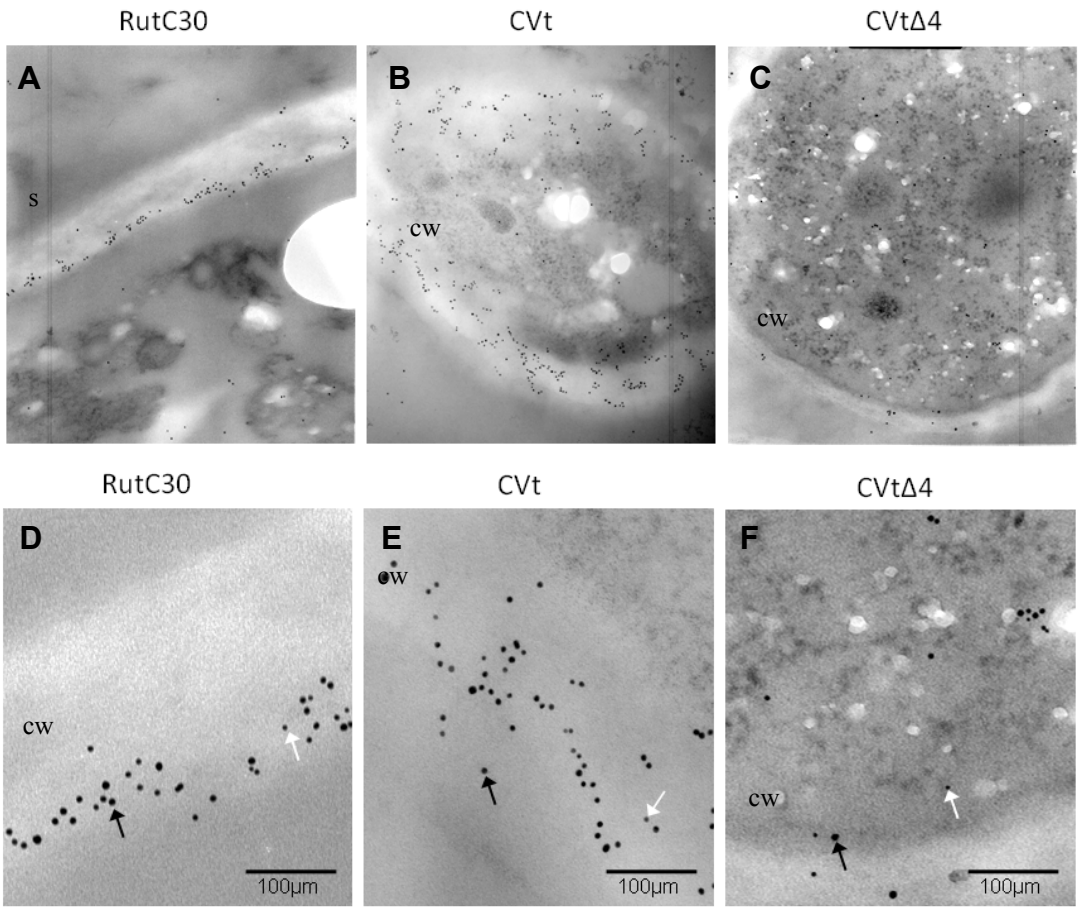

Figure 48. TEM images of ultrathin sections of *T. reesei* hyphae. Pictures in the upper row represent an overview of the immunolabelled hyphae of each strain: A. Rut-C30, longitudinal section of septum area; B. CVt, vertical section of a hyphal tip and C. CVtΔ4, vertical section of the middle of a hypha. Pictures in the lower row represent a closer view of each strain. D. Rut-C30, vertical section of cell envelope region; E. CVt, vertical section of cell envelope region; F. CVtΔ4 vertical section of cell envelope region. Sections were embedded in White resin and double immunolabelled with yeast anti-20S pAb with anti-IgG-5 nm gold particles (white arrow) and mAb anti CBHI with anti IgG- 10 nm gold particles (black arrow). S = septum; cw = cell wall; Bar = 100 µm.

Figure 48 shows a selected sample of all the images obtained. Consistent with the previous observations from the fluorescence microscopy studies (section 3.7.1), the 20S proteasome was immunologically detected in the cell envelope regions and hyphal tips (Fig. 45 B), and CBHI was located at the periphery of the cytoplasm in strains Rut-C30 and CVt (Fig. 45A and B).

It has been established earlier that the default route of a protein secreted from a fungal hyphae is through the growing tip with some secretion occurring at older parts of the hyphal wall. This notion has been supported by Sprey (1988) in immunoelectron

microscopy studies which showed that endogenous endoglucanases were localised in the hyphal tips and hyphal wall of *T. reesei*. When the recombinant EPB (Barley cysteine endopeptidase B) was co-expressed with the endogenous CBHI in *T. reesei,* the EPB protein was also localised throughout the mycelium, suggesting that the secretion may not be restricted to the hyphal tips (Nykänen *et al*., 1997). In studies with fusion proteins, *Aspergillus* glucoamylase fused with EGFP (enhanced green fluorescent protein) seemed to have localised to the septal region suggesting targeting of the protein to the septa in addition to hyphal envelope (Gordon *et al*., 2000; Masai *et al*., 2003). In the current study, septal localisation of CBHI was shown in the non-transformant Rut-C30 strain (Fig. 45A).

