Genome-wide expression analysis upon constitutive activation of the HacA bZIP transcription factor in *Aspergillus niger* reveals a coordinated cellular response to counteract ER stress

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Chapter 5

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Abstract

HacA/Xbp1 is a conserved bZIP transcription factor in eukaryotic cells which regulates gene expression in response to various forms of secretion stress. In the present study, we genetically engineered an Aspergillus niger strain that expresses only the activated form of the HacA transcription factor (HacA\textsuperscript{CA}) and used transcriptomic analysis to identify genes and processes that are affected under this condition. Transcription profiles for the wild-type strain (HacA\textsuperscript{WT}) and the HacA\textsuperscript{CA} strain were obtained using Affymetrix GeneChip analysis of three replicate batch cultivations of each strain. In addition to the well known HacA targets such as the ER resident foldases and chaperones, GO enrichment analysis revealed up-regulation of genes involved in protein glycosylation, phospholipid biosynthesis, intracellular protein transport, exocytosis, and protein complex assembly in the HacA\textsuperscript{CA} mutant. Biological processes overrepresented in the down-regulated genes include those belonging to central metabolic pathways, translation and transcription. A remarkable transcriptional response in the HacA\textsuperscript{CA} strain was the down-regulation of the AmyR transcription factor and its target genes. The results indicate that the constitutive activation of the HacA leads to a coordinated regulation of the folding and secretion capacity of the cell, but with consequences on growth and fungal physiology.
5.1. Introduction

The secretion of extracellular proteins is very important to the natural saprophytic lifestyle of *Aspergillus niger*. The inherent ability of efficient protein secretion, found among several *aspergilli* such as *A. niger* and *A. oryzae*, has led to their biotechnological exploitation as hosts for homologous and heterologous protein production (Jeenes et al., 1991; Gouka et al., 1997; Braaksm et al., 2009; Nemeto et al., 2009; Lubertozi and Keasling, 2009). As protein yields for heterologous proteins are often reported as low, efforts have been made in order to describe and understand the processes that limit their secretion (Conesa et al., 2001, Jacobs et al., 2009), as well as efforts to prevent proteolytic activity outside the cell (Punt et al., 2008; Nemoto et al., 2009; Yoon et al., 2009).

Secretory proteins begin their journey by entering the endoplasmic reticulum (ER) where they are assembled, folded and modified. Then, they are packed into COPII coated vesicles and transported into the Golgi-like structures where further modifications take place. Proteins destined for secretion are packed into secretory vesicles to be transported to the tip of the growing hyphae, where the proteins are released extracellularly (Conesa et al., 2001; Shoji et al., 2008; Tahari-Talesh et al., 2008). Among the factors that disturb efficient secretion of heterologous proteins is the misfolding of these proteins in the ER or the fact that the proteins are recognized as misfolded by the Quality Control system present in the ER (Sagt et al., 2002; Rakestraw and Wittrup, 2006). The presence or accumulation of aberrant proteins in the ER may become fatal to the cell and to deal with the presence of misfolded proteins in the ER, eukaryotic cells react with the expression of several genes related to protein folding and degradation, a response termed the Unfolded Protein Response (UPR) (Travers et al., 2000). The basic sensing pathway to detect ER stress or an increase in folding load is highly conserved from yeast to man. In *Saccharomyces cerevisiae*, the sensor protein is Ire1p which is an ER resident transmembrane protein that contains a luminal domain that functions as the sensor of the proteins folding state, and a RNase domain at the C-terminal (Gonzalez and Walter, 2001; Lee et al., 2008). The accumulation of unfolded proteins is sensed through a dynamic interaction between Ire1 and the chaperone Bip1 or by direct sensing by Ire1 (Bertolotti et al., 2000; Kimata et al., 2003; Credle et al., 2005). As Bip1 is recruited to help with the folding of the ER accumulating proteins, its release from Ire1p leads to the oligomerization of Ire1p proteins. In turn, the formed Ire1p oligomer is activated by autophosphorylation and a site-specific endoribonuclease (RNase) domain is responsible for the splicing of a 252 nt intron present in mRNA of the bZIP transcription factor Hac1p (HacA in filamentous fungi and XBP-1 in the mammalian system), a process well characterized in fungi (Sidrauski and Walter, 1997; Kawahara et al., 1998; Valkonen et al., 2004) and higher eukaryotes (Shen et al., 2001; Calfon et al., 2002; Lee et al., 2002; Plongthongkum et al., 2007). Alternatively, from the known structures of the yeast and human luminal and cytoplasmic domains of Ire1 (Credle et al., 2005; Zhou et al., 2006; Korennykh et al., 2009; Lee et al., 2008) a model for direct binding of Ire1 to unfolded proteins is postulated that leads to structural changes in Ire1, oligomerization and activation of the kinase and endoribonuclease domains. In *A. niger*, the hacA mRNA splicing event results in the
excision of a 20 nt intron (Mulder et al., 2004), releasing it from a translational block (Mulder and Nikolaev, 2009). Although it has not yet been shown in the S. cerevisiae or mammalian homologues, in addition to the intron splicing, the hacA mRNA of A. niger, Aspergillus nidulans and Trichoderma reesei is truncated at the 5’-end during UPR induction (Saloheimo et al., 2004; Mulder et al., 2006). However, Mulder and Nikolaev (2009) showed that in A. niger truncation of hacA is not a requirement for induction of the pathway. Once translated, HacAp migrates into the nucleus where it binds to palindromic UPR elements at the promoter regions of UPR targets (Mulder et al., 2006).

Transcriptome analysis under UPR inducing conditions in both fungi and mammalian cells has revealed a subset of genes involved in folding, secretion, phospholipid biosynthesis and protein degradation (Travers et al., 2000; Lee et al., 2003; Shaffer et al., 2004; Arvas et al., 2006). Most of the UPR studies performed have induced this pathway through the presence of harsh chemicals (DTT or tunicamycin), which by itself may impose collateral responses that might not only provoke ER stress and by expressing heterologous proteins such as tPA and chymosin (Sims et al., 2005; Arvas et al., 2006; Guillemette et al., 2007). However, a recent study has illustrated that the induction of UPR-target genes may not be a stress response only induced by the presence of misfolded proteins, but may represent a more physiologically normal mechanism required and induced under conditions where there is a demand for an increased secretion capacity (Jørgensen et al., 2009). Although no indications for alteration in the amount of spliced HacA mRNA was detected in this study, a role for HacA in mediating differential gene expression cannot be excluded.

Manipulation of the UPR pathway and its components, like Bip1 and PDI (Robinson et al., 1994; Harmsen et al., 1996; Shusta et al., 1998), has been a common approach to improve the secreted production of heterologous proteins. Valkonen et al. (2003a) have shown, in S. cerevisiae, that controlling Hac1 expression has effects on native and foreign protein production; hac1 deletion led to a decrease of heterologous α-amylase and endoglucanase production whereas overexpression of this transcription factor resulted in an increase in the production of these proteins when compared to the respective parental strains. Similar results have been demonstrated in A. niger var awamori, where a constitutive induction of the UPR pathway enhanced the production of heterologous laccase and of bovine preprochymosin (Valkonen et al., 2003b). The UPR is activated to alleviate the stress caused by the accumulation of misfolded protein in the ER lumen by improving protein folding, degrading unwanted proteins (Travers et al., 2000; Guillemette et al., 2007) and reducing the entry of secretory proteins into the ER, a mechanism known as REpression under Secretion Stress (RESS) (Pakula et al., 2003). Studies have shown in the presence of chemicals that inhibit protein folding there is a selective down-regulation of genes coding extracellular enzymes (Pakula et al., 2003; Martínez and Chrispeels, 2003; Al-Sheikh et al., 2004).

In this study, we present a genome-wide overview of the HacA responsive genes by comparing the transcriptomic profiles of A. niger strain expressing the wild-type hacA gene with a genetically engineered A. niger strain that expresses only the activated form of the HacA
transcription factor. The comparison revealed that HacA is a master regulator, coordinating several processes within the secretory pathway such as the induction of protein folding, protein glycosylation and intracellular transport. Additionally, we discovered that constitutive activation of HacA results in the down regulation of the AmyR transcription factor and the AmyR regulon, which includes the most abundantly produced extracellular glycoproteins, thereby reducing import of new proteins into the ER. The downregulation of the AmyR regulon revealed by the genome wide expression analysis was phenotypically confirmed as the HacA\textsuperscript{CA} mutant displayed a strongly reduced growth phenotype on starch plates.

5.2. Material and Methods

5.2.1. Strains and culture conditions

Aspergillus niger strains used throughout study (Table 1) were cultivated in minimal medium (MM) (Bennett and Lasure, 1991) containing 1% (w/v) of glucose (or other as indicated) as a carbon source, 7 mM KCl, 11 mM KH\textsubscript{2}PO\textsubscript{4}, 70 mM NaNO\textsubscript{3}, 2 mM MgSO\textsubscript{4}, 76 nM ZnSO\textsubscript{4}, 178 mM H\textsubscript{3}BO\textsubscript{3}, 25 mM MnCl\textsubscript{2}, 18 mM FeSO\textsubscript{4}, 7.1 mM CoCl\textsubscript{2}, 6.4 mM CuSO\textsubscript{4}, 6.2 nM Na\textsubscript{2}MoO\textsubscript{4}, 174 mM EDTA; or in complete medium (CM) containing, in addition to MM, 0.1% (w/v) casamino acids and 0.5% (w/v) yeast extract. When required, 10 mM uridine was added. The glucose minimal medium used for bioreactor cultivations has been described previously (Jorgensen \textit{et al.}, 2010).

Table 1. \textit{Aspergillus niger} strains used in this study.

| Strain | Genotype | Reference |
|--------|----------|-----------|
| N402   | cspA\textsuperscript{1} derivative of ATCC9029 | Bos \textit{et al.}, 1988 |
| MA70.15| ΔkusA\textsuperscript{-}:amdS\textsuperscript{+} in AB4.1 pyrG\textsuperscript{-} | Meyer \textit{et al.}, 2007 |
| NC1.1  | Wild type hacA in MA70.15, pyrG\textsuperscript{+} | This study |
| NC2.1  | Constitutive active hacA in MA70.15, pyrG\textsuperscript{+} | This study |
| YvdM1.1| ΔamyR in AB4.1 pyrG\textsuperscript{+} | Yuan \textit{et al.}, 2008a |
| XY3.1  | ΔnudR in AB4.1 pyrG\textsuperscript{+} | Yuan \textit{et al.}, 2008b |

5.2.2. Construction of the constitutive active hacA strain and the hacA reference strain

To replace to endogenous hacA gene on the hacA locus with a constitutive activated allele of the hacA gene, a replacement cassette was constructed. As a control, a similar replacement cassette was made with the wild-type hacA gene. To construct the hacA reference strain, three PCR fragments consisting of the hacA gene including promoter and terminator regions, the \textit{Aspergillus oryzae} pyrG selection marker and a hacA terminator region were cloned into pBluescript-SK. Subsequently, this plasmid was used as template to introduce the mutations
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that led to a constitutive active hacA allele by site directed mutagenesis (according to Quick Change II site directed mutagenesis protocol, Stratagene). To construct the wild-type hacA replacement construct the A. niger hacA gene (accession number: AY303684), including about 0.6 kb promoter and 0.6 kb of terminator regions, was amplified by PCR using N402 genomic DNA as template and primers NC8 and NC11 (Table 2) to which NotI and XhoI restriction sites were added, respectively. The amplified gene was cloned into pTZ57R/T (Fermentas) and sequenced. The hacA terminator region (≈1 kb) was amplified by PCR using N402 genomic DNA as template and primers NC1 and NC2, to which SalI and KpnI restriction enzymes were added, respectively. The fragment was cloned into pGEM-T easy (Promega) and sequenced. For PCR amplification, Phusion™ High-Fidelity PCR Kit (Finnzymes) was used according to manufacturer’s instructions. The AopyrG gene (≈2kb) was PCR amplified using pAO4-13 (de Ruiter-Jacobs, 1989) as template DNA and primers NC7 and pAOpyrG-GA5rev, to which XhoI and SalI restriction sites were added, respectively. The fragment was cloned into pGEM-T easy (Promega) and sequenced. The fragments corresponding to the hacA terminal region and pyrG were digested from the plasmids using the respective restriction enzymes mentioned above and cloned in a 3-way ligation step into pBlue-SK, previously digested with XhoI-KpnI to give pBS-pyrG-3’hac. To obtain the final construct, the hacA gene was digested from pTZ57R/T using NotI and cloned into pBS-pyrG-3’hac, previously digested with the same enzymes. The final construct, named pHAC, was linearized with NotI and transformed into the A. niger MA70.15 strain. Transformants with a targeted integration of the construct at the hacA locus were screened by Southern blot analysis.

To obtain a strain only expressing the constitutively active hacA gene, a construct was made lacking the 20 nucleotide intron (see introduction for details) using the site-directed mutagenesis technique. Mutagenic oligonucleotide primers NC31 and NC32 (Table 2) were designed, surrounding each side of the intron region. PCR was performed using PfuUltra HF DNA polymerase (Stratagene), the pHAC (≈10 ng) as template and conditions as follows: initial denaturation of 1 min at 95°C, 18 cycles of 30 sec denaturation at 95°C, annealing at 55°C for 30 sec and elongation for 8 min and 30 sec at 68°C. Afterwards, PCR products were digested with DpnI for one hour at 37°C, for destruction of parental methylated and hemimethylated plasmid DNA. The mixture was directly used for E. coli transformation. Plasmid pConstHac was analyzed by restriction enzymes and sequencing, confirming the absence of the 20 nt intron. This construct was linearized with NotI and then transformed into A. niger MA70.15. Southern analysis of putative transformants carrying the wild-type hacA and the constitutively active hacA was performed by digesting the genomic DNA with NheI and probing with a 0.6 kb probe corresponding to the hacA 3’-flanking region. Transformants NC1.1 containing expressing the wild-type hacA and NC2.1 expressing the activated hacA form at the endogeneous hacA locus were chosen for further studies and we will refer to these strains as the HacA<sub>WT</sub> (wild-type) and HacA<sub>CA</sub> (Constitutive Active) strains, respectively. The absence of the intron in the NC2.1 strain was further confirmed by PCR analysis using genomic DNA as template, together with primers phac1 and phac2 (Table 2) using Taq polymerase (Fermentas).
5.2.3. Bioreactor cultivation conditions

Conidia for inoculation of bioreactor cultures were harvested from solidified CM with a sterile detergent solution containing 0.05% (w/v) Tween80 and 0.9% (w/v) NaCl. Batch cultivation of HacA<sup>WT</sup> and HacA<sup>CA</sup> was initiated by inoculating 5L MM with conidial suspension to give 10<sup>9</sup> conidia L<sup>-1</sup>. Glucose was sterilized separately and added to sterile MM to give a final concentration of 0.75% (w/v). During cultivation at 30 °C, pH 3 was maintained by computer-controlled addition of 2 M NaOH or 1 M HCl. Sterile air was supplied at 1 L min<sup>-1</sup> through a ring-sparger. Dissolved oxygen tension was above 40% of air saturation at any time, ensuring sufficient oxygen for growth. After spore germination 0.01% (v/v) polypropylene glycol P2000 was added as antifoam agent. Submerged cultivation was performed with 6.6 L BioFlo3000 bioreactors (New Brunswick Scientific, NJ, USA). A more detailed description of the medium and batch cultivation protocol is given in Jørgensen et al. (2010).