The appearance of the 20S proteasome label in the cell envelope region (Fig 48D-F) was expected, because the ER has been shown to be located near the plasma membrane, and the 26S proteasome interacts with integral and ER membrane bound proteins during the process of protein ubiquitination and degradation (Schuberth and Buchberger, 2005; Shoji *et al*., 2008).

In the strain CVtΔ4 the proteasome label was observed in the periphery of the cytoplasm (Fig. 48C). In CVtΔ4 relatively less immunolabelling of CBHI occurred at the cell envelope (Fig. 48F) when compared to the two other strains studied. Proteasome label attached to the CBHI label was seen in clusters more often in the cytoplasm in strain CVtΔ4 (Fig. 48C and 48F) when compared to Rut-C30 (Fig. 48A and D) and CVt (Fig. 48B and 48E), indicating co-localisation of the mutant CBHI by the proteasome.

The quantitation data based on the calculation of the gold particles from images obtained by TEM indicated that a lower amount of mutant CBHI was produced in CVtΔ4 since approximately half of the amount of CBHI label was detected in CVtΔ4 compared to the label contained in Rut-C30 and CVt (Table 13). Furthermore, the quantity of the 20S proteasome label was higher in CVtΔ4 compared to CVt (68 %) and Rut-C30 (26 %) (Table 13); this was probably due to an increased need for protein eradication in the CBHI mutant strain.

Table 13. Quantitation of gold particles. Average amount of particles in each image and ratio of 5 nm versus 10 nm gold particles in thin sections of *T. reesei* Rut-C30, CVt and CVtΔ4 hyphae grown for 2 days in the CLS medium. a, $p<0.01$ CVtΔ4 vs. Rut-C30; b, $p<0.05$ CVtΔ4 vs. Rut-C30; c, $p<0.01$ CVtΔ4 vs. CVt. SD, standard deviation.

	Rut-C30		*CVt*		*CVtΔ4*	
	5 nm	10 nm	5 nm	10 nm	5 nm	10 nm
Average per image	2.00	6.79	5.25	7.28	7.69 a	2.54 b,c
SD	2.06	12.11	5.05	5.94	5.95	2.22
Ratio	0.29		0.72		3.03	

In summary the distribution of the gold particles in the indirect immunolabelling of CBHI and the 20S proteasome was different in the mutant strain CVtΔ4 compared to the non-mutant CVt and the non-transformant Rut-C30. The ratio of the proteasome to CBHI label was considerably higher in CVtΔ4 and the two labels showed co-localisation. Difference in the amount of proteasome label between CVtΔ4 and Rut-C30 was significant ($p<0.05$) and the difference in the amount of CBHI label between CVtΔ4 and Rut-C30 was highly significant ($p<0.01$). Also, there was a highly significant difference in the amount of CBHI label in CVtΔ4 compared to CVt. These findings would indirectly point to degradation of the mutant CBHI by the proteasome.

The majority of the label hybridised to the non-mutant CBHI in the Rut-C30 and CVt strains was localised on the cell envelope which points to the possibility that the protein was on its way out of the hyphae. Localisation of the proteasome in the cell envelope could provide the final point of quality control before protein externalisation.

The findings from the fluorescence microscopy and immunoelectron microscopy are in strong agreement with each other and support the results obtained from the microarray analysis. All data provide support to the view that degradation of the mutant CBHI produced in CVtΔ4 occurred at the fungal proteasome. This may also be the case with CVtΔ5, which, however, was not subjected to the TEM analysis.

4 General discussion and main findings

Trichoderma reesei has a naturally high capacity for protein secretion and is currently employed for industrial production of a range of enzymes and recombinant gene products for a variety of biotechnological applications. A major impediment for the use of *T. reesei* as a universal production host is its limited capacity to secrete heterologous proteins. One reason for the low secretion yields of heterologous proteins is their improper folding and consequent elimination from the cell by the protein quality control mechanisms mediated by the unfolded protein response (UPR; Punt *et al*., 1998; Yoder and Lehmbeck, 2004) and ER-associated degradation (ERAD).

In order to explore the effect of expression of misfolded proteins, three different mutant strains of *T. reesei* carrying the *cbh1* gene with two, four and five mutations were constructed. Cellobiohydrolase I (CBHI) is the major cellulase in *T. reesei* and comprises 60 % of the total secreted protein. The response of *T. reesei* to the expression of the mutant CBHI forms was analysed at the physiological, transcriptional and protein secretion levels with a particular view into the role of the proteasome in the protein quality control in *T. reesei*.

4.1 Purification of the *Trichoderma reesei* proteasome

A new and rapid purification method for the *T. reesei* proteasome was developed during this study reducing the purification time from 5 to 2.5 days compared to the traditional method used earlier. The new method also facilitated effective purification of the entire 26S proteasome, which could be seen in the TEM images together with the 20S and 19S particles (section 3.1.3).

All the 20S proteasome subunits and four of the 19S proteasome subunits were identified by mass spectrometry, cross species identification (CSI) or through *T. reesei* database searches. A potential reason for not detecting the rest of the 19S subunits on the 2D-display

was that they were hidden under other spots due to a similar molecular weight or pI. One way to solve this problem would be a direct mass spectrometry approach such as LC□ MS/MS without 2D-gel electrophoresis.

Proteasome purification was carried out with the Poros HQ column which has a high protein binding capacity allowing the purification of the entire 26S complex as indicated by electron microscopy. However, at least some part of the 26S proteasome appeared to have dissociated during purification. Dissociation of the 20S core particle from the 19S regulatory particle may have been due to dilution of the sample concentration carried out to make it suitable for TEM (Cascio *et al*., 2002). This may have resulted in a decrease of the ATP concentration necessary to keep the complex intact.

There may also be additional factors that affect the association between the 20S particle and the 19S particle *in vivo*. For example, it has been proposed that the 19S regulatory particle mediates the gating and translocation of the substrate into the central proteolytic chamber, and most likely associates with the 20S particle in an ATP dependent manner just after recognition of the target substrate making the 20S complex ready for the degradation process. This association is probably very quick and dissociation would occur rapidly after degradation of the substrate. The 19S proteasome subunits have also been found to play a role in a range of other cellular processes without partnering with the 20S particle (Ferdous *et al*., 2005). Therefore, purification of the intact proteasome, when it has been taken out from its natural environment, is very challenging. In support of the above, our results showed a lower abundance of the 26S proteasome compared to 20S particles in the fungal hyphae. It has also been shown that the molar ratio of 20S and 26S particles in human erythrocytes was 11.5 (Majetshak and Sorell, 2008).

There is also a possibility that at least part of the 19S particle was dissociated as a result of preparation and lysis of the fungal hyphae. Purification of the proteasome was followed by monitoring the fractions by measuring chymotrypsin-like activity associated with the β3□ subunit. Therefore, a portion of dissociated 19S subunits present in non-active fractions could have been discarded before 2D-analysis.

Due to reasons discussed above, native PAGE would propably be a better way to analyse the quantity of the 20S and 26S proteasomes than TEM.

Proteasome interacting proteins (PIPs)

Verma *et al*. (2000) found 52 PIPs using direct analysis of large protein complexes by mass spectrometry, which enabled rapid compositional analysis of complex protein mixtures. Many PIPs co-purified with the 26S proteasome in this study were similar to those found by Verma *et al*. (2000). Among them were chaperones such as Hsp70 and Hsp90, which are known to participate in refolding of aberrant proteins in the ER and in protein translocation into organelles (Frydman and Hartl, 1994). Furthermore, this study indicated association of the 26S proteasome with a large number of proteins involved in transcription/translation which suggests that the proteasome performs multiple tasks in the cell (Murata and Shimotohno, 2006). Many PIP proteins associated with the 26S proteasome also have a role in the ERAD and UPR processes. For example, the negative regulator of UPR, serine/threonine phosphatase, and CDC48 (Bays and Hampton, 2002) and BiP (Wang and Johnsson, 2005) also participate in the translocation of misfolded proteins.

The availability of a species-specific database for peptide mass fingerprinting (PMF) searching greatly improved the number of protein identifications from the purified proteasome from 41 % (by CSI) to 66 %.

4.2 Three different mutant versions of CBHIs caused physiological changes and secretion stress in *Trichoderma reesei*

The well established fact that ER-stress induces the unfolded protein response (UPR) inspired the idea to design a misfolded secreted endogenous protein, here the main cellobiohydrolase CBHI, to study cellular effects of its expression in *T. reesei*. Expression of a mutant version of a native highly expressed and efficiently secreted protein would present a more natural means to explore stress in the ER. Previously secretion stress has been introduced by treating fungal cultures with drugs such as DTT or tunicamycin.

Mutated CBHIs

Three different mutant strains carrying a mutant *cbh1* core gene were generated with the view of making a misfolded CBHI protein. The strategy seemed effective in causing stress, based on various changes in transcription and protein expression recorded for each CBHI mutant strain. The effects were evident even though not all the cysteines substituted by proline residues were in a sheet, which is known to be the most important secondary structural element concerning protein folding (personal information, B. Mabbutt, Macquarie University, Sydney, Australia).

All CBHI mutant strains were derived from the stain CVt containing the intact CBHI core linked with the Venus fluorescent protein (section 2.2.1). Fusion of the *cbh1* core gene to DNA encoding the fluorescent protein Venus was originally performed for tracking the secretion of the CBHI protein throughout the fungal hyphae (Yu *et al*., unpublished) and to use the constructs in FRET studies to explore interaction of the mutant CBHI with the fungal proteasome. Importantly, it was established that the heterologous fusion partner Venus did not cause secretion stress at any of the time points used in the microarray analysis when expressed as a fusion to a non-mutant CBHI (section 3.5.1).