5.2.4. Biomass concentration and substrate determination

Dry weight biomass concentration was determined by weighing lyophilized mycelium separated from a known mass of culture broth. Culture broth was filtered through GF/C glass microfibre filters (Whatman). The filtrate was collected and frozen for use in solute analyses. The mycelium was washed with demineralised water, rapidly frozen in liquid nitrogen and stored at -80°C until lyophilization. Glucose was determined according to the method of Bergmeyer et al. (1974) with a slight modification: 250mM triethanolamine (TEA) was used as buffer (pH7.5).

5.2.5. RNA isolation and quality control

Mycelium intended for gene-expression analyses was separated from culture medium and frozen in liquid nitrogen within 15-20 s from sampling RNA was extracted from mycelium and
snapfrozen in liquid nitrogen using TRIzol reagent (Invitrogen). Frozen ground mycelium (≈200 mg) was directly suspended in 800 µl Trizol reagent and vortexed vigorously for 1 min. After centrifugation for 5 min at 10000 × g, 450 µl of the supernatant was transferred to a new tube. Chloroform (150 µl) was added and after 3 min incubation at room temperature, samples were centrifuged and the upper aqueous phase was transferred to a new tube to which 400 µl of isopropanol was added, followed by 10 min incubation at room temperature and centrifugation for 10 min at 10000 × g. The pellet was washed with 75% (v/v) ethanol and finally dissolved in 100 µl H2O. RNA samples for micro-array analysis were additionally purified on NucleoSpin RNA II columns (Machery-Nagel) according to the manufacturer’s instructions. RNA quantity and quality was determined on Nanodrop spectrophotometer.

5.2.6. Microarray analysis

Probe synthesis and fragmentation were performed at ServiceXS (Leiden, The Netherlands) according to the GeneChip Expression Analysis Technical Manual (Affymetrix, 2002). DSM (Delft, The Netherlands) proprietary A. niger GeneChips were hybridised, washed, stained and scanned as described in the GeneChip Expression Analysis Technical Manual (Affymetrix inc., 2002). MAS5 condensation was used. The 3’ to 5’ signal ratio of probe sets of internal control genes, like gpdA (glyceraldehyde-3-phosphate dehydrogenase), pkiA (pyruvate kinase), hxk (hexokinase) and actin, were below 3 on all 12 arrays.

5.2.7. Transcriptomic data analysis

Bioconductor, a collection of open source and open development packages for the statistical programming language R, was used for data analyses (Gentleman et al., 2004; Team RDC, 2010). The transcriptomic data set comprises 12 arrays representing independent triplicates for each of the following four conditions: HacA WT, HacA CA-1, HacA CA-2 and HacA CA-3. Using the robust multi-array analysis (RMA) package (Irizarry et al., 2003), RMA expression values were computed from the perfect match probes only. Background correction, normalization and probe summarization steps were performed according to the default settings of the RMA package. Defining the following contrast matrix (HacA CA-1 - HacA WT, HacA CA-2 - HacA WT, HacA CA-3 - HacA WT), three sets of differentially expressed genes were determined by moderated t-statistics using the Limma package (Smyth, 2004). The Benjamini and Hochberg False Discovery Rate (Benjamini and Hochberg, 1995) (FDR) was controlled at q < 0.005. RMA expression values (log2 scale) for each array, mean expression values (normal scale) for each condition, fold-changes and FDR q-values for each of the three comparisons as well as classifiers for the moderated t-statistics are summarized in Supplementary Table S1A.

5.2.8. Enrichment analysis of Gene Ontology (GO) terms

Controlling the FDR at q < 0.05, over-represented GO terms in sets of differentially expressed genes were determined by Fisher's exact test (Fisher, 1922). An improved GO
annotation for the \textit{A. niger} CBS 513.88 genome was based on orthology mappings from \textit{A. nidulans} FGSC A4 (Nitsche, unpublished results).

\section*{5.3. Results}

\subsection*{5.3.1. Construction and analysis of a strain expressing a constitutively activated form of \textit{hacA}}

To obtain an \textit{A. niger} strain with a constitutively activated HacA transcription factor, the wild-type \textit{hacA} gene was replaced by the spliced form of \textit{hacA} that lacks the 20 nucleotide intron. For the construction of a reference strain and a strain only expressing the \textit{hacA} induced form, plasmids pHac\textsuperscript{WT} (Fig. 1A) and pHac\textsuperscript{CA} (Fig. 1B) were used.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic representation of the plasmids pHAC (A) and pConstHac (B) (Note: fragment sizes are not on scale). (C) Sequence alignment of pHAC and pConstHAC showing the absence of the 20 nt intron on pConstHac. (D) PCR amplification of gDNA of HacA\textsuperscript{WT} (NC1.1) and HacA\textsuperscript{CA} (NC2.1) transformants. Primers were designed about 100 bp upstream and 100bp downstream of the \textit{hacA} intron region, giving rise to a band of 200 bp for HacA\textsuperscript{CA} and 220 bp for HacA\textsuperscript{WT}. Sizes of the DNA Marker (M) are indicated.}
\end{figure}

Both plasmids were sequenced, confirming the absence of the intron in pHac\textsuperscript{CA} (Fig. 1C). \textit{A. niger} was transformed with either pHac\textsuperscript{WT} or pHac\textsuperscript{CA} and 10 putative transformants from each transformation were purified and analyzed by Southern blot (data not shown). Transformants with the correct integration pattern for each plasmid were obtained and strains NC1.1 (HacA\textsuperscript{WT}),
and NC2.1 (HacA\textsuperscript{CA}) were chosen for the further experiments. To further confirm that HacA\textsuperscript{CA} did not contain the intron, a PCR analysis was performed on genomic DNA isolated from both strains revealing the 20-nt difference expected among them, as shown in Fig. 1D.