Stable homologous integration of the whole expression cassette containing a mutated *cbh1* core gene seemed to have occurred successfully in strains CVtΔ4 and CVt only, as evidenced by the Northern analysis (section 3.3). Even though it was evident that the strains CVtΔ2 and CVtΔ5 also contained one copy of the entire expression cassette integrated into the *cbh1* locus as shown by PCR and Southern blotting, they seem to have lost the *venus* gene as shown by Northern analysis carried out two years later and confocal microscopy (Fig 27., p.110).

A possible explanation for findings discussed above could be that the *venus* gene had been spliced out of the genome at some point during the repeated culturing on a PDA medium without hygromycin B selection. It was confirmed later that the strain CVtΔ5 was not growing on hygromycin plates anymore indicating the at least the *hph* marker had been lost.

Loss of a gene integrated into the *cbh1* locus could be a locus specific phenomenon reported to have occurred in fungi in some earlier studies but not recorded in publications (personal information, T. Pakula, VTT Biotechnology, Finland). Sequencing of the genomic DNA in the affected area, now in progress, will confirm the mutations and the integration of the transforming DNA into the *cbh1* locus.

Cellulase activity

When the cellobiohydrolase activity was measured from the culture supernatants, large differences were observed between all strains studied. A higher cellobiohydrolase activity in strain CVtΔ5, compared to CVtΔ4 and CVtΔ2. Peptide mapping and nuclear magnetic resonance (NMR) spectroscopy analyses on purified CBHI mutant proteins could confirm that the native disulfide pairings were destroyed, which presumably also accounted for the mutant CBHIs lack of activity, especially in CVtΔ4. We did not have the opportunity to perform NMR spectroscopy during this study, but had to rely on the theoretical deduction on how to interfere with the formation of disulfide bridges based on the location of the disulfide bridges according to the published CBHI crystal structure (Divne *et al*., 1994).

Physiological stress

The number of mutations in the *cbh1* core gene was found to have a significant effect on secretion of the mutant CBHI forms produced in *T. reesei*. The mutation in the *cbh1* gene seemed to have caused stress in *T. reesei* based on physiological changes resembling starvation stress after 36 h of cultivation. These changes were observed in all mutant strains but not in CVt expressing a non-mutant CBHI or the non-transformant Rut-C30.

Microarray results provided further evidence for physiological stress (section 3.5.3 and 3.5.4). This physiological stress was reflected by expression of genes encoding the synthesis of cell membrane components, such as glucan and chitin which are the main components of the fungal cell wall (see supplementary data, Tables 1, 2, and 3). Genes encoding several proteins involved in cell wall proliferation and growth, such as α-tubulin, chitin synthetase, cell division control protein, hydrophobin I and II were also affected.

The cell wall is a highly dynamic structure and has been shown to adapt to various developmental or environmental changes. In fungi, the cell wall provides structural support, mediates cell-cell interactions and serves as a filter for controlling protein secretion and uptake of molecules. It is also required to resist the external and internal pressure to prevent cell lysis and protect the cell from potentially damaging enzymes in the environment (Adams, 2004).

Transcription and protein secretion

The mutations introduced into the *cbh1* gene resulted in a reduction of the rate of both mRNA transcription and production of CBHI in the mutant strains when compared to the non-mutant strain CVt. The overall expression of CBHI mRNA increased with time in each mutant strain (Chapter 3.3, Fig.26). There is a mechanism, not yet fully characterised, in which the presence of abnormal or damaged DNA bases usually halts RNA transcription until the "wrong" base has been excised and the DNA becomes repaired by special enzymes in the cell (Viswanathan *et al*., 1999). In the current study, the microarray results showed altered expression of genes coding for DNA repair related proteins and enzymes such as topoisomerases and helicases in strain CVtΔ2 and CVtΔ5 at early time points.

Even though the CBHI protein was tagged with Venus in all mutant strains and the non-mutant CVt, only CVt and CVtΔ4 were able to secrete the fluorescent protein Venus into the culture supernatant (reasons discussed above). In the other two mutant strains, CVtΔ2 and CVtΔ5, the Venus protein was not detected inside the mycelia at 3d, neither was it secreted into the culture medium (p. 107, 112). It also appeared that the *venus* gene was not transcribed in these mutants.

Pulsing in protein secretion

A novel and intriguing finding in this study was that protein secretion seemed to be "pulsing" in 22-24 h cycles. When total protein secretion was followed over seven days, the "pulsing" was observed in all strains. Transcription of the CBHI mRNA also showed a similar "pulsing" pattern, although this was determined only up to 48 h (Section 3.3.2). These findings are supported by other research into protein secretion carried out in our laboratory (Godlewski *et al*., manuscript in preparation).