Growth assays were performed with both strains at different temperatures (Fig. 2A and B). At each temperature tested, radial growth rate (colony size) of HacA\textsuperscript{CA} strain was reduced compared to HacA\textsuperscript{WT}, and this growth impairment was more pronounced at 37 and 42°C (Fig. 2A). Differences in phenotype between both strains were also apparent as HacA\textsuperscript{CA} showed a delay in growth and sporulation in comparison to HacA\textsuperscript{WT} (Fig. 2B). As no phenotypic differences were found between our reference strain HacA\textsuperscript{WT} and N402 (data not shown), we conclude that the phenotypic effects observed in HacA\textsuperscript{CA} are due to the presence of only the UPR-induced form of hacA. The effects of having a constitutive activation of the UPR are different from the absence of a functional UPR. The deletion of the HacA transcription factor in\textit{A. niger} has a profound effect on this fungus growth and morphology, resulting in small, compact colonies that hardly sporulated (Mulder\textit{et al.}, 2009; Carvalho\textit{et al.}, 2010).

Figure 2. (A) Differences on colony size (diameter) of HacA\textsuperscript{WT} and HacA\textsuperscript{CA} strains growing at different temperatures. 10\textsuperscript{4} spores were spotted on solid CM plates and growth was monitored for 6 days. (B) Strains phenotype on CM after 3 and 6 days of growth at 30°C. HacA\textsuperscript{CA} phenotype is characterized by a slower growth/colony size as well as a delay in sporulation compared to the HacA\textsuperscript{WT}. Bars indicate standard deviations from three individual measurements.

5.3.2. Physiological consequences of the constitutive hacA activation in batch cultivations

Growth of triplicate batch cultures of HacA\textsuperscript{WT} and HacA\textsuperscript{CA} was characterized as filamentous and highly reproducible. The growth kinetics of a representative culture of each strain is shown in Fig. 3 and results from all cultures are given in the supplemental material (Suppl. Fig. 1).
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Cultures of the HacA<sup>WT</sup> strain exhibited exponential growth with a specific growth rate ($\mu$) of $0.22 \pm 0.01$ h<sup>-1</sup> (n=4) from exit of lag phase to depletion of glucose (Fig. 3A). Initial growth of HacA<sup>CA</sup> was similar to that of the HacA<sup>WT</sup>; it was exponential with a $\mu$ of $0.21 \pm 0.01$ h<sup>-1</sup> (n=3). However, after 21-22 h of batch cultivation, when half of the glucose was consumed, the growth kinetics shifted from exponential to apparently linear (Fig. 3B). It was not clear from the

Figure 3. Growth profiles of one of the triplicate A. niger HacA<sup>WT</sup> (A) and HacA<sup>CA</sup> (B) batch cultures. Dry weight biomass concentration (g<sub>DW</sub> kg<sup>-1</sup>) as a function of time (h) illustrates the growth of the cultures. The maximum specific growth rate for each culture was determined from the slope ($\alpha$) of the ln transformation of biomass ($C_{\text{biomass}}$) in the exponential growth phase as a function of time (h), as well from log transformation of alkali addition as a function of time (h). Dash-line represents the end of the exponential growth phase (depletion of glucose). Arrows indicate time-points where mycelium was harvested for transcriptomic analysis.
relatively few determinations of biomass concentration whether growth was truly linear in the second phase but this was strongly supported by analysis of the growth-dependent alkali addition (inset Fig. 3A, B). We established a concordance between growth and alkali added to maintain constant pH in the cultures (not shown), and used this as an indirect measure of growth as described previously by Iversen et al. (1994). Linearity was then confirmed by log-transformation of alkali addition rates using the computer recorded titrant addition data and the LOS program (Poulsen et al., 2003). During exponential growth, growth yield on substrate \( (Y_{ss}) \) was comparable in both strains: 0.53±0.02 for HacA\(^{\text{WT}}\) and 0.52±0.04 for HacA\(^{\text{CA}}\).

5.3.3. Impact of the constitutive activation of hacA on the transcriptome of A. niger

Three independent bioreactor cultures with the HacA\(^{\text{WT}}\) strain were performed. From each cultivation experiment, biomass was harvested from the mid-exponential growth phase (biomass concentration 1.5 gr/kg (Fig. 3A)) and used for RNA extraction and subsequent microarray analysis. Likewise, for the HacACA strain three bioreactor cultivations were performed and from each culture biomass was harvested and RNA was isolated from the mid-exponential time point (time point 1; HacA\(^{\text{CA-1}}\)) (Fig. 3B). For the HacA\(^{\text{CA}}\) cultures, RNA was extracted from two additional time points subsequent to the shift to linear growth and the RNA was also analyzed (time point 2 and 3; HacA\(^{\text{CA-2}}\) and HacA\(^{\text{CA-3}}\) (Fig. 3B). Thus, the data set in this study consists of four groups of triplicate biological replicates of HacA\(^{\text{WT}}\) and HacA\(^{\text{CA}}\) at three timepoints (HacA\(^{\text{CA-1}}\), HacA\(^{\text{CA-2}}\) and HacA\(^{\text{CA-3}}\)). The reproducibility of the triplicate array analyses was high with a mean coefficient of variation (CV) ranging from 0.12 to 0.14 for transcripts rated as present or marginal.

The number of differentially-expressed genes (FDR <0.005) in a pairwise comparison is given in Table 3. In response to constitutive activation of hacA at time point 1 (HacA\(^{\text{CA-1}}\)) 1235 genes were differentially expressed. The number of differentially expressed genes increased when comparing the later time points (HacA\(^{\text{CA-2}}\) and HacA\(^{\text{CA-3}}\) to the wild-type strain to give a total number of 1698 and 1978 differentially expressed genes. Table 3 also shows that the transcriptomic differences between the different time points of the HacA mutant (HacA\(^{\text{CA-1}}\), HacA\(^{\text{CA-2}}\) and HacA\(^{\text{CA-3}}\)) were relatively minor (48 and 179 differentially expressed genes comparing HacA\(^{\text{CA-2}}\) vs. HacA\(^{\text{CA-1}}\) and HacA\(^{\text{CA-3}}\) vs. HacA\(^{\text{CA-1}}\) respectively).

Table 3. Overview of the number of differentially expressed genes.

|       | HacA\(^{\text{WT}}\) | HacA\(^{\text{CA-1}}\) | HacA\(^{\text{CA-2}}\) |
|-------|---------------------|----------------------|----------------------|
| HacA\(^{\text{CA-1}}\) | 1235 | 668 ↑ | 567 ↓ |
| HacA\(^{\text{CA-2}}\) | 1698 | 973 ↑ | 725 ↓ | 48 ↑ | 43 ↑ | 5 ↓ |
| HacA\(^{\text{CA-3}}\) | 1978 | 1109 ↑ | 869 ↓ | 179 | 155 ↑ | 24 ↓ | 0 ↑ | 0 ↓ |

↑ up-regulated ; ↓ down-regulated
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Comparison of HacA<sup>CA-2</sup> with HacA<sup>CA-3</sup> revealed that the transcriptomes were very similar and with the stringent FDR of <0.005, no differentially expressed genes were detected. As a start to analyse the expression data, Venn diagrams were made to identify genes that were differentially expressed in HacA<sup>CA</sup> at all three time points when compared to the wild-type strain. As shown in Fig. 4A, 616 genes were up-regulated in the HacA mutant at all three time points and 433 genes were down-regulated (Fig. 4B). A complete list of all expression data and the FDR values for the pair wise comparison of the different strains and time points is given in Supplementary Table S1A (available upon request: A.F.J.Ram@biology.leidenuniv.nl).

![Figure 4](image)

**Figure 4.** Venn diagrams of the number of overlapping and non-overlapping induced (A) or repressed (B) genes on *A. niger* HacA<sup>CA</sup> mutant strain at different time points in comparison to HacA<sup>WT</sup> strain.