4.3 Not all misfolded CBHIs induced secretion stress at the transcriptional level

This is the first reported genome-wide investigation into the effects of expression of an endogenous mutant protein in *T. reesei*. The *T. reesei* genome has been recently sequenced for the second time (Martinez *et al*., 2008). Even though the database is continually being corrected for sequence information, splicing and other fundamental features, there are still challenges to be met in relation to protein identification.

The genome wide microarray data was produced with two different sets of genes. The first set (Set1) included genes related to the UPR and ERAD pathways and were spotted as four to eight replicates on the slides. The results from these data which also contained dye swap replicate slides had good reproducibility. The second set of genes (Set2) were spotted only once or twice on the slide, and therefore the results from this data set can be only taken as a general guide, even though dye swap replicates as a technical replicates were performed. Statistically relevant data would further require biological replicates to determine the reproducibility of data, which was missing from our study. However, there were other measures to add confidence in the approach and results obtained as discussed above and in section 3.5.

The microarray experiment was carried out using CustomArray slides from CombiMatrix, USA. These slides were re-useable at least three times and allowed dual labelling of the samples with two different Cy-dyes, Cy3 and Cy5. A problem accounted with dual labelling is that the dyes are not equally strong which may cause problems in the interpretation of the results (gene expression versus dye effect). This problem can be avoided when the signal from the channels detecting the two Cy-dyes is normalised to correct the dye bias (Martin-Magniette *et al*., 2008).

Of all the differentially expressed genes identified in this work, 23-50 % had no match to known genes or proteins, or hypothetical genes or proteins. Predictions of function, based on the genome sequence analysis, will become more and more accurate as the number and

diversity of sequenced fungal genomes increases. The predicted functions of some fungal sequences may, however, remain unknown, because some classes of genes in filamentous fungi are almost absent from other organisms. Integration of additional datasets from various microarrays, proteomic and metabolomic studies will facilitate further identification of gene functions.

This study has been performed to fulfil the requirements of "minimum information about a microarray experiment" (MIAME; Brazma *et al*., 2001). Information in the supplementary data has been presented in a simplified format for easier reading. For publication, all data will be submitted in Arrayexpress (www.ebi.ac.uk/microarray-as/ae/).

The microarray analysis showed that the addition of the heterologous protein Venus did not cause any stress to the strain in the culture conditions used when compared to the non-transformant Rut-C30. Therefore the loss of Venus in strains CVtΔ2 and CVtΔ5, discussed in section 3.3 did not cause differences in gene expression related to UPR stress and thus CVt could be used as a control for them.

Expression of genes related to UPR and ERAD

Yeast cells have been shown to respond to ER-stress by transcriptional up-regulation of genes involved in ER-associated processes, such as translocation, *N*-glycosylation, ERAD and anterograde vesicle transport (Jørgensen 2009). In this study, from the known ER-stress-induced chaperones, BiP, PDI and PPI were found to be affected in strain CVtΔ4, which showed ER stress and activation of UPR at 48 h. From the genes related to translocation of the misfolded proteins to degradation by the proteasome, genes encoding Sec61 and Der1 were also up-regulated in strain CVtΔ4 exhibiting considerable physiological stress (section 3.5.3).

The strain CVtΔ5 showed transcriptional activation of the ER-associated degradation pathway at 48 h by showing up-regulation of genes encoding the β6 subunit of the 20S proteasome and proteasome maturation protein Ump1. Although there were no signs of up-regulation of ER-stress chaperones related to UPR stress in this strain at 12 h, some genes

encoding the 19S regulatory particle of the 26S proteasome were up-regulated indicating activation of the ERAD pathway at this early time point.

The calnexin encoding gene was up-regulated in strains CVtΔ4 and CVtΔ5 at 48 h. Calnexin is an ER resident integral membrane chaperone protein that plays an important role in the biogenesis and quality control of glycoproteins (Ellgaard *et al*., 1999). Calnexin specifically interacts with glycan moieties of the glycoproteins and associates transiently with newly synthesised glycoproteins until they fold properly. If proteins do not fold correctly, the interaction between calnexin and the misfolded protein is prolonged, leading to its retention in the ER (Dickson *et al*., 2002). It is possible, that the mutant CBHI with several incomplete disulfide bridges, expressed in CVtΔ4 and CVtΔ5, caused the protein to spend a longer time in the refolding cycle. Cyclophilin is known to accelerate protein folding (Matouschek *et al*., 1995) and expression of this gene was up-regulated in CVtΔ5 with five mutations in the *cbh1* gene indicating prolonged retention of the mutant protein in the ER.

A clear connection has been established between UPR and ERAD by earlier studies carried out with yeast (Travers *et al*., 2000). In the current study, the connection between UPR and ERAD was apparent in strain CVtΔ4; the accustomary UPR signals were evident at 48 h, along with induced expression of the ubiquitin activating enzyme E1, indicating the need to engage with the ERAD pathway. In contrast to others, in the strain CVtΔ5, ERAD activation was evident but UPR seemed not to be activated. It may also be possible that activation of the UPR in strain CVtΔ5 had already occurred before 12 h (first time point for sample collection) and was not detected here.