From the 616 up-regulated genes (Supplementary Table S1B) we were able to retrieve 598 upstream regions. These upstream regions were analysed for the presence of UPRE sequences (5’-CAN(G/A)NTGT/GCCT-3’, Mulder *et al.*, 2006). From the up-regulated genes in the HacA<sup>CA</sup> strain, we found 47 genes that contained at least one UPRE sequence within the 400 bp region up-stream their start codon (Supplementary Table S1C). Compared to the frequency of UPRE in the 400 bp upstream region of the remaining non up-regulated genes (457 out of 13156) a statistical significant enrichment (p ≤ 5.4×10<sup>-7</sup>) was assessed with the Fisher's exact test (one-sided). Although this analysis indicates a statistical enrichment for genes containing a HacA binding site in the promoter region of HacA induced genes, it shows that only about 10% of the HacA<sup>CA</sup> induced genes contain an UPRE. It suggests that either the currently used HacA binding consensus site is too stringent and that additional sequences allow HacA to bind, or that additional transcription factors are involved in the induction in response to the constitutive activation of HacA. The data set of HacA induced genes with a putative UPRE site include genes related to protein folding (as previously described by Mulder *et al.*, 2006), lipid metabolism, transport within the cell, glycosylation, ER quality control as well as a large set of genes that code for hypothetical and unknown function proteins (Supplementary Table S1C).
5.3.4. Identification of biological processes enriched in the transcriptomic profiles of HacA\textsuperscript{CA} strain

To obtain an overview of the processes affected at the transcriptional level between the HacA\textsuperscript{WT} and the HacA\textsuperscript{CA-1} mutant, overrepresented GO terms were identified of differentially expressed genes. For this analysis, we have used an improved version of the GO annotation for *A. niger*, by using the *A. nidulans* computational and manual annotations efforts at the *Aspergillus* Genome Database (Nitsche *et al.*, manuscript in preparation). Network maps of related GO-terms (Biological Processes), over- or under-represented in the HacA\textsuperscript{CA} strain, are given as supplementary figures (S2 and S3). To present the results, two complementary approaches were taken. Firstly, we rationally defined GO-terms of higher order that include several GO-terms. Secondly, we looked specifically at GO-terms that are terminal in the network, as these annotations are the most detailed (Suppl. Fig. S2 and S3). These approaches enabled us to identify four major categories to describe the most relevant up-regulated biological processes in the HacA\textsuperscript{CA} strain (Fig. 5). The four main categories included those related to I) ER translocation and protein folding (Table S4), II) intracellular vesicle trafficking (Table S5), III) protein glycosylation (Table S6) and IV) lipid metabolism (Table S7).

In the HacA\textsuperscript{CA} strain we found enriched GO terms linked to ER processes, such as those related to entry in the ER: signal particle recognition, cleavage of signal sequence, and translocation (e.g. Sec61 and related subunits). In parallel to the processes that mediate the recognition, targeting and entering the ER, enrichment of GO terms that include a large number of genes involved in the subsequent events of protein folding and quality control are also observed. The category of protein folding includes the well known HacA targets such as *bipA*, *pdiA*, *tigA* and *prpA* (Mulder *et al*. 2006). After being synthesized and folded properly in the ER, proteins are packed in vesicles and transported to the Golgi and from there on, further transported to reach their final intra- or extra-cellular destination. Our analysis identified a number of genes that encode proteins that take part in the vesicle/trafficking machinery such as those involved in ER-to-Golgi (COPII associated components), Golgi-to-ER (COPI transport vesicles, Sec components) and Golgi to endosome transport. Additionally, genes involved in exocytosis were also induced (Fig. 5). GO-terms related to processes involving protein glycosylation, were up-regulated in the HacA\textsuperscript{CA} strain. The processes include genes involved in sugar nucleotide synthesis, oligosaccharyl synthesis (ALG-genes) and transfer (OST-complex) of the preassembled oligosaccharide to certain asparagine residues (*N*-glycosylation). In addition, genes related to the addition of O-glycans (genes homologous to the *S. cerevisiae* Pmt-family and Kre2-family of mannosyltransferases) were also up-regulated. Finally, several genes related to the synthesis and transfer of Glycosylphosphatidylinositol (GPI) anchors to proteins were found to be up-regulated. Supplementary Table 6 lists the differentially expressed genes with a proposed function in relation to protein glycosylation or GPI-anchor attachment. In addition, the constitutive activation of HacA has a pronounced effect on the transcription of genes involved in phospholipid metabolism and includes proteins that are homologous to proteins involved in ergosterol biosynthesis as well as proteins involved in fatty acids and inositol metabolisms.
Genome-wide expression analysis upon constitutive activation of the HacA bZIP transcription factor in *Aspergillus niger* reveals a coordinated cellular response to counteract ER stress (Table S7). Categories containing less GO-terms included terms related to intracellular pH regulation and terms related to glutathione catabolic processes (Table S8).

Concerning the biological processes over-represented in the down-regulated set of genes we found one major category linked to the central metabolic pathways (Fig. 5 and Table S9). This category includes the down-regulation of genes within Glycolysis/Gluconeogenesis; Alcohol catabolic/metallic process; Carboxylic Acid Cycle and Carbon Metabolic/Cellytic metabolism. Categories containing fewer GO-terms included terms related to transporters and response to oxidative stress. The down-regulation of genes in central metabolic pathways may reflect the growth limitation observed in the HacA<sup>CA</sup> mutant (Figs. 2 and 3).

**Figure 1.** Main significant induced and repressed biological processes in the HacA<sup>CA</sup> mutant strain in comparison to HacA<sup>WT</sup> strain.

### 5.3.5. Common and different features of the constitutive activation of HacA and the UPR induction by chemicals or heterologous protein expression.

To gain a broader overview of the impact of a constitutive activation of HacA on *A. niger* we compared our data set (HacA<sup>CA-1</sup>/HacA<sup>WT</sup>) with the data of Guillemette and co-workers (2007; additional files 5 and 6) in which the genome-wide transcriptional protein secretion-related stress responses was analyzed. In this study (Guillemette *et al.*, 2007) transcriptional
targets of the UPR pathway were identified by treatment of *A. niger* with the ER-disturbing chemical agents tunicamycin and dithiothreitol (DTT) and using a strain producing the recombinant tissue plasminogen activator (t-PA) as a model for heterologous protein production. As shown in Fig. 6, in the induced set of genes, 13 genes are commonly unregulated in both studies (all conditions) and 80 genes that are differentially expressed in HacA\(^{CA-1}/\)HacA\(^{WT}\) and in at least two of the three conditions performed by Guillemette *et al.* (2007). These 93 commonly induced genes include all the genes identified in the Guillemette study related to protein folding, translocation/signaling peptidase complex and glycosylation and most of the genes that belong to the categories of vesicle trafficking and lipid metabolism (Supplementary Table S10). However, more genes belonging to each of these categories have been identified in HacA\(^{CA-1}/\)HacA\(^{WT}\) (Fig. 5 and Supplementary Tables 4-7). Unique genes found in at least two of the conditions tested (56) and not in our data set relate mainly to the categories of cellular transport, stress related, amino acid metabolism, carbohydrate metabolism and unclassified genes.