Genes related to ribosome structure and biogenesis

Many of the earlier studies into the UPR in *T. reesei* have been performed by treating the cultures with chemical agents such as DTT, brefeldin A, A23187 or tunicamycin which interfere either with protein folding or transport (Pakula *et al*., 2003; Schawalder *et al*., 2004; Sims *et al*., 2005).

In this study, secretion stress was applied to the hyphae by expression of a misfolded endogenous protein. Based on our findings discussed below, it seems that the feedback from the secretory pathway to the translation machinery is different in a drug induced UPR compared to UPR induced by a misfolded protein. In our study, all genes related to "ribosome structure and biogenesis" were up-regulated throughout all time points and all mutant CBHI strains when compared to a non-mutant CBHI strain CVt. Differently to our findings, in *A. nidulans* (Sims *et al*., 2005) and *A. niger* (Guillemette *et al*., 2007), the corresponding genes were down-regulated when UPR was induced by chemicals. In studies with *S. cerevisiae,* 16 ribosomal genes showed a down-regulation response to DTT treatment (Payne *et al*., 2008).

The use of chemicals such as DTT to induce UPR may lead to a strong block in the secretory pathway to which the cell may respond by slowing down protein translation to avoid further stress. For example, exposure of cells to DTT will affect all disulfide bonds coming into contact with the drug. Differently to this, hyphae expressing individual misfolded proteins such as mutant CBHI might up-regulate the protein translation machinery to compensate for the loss of misfolded proteins destined to degradation by ERAD. Up-regulation of the expression of genes involved in "ribosome structure and synthesis" could form part of this response.

HAC1 expression

UPR signalling in filamentous fungi has been found to be rather similar to that in yeast. However, there are some differences; the ER transmembrane protein IRE1 induces a different type of splicing and truncation of a HAC1 mRNA in filamentous fungi compared to yeast (Valkonen *et al*., 2004). Surprisingly, neither IRE1 nor HAC1 encoding genes were shown to be affected in this study in any of the strains or any time point. This may indeed be the case or it could also be possible that the expression of *ire1* and *hac1* genes occurred earlier than the time of collection of the first samples in this study. This view is supported by the fact that in a previous study HAC expression was shown to increase in *T. reesei* Rut-C30 by four fold after 1 h of DTT addition to the cultures and then decrease at 3h which was the last sample collection point studied by Pakula *et al*. (2003).

RESS

Down-regulation of genes encoding secreted proteins protecting the hyphae from the ER stress (called RESS, Pakula *et al*., 2003) was observed at an early time point in all strains carrying a mutant version of *cbh1* core gene which is in accordance with earlier studies.

Autophagy genes

Fujita *et al*. (2007) reported about another ERAD pathway in addition to the conventional ubiquitin-proteasome pathway in human cells. The study showed that mutant dysferin, aggregated in the ER and stimulated autophagosome formation to engulf the mutated protein. This occurred via activation of ER stress-eIF2α phosphorylation pathway triggered autophagy/lysosome degradation, ERAD (II), while the non-mutant dysferin was demonstrated to be a substrate for ubiquitin/proteasome ERAD (I). A similar ER stress-eIF2α phosphorylation pathway has not yet been found in fungal cells. However, autophagy has been shown to function as a protective measure against cell death and have significant effects on cellular growth and development. Putative autophagy proteins characterised in filamentous fungi so far are Atg1 and Atg8 (Pollack *et al*., 2009). Interestingly, two genes coding for autophagy proteins, autophagy protein Atg13 and autophagy related protein 18, were down-regulated in the current study in strain CVtΔ5 at 24 h, contributing to the emerging knowledge on this group of genes and their function in filamentous fungi.

Proteasome inhibition

Addition of the proteasome inhibitor MG132 to the *T. reesei* cultures for 2h after 46h cultivation showed that the proteasome activity could be efficiently inhibited. This treatment affected mainly expression of the proteasome genes. The inhibition effect was stronger in the strain CVtΔ4 already showing secretion stress (section 3.6.1). Interestingly, the inhibition of proteasome activity lead to strong expression changes in the proteasome genes in a non-transformant strain Rut-C30, but not in CVt, which was carrying a non-mutant *cbh1* gene tagged with *venus*. Rut-C30 is a protease deficient, high cellulase-secreting strain (Montenecourt and Eveleigh, 1979). Up-regulation of the proteasome genes in the Rut-C30 strain may relate to the fact that this strain produces secreted proteins faster than the CBHI mutant strains and CVt, and would therefore require an active proteasome as

part of normal cell growth and development. Consequently, blocking of the proteasome would cause considerable adjustments in gene expression.

Overall summary of gene regulation changes as indicated by the microarrays

Several important features of the UPR signalling pathway and ER-associated degradation pathway were initially revealed through the studies into the production of misfolded CBHI in *T. reesei*. Direct comparison of down-regulated or up-regulated genes between the strains could not been carried out, because of different timing in growth, transcription, translation and protein production. The timing diffrences were reflected by regulation changes in genes related to "ribosome structure and biosynthesis", "transcription", "translation", and also "metabolic pathways". Overall, the most significant gene regulation changes were observed in strain CVtΔ5 with five mutations in the CBHI gene.