For the repressed set of genes we found 45 common genes to our study and Guillemette *et al.* (2007) which are evenly distributed throughout the categories established by the authors (additional file 6 in Guillemette *et al.*, 2007). The fact that the number of commonly down-regulated is small between the two studies suggests important differences and heterogeneous responses to the induction of the UPR indirectly (chemicals and heterologous protein) and the manipulation of the transcription factor that regulates this pathway in the overall cell metabolism.

### 5.3.6. The constitutive activation of HacA triggers the induction of ERAD genes

Secretory proteins that fail to fold properly usually accumulate in the ER and are sooner or later targeted to destruction by the proteasome, a process termed ER-associated degradation (ERAD) (Nishikawa *et al.*, 2005). Genes encoding proteins that are putatively involved in ERAD have been identified in the *A. niger* genome (Pel *et al.*, 2007; Carvalho *et al.*, 2011) and the
expression of these gene was examined in the microarray data set. As highlighted in Table 4, the expression of several putative ERAD components was induced in the HacA\textsuperscript{CA} mutant.

### Table 4. Expression values of \textit{A. niger} ERAD genes.

| Gene ID       | Gene name   | Description                                      | Fold change | HacA\textsuperscript{CA}/HacA\textsuperscript{WT} | HacA\textsuperscript{CA}/HacA\textsuperscript{WT} | HacA\textsuperscript{CA}/HacA\textsuperscript{WT} |
|---------------|-------------|--------------------------------------------------|-------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| An15g00640    | \textit{derA} | strong similarity to hypothetical protein GABA-A receptor epsilon subunit – \textit{C. elegans}       | 4.0         | 6.0                                               | 6.4                                               |                                                   |
| An01g12720    | \textit{hrdC} | similarity to tumour suppressor TSA305 protein of patent W09928457-A1 – \textit{H. sapiens}              | 3.3         | 3.9                                               | 4.0                                               |                                                   |
| An01g14100    | \textit{mifA} | weak similarity to stress protein \textit{Herp} – \textit{M. musculus}                                   | 3.1         | 4.3                                               | 4.6                                               |                                                   |
| An18g06220    | \textit{mnsA} | strong similarity to alpha-mannosidase MNS1 – \textit{S. cerevisiae}                                     | 4.2         | 4.7                                               | 5.0                                               |                                                   |
| An08g09000    |             | strong similarity to ubiquitin-like protein DSK2 – \textit{S. cerevisiae}                                  | 1.8         | 1.7                                               | 1.9                                               |                                                   |
| An16g07970    |             | similarity to autocrine motility factor receptor Amfr – \textit{M. musculus}                                      | 2.9         | 2.9                                               | 3.1                                               |                                                   |
| An03g04340    |             | strong similarity to ER membrane translocation facilitator Sec61 – \textit{Y. lipolytica}                                                | 2.6         | 2.6                                               | 2.6                                               |                                                   |
| An04g01720    |             | similarity to DnaJ protein SIS1 – \textit{C. curvatus}                                                        | 1.8         | 2.3                                               | 2.2                                               |                                                   |
| An12g00340    |             | similarity to alpha 1,2-mannosidase IB – \textit{H. sapiens}                                                     | 3.2         | 2.9                                               | 3.1                                               |                                                   |
| An04g00360    |             | strong similarity to transport vesicle formation protein Sec13p – \textit{S. cerevisiae}                                 | 2.1         | 2.1                                               | 2.1                                               |                                                   |
| An09g06110    |             | strong similarity to ubiquitin conjugating enzyme ubcp3p – \textit{S. pombe}                                              | 1.4*        | 1.6                                               | 1.7                                               |                                                   |

*Not significantly differentially expressed

For instance, the \textit{derI} homologue (\textit{derA}, An01g00560), involved in transport of unfolded proteins out of the ER (Ye \textit{et al.}, 2001), is 4.0-fold induced; \textit{hrd3} (\textit{hrdC}, An03g04600), involved in recognition and presentation of the substrate for degradation (Plemper \textit{et al.}, 1999), is 3.3-fold induced. The \textit{mifA} (An01g14100) gene, a homologue of mammalian \textit{herp1/mif1} protein and suggested as the link between the UPR and ERAD pathways (van Laar \textit{et al.}, 2001), 3.1-fold induced. Furthermore, \textit{mnsI} (\textit{mnsA}, An18g06220), a mannosidase that by removal of 1,2 α-mannose units targets the substrate to degradation (Tremblay and Herscovics, 1999), is 4.2-fold induced. In comparison to Travers \textit{et al.} (2000), our study allowed us to unravel the regulation of other ERAD related genes in relation to UPR, such as \textit{mnsI}, \textit{mif1}, a DSK2 homologue (An08g09000, putatively encoding a ubiquitin-like protein) (1.8-fold induction) and another putative α-mannosidase (An12g00340, 3.2-fold induced).

### 5.3.7. Constitutive activation of HacA leads to the down-regulation of the AmyR regulon

Although an increase in expression of secretion related processes (folding, glycosylation, vesicle transport) is observed in the HacA\textsuperscript{CA} strain, the expression of several genes encoding secreted proteins is down-regulated (Supplementary Table 9). In addition, expression of the AmyR transcription factor was repressed under these conditions (-3.3 fold, FDR < 10\textsuperscript{-5}). Starch is a polymeric carbon source consisting of glucose units joined together by alpha1,4- and
alpha1,6-glycosidic bonds and naturally synthesized by plants. *A. niger* is able to degrade starch by secreting various amylases that convert starch into maltose and glucose (Yuan *et al*., 2008a). The transcription of these amylolytic enzymes is mediated by AmyR (Petersen *et al*., 1999; Gomi *et al*., 2000). The AmyR regulon has been defined and consists of several alpha-glucosidases as well as two sugar transporters (Yuan *et al*., 2008a). Our transcriptome profiles show that the enzymes and sugar transporters in the AmyR regulon are commonly down-regulated (Table 5).

Table 5. Expression values of genes involved in starch metabolism.

| Gene ID      | Gene name | Description | Fold change |
|--------------|-----------|-------------|-------------|
|              |           |             | HacA<sup>CA</sup>-<i>y</i>/HacA<sup>WT</sup> | HacA<sup>CA</sup>-<i>y</i>/HacA<sup>WT</sup> | HacA<sup>CA</sup>-<i>y</i>/HacA<sup>WT</sup> |
| Starch regulation |           |             |             |             |             |
| An04g06910 | amyR      | transcription regulator of maltose utilization AmyR – *A. niger* | -3.3 | -3.3 | -3.3 |
| An01g06900 | weak similarity to transcription activator AmyR – *A. oryzae* | -1.7* | 1.4* | 2.1 |
| An09g03100 | amyA      | strong similarity to alpha-amylase precursor AMY – *A. shirousamii* | -5 | -5 | -5 |
| Starch degradation |           |             |             |             |             |
| An11g03340 | aamA      | acid alpha-amylase – *A. niger* | -370 | -50 | -50 |
| An04g06920 | aggA      | extracellular alpha-glucosidase – *A. niger* | -5 | -10 | -10 |
| An01g10930 | aggB      | extracellular alpha-glucosidase – *A. niger* | -10 | -10 | -10 |
| An03g06550 | glA       | glucan 1,4-alpha-glucosidase – *A. niger* | -10 | -25 | -25 |
| An04g06930 | amyC      | extracellular alpha-amylase – *A. niger* | -10 | -25 | -25 |
| Sugar uptake |           |             |             |             |             |
| An02g03540 | mstC      | strong similarity to hexose transport protein HX3 – *S. cerevisiae* | -2 | -2 | -2 |
| An15g03940 | mstA      | strong similarity to monosaccharide transporter Mst-1 – *A. muscaria* | -2.5 | -2 | -1.7 |
| An09g04810 | strong similarity to high affinity glucose transporter HGT1 - *K. lactis* | -5 | -10 | -10 |
| An11g01100 | strong similarity to high-affinity glucose transporter HGT1 - *K. lactis* | -5 | -5 | -5 |
| An12g07450 | mstA      | Sugar/H+ symporter | -5 | -10 | -10 |

*Not significantly differentially expressed.

The down-regulation of genes involved in starch degradation and uptake suggested that the HacA<sup>CA</sup> mutant may not be able to grow on starch as sole carbon source. In order to test this, we performed growth tests of HacA<sup>CA</sup> together with HacA<sup>WT</sup> and a ΔamyR strain in which the AmyR-encoding gene has been deleted (Yuan *et al*., 2008a) on solid media containing starch or its derivatives in a range of different complexity (Fig. 7).

As predicted from the transcriptomic and similar to the ΔamyR strain, HacA<sup>CA</sup> was unable to grow on the plate containing starch as sole carbon source. With the aim of testing if this reduced growth was specific for growth on starch or if it would apply to other complex carbohydrates, we performed a similar test on other polymers, inulin, xylan and pectin and respective monomeric substrates, fructose, xylose and galacturonic acid (Fig 8). These results show that the HacA<sup>CA</sup> strain is growth impaired when challenged to assimilate nutrients from complex substrates, although this was not so evident when grown on inulin, but growth of the
HacA\textsuperscript{CA} strain was clearly further reduced on xylan and pectin, suggesting that the down-regulation of extracellular enzyme expression is not limited to the amylolytic genes, but also for xylanolytic and pectinolytic genes.

**Figure 7.** Effects of the constitutive activation of the UPR on the utilization of starch and starch related carbon sources. The wild-type strain (HacA\textsuperscript{WT}), the strain containing a constitutive active form of hacA (HacA\textsuperscript{CA}) and the AmyR disruptant (ΔamyR) strain were grown on MM containing 1% of the different carbon sources indicated at 30°C for 3 days.

**Figure 8.** Effects of the constitutive activation of the UPR on the utilization of different polimeric and monomeric carbon sources. The wild-type strain (HacA\textsuperscript{WT}), the strain containing a constitutive active form of hacA (HacA\textsuperscript{CA}) the amyR disruptant (ΔamyR) and inuR disruptant (ΔinuR) strains were grown on MM containing 1% of the different carbon sources indicated at 30°C for 3 days.
5.4. Discussion

5.4.1. Genome-wide gene expression variations upon constitutive activation of HacA

Using a defined A. niger strain bearing a constitutively active form of HacA (HacA\textsuperscript{CA}), the key regulator of the UPR pathway in eukaryotic cells, together with Affymetrix GeneChips technology, we have defined a large set of HacA-responsive genes. Unlike other studies, in which the hacA mRNA splicing is stimulated by the presence of unfolded proteins in the ER by chemicals or by expression of heterologous proteins (Mulder et al., 2004; Guillemette et al., 2007), we used a different approach by creating a strain lacking the 20 nt intron in the hacA gene. To minimize additional effects of expressing the constitutive form of hacA, the hacA\textsuperscript{CA} gene was targeted to its endogenous locus. This contrasts to previous studies in which the constitutive hacA was expressed from a highly-expressed promoter (Valkonen et al., 2003) or expressed from the pyrG locus (Mulder et al., 2009). The microarray data revealed, even under stringent criteria (Benjamini and Hochberg False Discovery Rate at q < 0.005), a large number of differentially-expressed genes (1235 to 1978) upon HacA activation (Table 3). The transcriptomic data obtained in our study reflects the consequences of a constitutive activation of the HacA transcription factor that results in the induction of many genes associated with the secretory pathway (Fig. 5) and related to ER translocation, glycosylation, folding, quality control, ERAD, GPI anchor biosynthesis, vesicle-mediated transport between organelles (ER-Golgi), lipid metabolism, endocytosis, vacuolar sorting. Because of the highly defined conditions (both the defined mutants and the bioreactor controlled cultivations), this study revealed new categories of differentially-expressed genes as well as a much larger number of genes related to each category. Our data are however consistent with previous UPR-related studies in fungal and mammalian cells where many secretory functions are up-regulated by Hac proteins, either directly or indirectly (Travers et al., 2000; Lee et al., 2003; Shaffer et al., 2004; Arvas et al., 2006; Guillemette et al., 2007).

Our results from the transcriptomic study also revealed that constitutive activation had a negative effect on central metabolism as well as on the production of extracellular enzymes. As the global mechanisms for energy generation and cell development are arrested or directed towards up-regulation of the protein secretion machinery, this might account for the unbalanced growth observed in HacA\textsuperscript{CA} in comparison to the HacA\textsuperscript{WT} (Fig. 3). These results suggest an implication for heterologous protein secretion if the protein causes ER stress. Studies on increasing heterologous protein production by enhancing UPR targets are contradictory and vary according to the protein expressed. Although protein-specific effects are likely, most studies were not controlled for the levels of chaperones or foldases co-expressed and it has been shown that there is an optimum level of both BipA (Lombràna et al., 2004) and PdiA (Moralejo et al., 2001).

GO enrichment analysis on the induced set of genes showed that all the well-known UPR target genes related to folding are represented in the HacA\textsuperscript{CA} dataset, and include genes encoding the chaperone BipA, and homologues of LhS1p (An01g13220), P58PK (An11g11250) and Scj1p (An05g00880), as well as the protein disulfide isomerases PdiA, PrpA and TigA.
Glycosylation also appeared as one of the enriched categories. Several aspects of protein glycosylation including the categories of oligosaccharide-lipid assembly, oligosaccharyl transferase complex, UDP-glucose transport, O-linked glycosylation and GPI anchor biosynthesis (Fig. 5), were up-regulated indicating that the cell responds to ER stress by increasing the capacity to glycosylate proteins. The induction of genes associated with lipid metabolism (Supplementary Table S7) suggests a proliferation of the ER to bear the increase of proteins that reside in this organelle, as also indicated in UPR studies of *S. cerevisiae* (Travers *et al.*, 2000).

The elimination of unfolded proteins from the ER involves the ERAD pathway (Nishikawa *et al.*, 2005). Travers and co-workers (2000) demonstrated that up-regulation of ERAD-related genes in *S. cerevisiae* is part of the UPR. These ERAD genes include *DER1* and *HRD3*, *UBC7*, the ubiquitin-related *DOA4*, the proteasome-related *PEX4* and translocon-related *SEC61* (Travers *et al.*, 2000). From the ERAD components defined in *A. niger* (Pel *et al.*, 2007), 11 out of 20 genes are induced in the HacA*CA* strain (Table 4). Furthermore, analysis of the 400 bp of the up-stream regions of *derA* (An15g00640), *sec61* (An03g04340) and An04g06990 (high similarity with a human 1,2-mannosidase) revealed that these genes contain at least one UPRE sequence (Supplementary Table S1C). These results support the connection between the two pathways, as previously suggested (de Virgilio *et al.*, 1999; Travers *et al.*, 2000; Wang *et al.*, 2010; Carvalho *et al.*, 2011) although the mechanistic connection between the two pathways is unresolved.