The finding that the cyclophilin (CVtΔ4) and the calnexin-gene (CVtΔ4 and CVtΔ5) were up-regulated in the CBHI mutant strains indicated that these proteins were retained in the refolding cycle from where they may have been directed to either the proteasome or secreted outside the hyphae. It is worth noting that the amount of total secreted protein and total cellulose activity in CVtΔ4 was considerably lower than in other strains; this strain also exhibited up-regulation of the UPR-related genes. The strain CVtΔ5 seemed to have responded by mainly up-regulating the genes involved in the ERAD pathway.

Chemical inhibition of the proteasome caused up-regulation of the genes encoding 20S and 19S subunits in the non-transorfmant Rut-C30 indicating that in principle, this blocking strategy was effective in a *T. reesei* strain exhibiting vigorous growth and producing and secreting a non-mutnat CBHI enzyme.

4.4 Misfolded CBHIs under microscopy

Understanding of the "mechanics" of the fungal secretion pathway has increased significantly in recent years. There is a general agreement that secretion of extracellular enzymes into the external medium occurs mainly from the hyphal tips (Gordon *et al*., 2000)

and subapical regions (Nykänen *et al*., 1997; Gupta and Heath, 2000; Valkonen *et al*., 2007). However, there are only a few studies that have localised the actual subcellular sites of protein secretion (Kuratsu *et al*., 2007). In this study, we have concentrated on establishing the proposed interaction of the mutant form of cellobiohydrolase I enzyme and the proteasome in *T. reesei*.

Confocal microscopy

To date there are no published studies on subcellular visualisation of the proteasome in fungal hyphae or co-localisation of the ER with the proteasome. Here we have shown a strong concentration of proteasomes at the growing tips of young hyphae and the wall of a germinating conidium in the non-transformant Rut-C30 (section 3.7.1). After two days of growth, the proteasome was also detected in the cytoplasm indicating that its cellular distribution is dynamic and changes according to the growth phase of the fungus.

The fluorescently labelled CBHI seemed to localise mainly in the cytoplasm in all mutant strains and the non-transformant Rut-C30 after 48 h cultivation; at the same time, the 20S proteasome was mainly localised in the cell envelope in the hyphal sections.

Similarly to the studies of Marquez and Helenius (1992), who showed that misfolded proteins tended to accumulate in the ER in mammalian cells, the CBHI label in the mutant strain CVtΔ2 showed accumulation in the cytoplasm and globular aggregation in CVtΔ4 carrying four mutations in the *cbh1* gene. Co-localisation of the proteasome with CBHI was clearly seen in the hyphae of the mutant CVtΔ4, indicating interaction and possible degradation of the misfolded CBHI by the proteasome. In another related study, there was a clear co-localisation of the mutant CBHI and ER in CVtΔ4 (Godlewski *et al*., unpublished) which provides further evidence that the mutant CBHI, ER and the proteasome can all be found in the same subcellular location in CVtΔ4.

In CVtΔ5 with five mutations in the *cbh1* gene, the mutant CBHI looked fuzzy and was dispersed inside the hyphae with no co-localisation with the proteasome (Fig. 47). This suggests that only part of the mutant CBHI in this strain and time point was degraded by the proteasome; alternatively, the protein may have escaped from ERAD or there may be

other mechanisms for protein degradation (e.g. lysosomes and other cytosolic and organellar proteases) active at different times of the development. Organisms have been shown to favour or choose a different degradation pathway depending on culture conditions and degree of stress (Fuertes *et al*., 2003; Chiechanover, 2005; Fujita *et al*., 2007). It is also possible that refolding of the mutant CBHI was successful and the protein was released to be secreted into the culture medium.

TEM studies

TEM studies were performed with the non-transformant Rut-C30, non-mutant CVt and the CVtΔ4 strain in which the mutant CBHI protein showed strong aggregation inside the hyphae. In the detailed view, the CBHI label was seen both in the cytoplasm and the cell envelope and the proteasome was mainly observed in the cell envelope in Rut-C30 and CVt. In CVtΔ4, the amount of the proteasome label was lower and was seen together with the CBHI label in the cytoplasm. The amount of both labels was confirmed by counting the different-sized gold particles (Table 13).

Immunogold-labelled CBHI detected in the hyphal envelope and in the hyphal tip in the non-transformant strain Rut-C30 and non-mutant strain CVt indicated that the CBHI protein was on its way out of the hyphae. However, in CVtΔ4, while some CBHI label was seen on the cell envelope, most of it seemed to have been retained in the cytoplasm together with the proteasome label. This provides strong evidence for the fact that most of the mutant CBHI was maintained in the cell and most probably degraded by the proteasome in this particular transformant. It is worth noticing that in the microarray analysis, the genes related to the ERAD pathway and some translocons were up-regulated in CVtΔ4, providing further support to the TEM findings and emphasising the acute stress the strain was under.