We compared our datasets with those in Guillemette *et al.* (2007) and found broad agreement with a wide range of up-regulated genes under ER stress conditions. However, Guillemette *et al.* (2007) showed trigger-specific responses that do not complicate our analyses with HacA*CA*. Additionally, we find putative translation initiation factors (Table S1), An18g06260 (highly homologous to the mammalian eIF3), repressed in HacA*CA-1* and An11g10630, An14g01030, An16g06850, An16g05260, An01g06230, An06g01710, An02g12320, An02g12420 and An04g01940 repressed in the other time points (HacA*CA-2* and/or HacA*CA-3*).

### 5.4.2. New leads on the RESS mechanism

The accumulation of misfolded protein in the ER leads to a selective down-regulation of genes encoding secreted proteins in fungi and plants (Pakula *et al.*, 2003; Martínez and Chrispeels, 2003; Al-Sheikh *et al.*, 2004; Wang *et al.*, 2010). This phenomenon is termed REpression under Secretion Stress (RESS). In these studies, associated with the UPR activation by chemical induction is the down-regulation of transcription encoding extracellular enzymes that include cellulases and xylanases in *T. reesei* (Pakula *et al.*, 2003) and glucoamylase in *A. niger* (Al-Sheikh *et al.*, 2004) amongst other genes encoding secreted proteins (Guillemette *et al.*, 2007). The mechanism by which the down-regulation is mediated is unknown, but *glaA* promoter studies in *A. niger* indicated that a promoter region between 1 and 2 kb upstream of translational start is important and a direct mediation of RESS through the UPR was questioned
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Genome-wide expression analysis upon constitutive activation of the HacA bZIP transcription factor in Aspergillus niger reveals a coordinated cellular response to counteract ER stress (Al-Sheikh et al., 2004). RESS has been recognized as an effort from the cells to prevent the entry and overload of newly synthesized proteins into the already “full” ER (Pakula et al., 2003; Al-Sheikh et al., 2004; Wang et al., 2010). In our study, the activation of UPR target genes by introducing the constitutive active form of the HacA transcription factor lead to the down-regulation of not only glucoamylase (glaA), but also other genes coding for starch-degrading enzymes that include acid α-amylase (aamA), α-glucosidases A and B (agdA and agdB) and α-amylase C (amyC), suggesting a down-regulation of the AmyR regulon and sugar transporters (Table 5). A phenotypic demonstration of this down-regulation was provided by the inability of the HacA\textsuperscript{CA} strain to grow on starch (Fig. 7). Growth assays on other polymeric substrates (Fig. 8) suggested that the down-regulation might not to be specific for starch but is relevant to other sugar polymers including xylan (Fig. 8). We speculate that HacA has a role in controlling the transcription of genes that encode the transcriptional activator AmyR (starch), and possibly XlnR (xylan). Such mechanisms include the possibility that HacA binds directly to the amyR promoter region (binding motif to be determined), and serves as a repressor. It will be of interest for future studies to determine the molecular mechanism that results in the downregulation of AmyR and AmyR targets genes in response to HacA activation.

5.4.3. Relation between yeast, filamentous fungi and mammalian UPR counterparts

The mammalian ER contains three types of transmembrane proteins – IRE1, PERK and ATF6 – which sense the accumulation of unfolded proteins and are responsible to activate three different branches of the UPR pathway (reviewed in Malhotra and Kaufman, 2007). Most of the players in the IRE1 pathway are conserved in fungi (Niwa et al., 1999) in which by activation of the transcription factor Hac1/HacA there is an induction of expression of UPR target genes related to the folding machinery (Sidrauski and Walter, 1997; Mulder et al., 2004), but protein homologous to PERK and ATF6 seems absent in fungal systems.

To prevent the influx of proteins into the ER in mammalian cells, a mechanism of translation attenuation is activated that is mediated by PERK. This transcription factor mediates the phosphorylation of eIF2 (eukaryotic translation initiation factor) which in turn leads to the arrest of protein translation. The eIF2 is also required for the translation of selective mRNAs such as the Activating Transcription Factor-4 (ATF4) (Vattem and Wek, 2004). ATF4 is involved in the regulation of UPR genes involved in ERAD, metabolism and apoptosis (Fels and Koumenis, 2006). Gcn4/CpcA are the ATF4 homologues of \textit{S. cerevisiae} and filamentous fungi, respectively. Both \textit{S. cerevisiae} and \textit{A. niger} lack an obvious PERK homologue. Gcn2p phosphorylates eIF2 leading to a global reduction on protein synthesis and stimulation of Gcn4 translation, that has been shown to control amino acid biosynthesis (Hinnebusch, 1993). Although this resembles the PERK function, Gcn2p–eIF2 phosphorylation is only attributed to amino acid starvation and not to ER stress (Harding et al., 2000). In \textit{S. cerevisiae}, the involvement of Gcn2 and Gcn4 in UPR has been shown (Patil et al., 2004). In our transcriptomic profiles, Gcn2 homologue (An17g00860) is not differentially expressed, whereas cpcA (An01g07900) shows \(\approx 2\) fold higher expression in comparison with the wild-type strain.
According to our results the activation of \textit{cpcA} is likely to occur in a Gcn2-independent way and it is tempting to speculate that in filamentous fungi a similar PERK-eIF2-ATF4 pathway may exist. ATF4 is involved in glutathione biosynthesis (Harding \textit{et al.}, 2003) and glutathione-s-transferases have been shown to be up-regulated under ER stress conditions (Gilmore and Kirby, 2004). According to our data, the homologue to human glutathione-s-transferases 3 (An12g03580) is 2-fold induced in HacA$^{CA-1}$ and 2.6 fold induce in the later time points. What we also observe is that as in the case of ATF4-regulated genes, not all the genes involved in glutathione metabolism are affected under secretion stress situation (Harding \textit{et al.}, 2003), as for example asparagine synthase (An01g07910) or glutathione reductase (An03g03660) that are not differentially expressed. Similar results have been observed in \textit{Trichoderma reesei} (Arvas \textit{et al.}, 2006). Another interesting observation is the 4-fold induction of the human homologue RNA-activated protein kinase inhibitor P58 (An11g11250). In mammals, P58 is induced via ATF6, a transcription factor also involved in the regulation of UPR chaperones and apoptosis (no homologue in fungi), and it is an important component on the regulation of PERK-eIF2-ATF4 pathway, attenuating the UPR (van Huizen \textit{et al.}, 2003). The up-regulation of P58 has been shown in studies characterizing the UPR under different conditions (Guillemette \textit{et al.}, 2007; Jørgensen \textit{et al.}, 2009); however, the role and (putative) involvement of a fungi P58 homologue in this pathway remains to be elucidated. ATF6, that induces XBPI (HacA homologue), also possesses the ability to impel lipid biosynthesis and expansion of the ER (Bommiasamy \textit{et al.}, 2009). The identification of these potential regulatory genes involved in mediating the HacA response in this study, has given multiple new leads for further research to better understand the mechanism of how \textit{A. niger} reacts to secretion stress.