4.5 Conclusions and future work

The proteasome was successfully purified and characterised from *T. reesei* during this study. A new method was developed allowing rapid purification of the entire 26S proteasome particle (Grinyer *et al*., 2007; Kautto *et al*., 2009).

The *T. reesei* proteasome seemed to have a very similar structure to proteasomes isolated from other eukaryotic cells. This study also revealed many proteasome interacting proteins (PIPs) and that the proteasome probably has multiple active roles in the cell beyond just the protein quality control. The microarray analysis showed that the protein quality control mechanism recognised the misfolded CBHI molecules and activated their degradation process mediated by the proteasome.

Previous attempts to identify genes related to the protein secretion pathway and recombinant protein secretion in filamentous fungi have featured induction of the UPR and inhibition of protein folding by using different drugs such as DTT (Pakula *et al.,* 2003), brefeldin A (Khalaj *et al*., 2001, Saloheimo *et al*., 2004) and tunicamycin (Mulder *et al*., 2004). Differently to these approaches, the strategy applied in this work featured expression of a misfolded endogenous secreted protein. Cellular effects of the expression and secretion of the mutant CBHI protein supported previous studies in that improper folding is a big problem for the cell and could well be the main reason to cause a bottleneck in the secretion of heterologous proteins from the fungal hyphae.

Transcription and production of the mutant CBHI protein seemed to be influenced by the number of amino acid replacements in the *cbh1* core gene; for example, multiple mutations in the *cbh1* gene prolonged the transcription time and *cbh1* specific mRNA was detected earlier in CVtΔ2 than in CVtΔ4.

In the microarray analysis UPR was not detected in CVtΔ2 with two mutations in the *cbh1* core gene at any of the time points included in the array, but there were physiological changes and some transcriptional signals of stress in this strain. Two additional cysteine substitutions in the *cbh1* core gene in CVtΔ4 caused significant activation of the UPR,

indicating that this strain had difficulties in folding CBHI correctly. In CVtΔ4, CBHI was found to aggregate in the cytoplasm of the fungal hyphae and there were indications that the mutant protein may have been degraded by the proteasome. CVtΔ4 also showed low total cellulase activity measured from the culture supernatant indicating that the CBHI enzyme was at least partially inactivated. The enzyme with one additional substitution at the C-terminal end of the *cbh1* core gene in strain CVtΔ5 seemed to be more active. It is possible, that the last replacement did cause conformational changes which allowed the substrate to bind to the active site. The CVtΔ5 strain did not show significant transcriptional changes related to the UPR pathway even though it showed activation of the proteasomal protein degradation pathway (ERAD).

There are no other previously published studies addressing ERAD function in filamentous fungi. In this study, the use of MG132 as an inhibitor of the proteasome activity demonstrated that degradation of misfolded proteasomal substrates can be blocked via this approach. The misfolded CBHI was seen to accumulate in the ER as evidenced by fluorescence microscopy (section 3.7) and the microarray results showing up-regulation of proteasome-related genes implied that the fungal proteasome was employed in the degradation of misfolded CBHI.

Putative autophagy proteins characterised in filamentous fungi so far are Atg1 and Atg8 (Pollack *et al*., 2009). Interestingly, two genes coding for autophagy proteins (autophagy protein Atg13 and autophagy related protein 18) were affected in the current study, which coulb be sign of activation of the lysosomal pathway during ER-stress in fungi, which should be futher investigated.

Functional analysis of *S. cerevisiae* apoptotic genes, and more recently of those in some filamentous species, has revealed that the apoptotic machinery in fungi is similar to that in mammals, but the apoptotic network is less complex and mode of action differs from the human mechanism (Amir *et al*., 2009). A more detailed view of the signalling pathways related to apoptosis in filamentous fungi would help understand the complex biological processes related to ER-stress. These studies may also contribute light to reasons for the low yields of heterologous proteins produced in over-stressed fungal strains.

Purification of three different mutant CBHI proteins and enzyme kinetics studies with the purified enzymes will offer more information on the biochemical and biological effects of the mutations introduced into the main secreted CBHI enzyme of *T. reesei*.

In summary, even though the microarray data generated was lacking statistically relevant replicates and therefore should be be taken as a guide, there were significant changes in transcriptomics related to the secretion stress caused expression of the mutant CBHI. This was reflected by up-regulation of genes encoding ER localised foldases such as Bip, PDI, PPI and Cal. Also many proteasome subunit encoding genes were affected along with the ubiquitin pathway genes and other ERAD-related genes such as Sec61, CDC48 and Der1, which warrants the suggestion that the mutant protein was degradated by ERAD.

Importantly, the current study has provided a new strategy for the induction of the cellular protein quality control mechanisms, such as UPR and ERAD, and therefore a more "natural" option to studies using chemicals to explore gene regulation and protein secretion in filamentous fungi under stress.