Methods for Investigating the UPR in Filamentous Fungi

ARTICLE *in* METHODS IN ENZYMOLOGY · DECEMBER 2011

Impact Factor: 2.09 · DOI: 10.1016/B978-0-12-385114-7.00001-5 · Source: PubMed

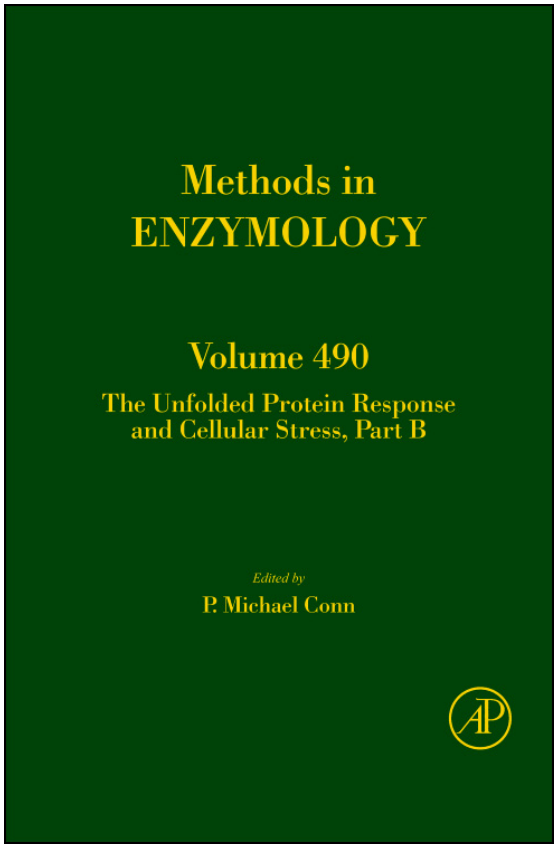
6 AUTHORS, INCLUDING:

Thomas Guillemette
University of Angers
22 PUBLICATIONS **901**
CITATIONS

SEE PROFILE

Arthur Ram
Leiden University
135 PUBLICATIONS **4,862**
CITATIONS

SEE PROFILE



From: Thomas Guillemette, Arthur F. J. Ram, Neuza D. S. P. Carvalho, Aymeric Joubert, Philippe Simoneau, and David B. Archer, Methods for Investigating the UPR in Filamentous Fungi. In Michael Conn, editor: Methods in Enzymology, Vol. 490,
Burlington: Academic Press, 2011, pp. 1-29.
ISBN: 978-0-12-385114-7

CHAPTER ONE

Methods for Investigating the UPR in Filamentous Fungi

Thomas Guillemette,* Arthur F. J. Ram,† Neuza D. S. P. Carvalho,† Aymeric Joubert,* Philippe Simoneau,* *and* David B. Archer‡

Contents

Abstract

Filamentous fungi have a high-capacity secretory system and are therefore widely exploited for the industrial production of native and heterologous proteins. However, in most cases, the yields of nonfungal proteins are significantly lower than those obtained for fungal proteins. One well-studied bottleneck appears to be the result of slow or aberrant folding of heterologous proteins in the ER during the early stages of secretion within the endoplasmic reticulum, leading to stress responses in the host, including the unfolded protein response (UPR). Most of the key elements constituting the signal transduction pathway of the UPR in *Saccharomyces cerevisiae* have been identified in filamentous fungi,

* UMR PAVE No 77, IFR 149 QUASAV, Angers Cedex, France
† Molecular Microbiology and Biotechnology, Kluyver Centre for Genomics of Industrial Fermentation, Institute of Biology Leiden, Leiden University, Leiden, The Netherlands
‡ School of Biology, University of Nottingham, University Park, Nottingham, United Kingdom

Methods in Enzymology, Volume 490
ISSN 0076-6879, DOI: 10.1016/B978-0-12-385114-7.00001-5

including the central activation mechanism of the pathway, that is, the stress-induced splicing of an unconventional (nonspliceosomal) intron in orthologs of the *HAC1* mRNA. This splicing event relieves a translational block in the *HAC1* mRNA, allowing for the translation of the bZIP transcription factor Hac1p that regulates the expression of UPR target genes. The UPR is involved in regulating the folding, yield, and delivery of secretory proteins and that has consequences for fungal lifestyles, including virulence and biotechnology. The recent releases of genome sequences of several species of filamentous fungi and the availability of DNA arrays, GeneChips, and deep sequencing methodologies have provided an unprecedented resource for exploring expression profiles in response to secretion stresses. Furthermore, genome-wide investigation of translation profiles through polysome analyses is possible, and here, we outline methods for the use of such techniques with filamentous fungi and, principally, *Aspergillus niger*. We also describe methods for the batch and controlled cultivation of *A. niger* and for the replacement and study of its *hacA* gene, which provides either a UPR-deficient strain or a constitutively activated UPR strain for comparative analysis with its wild type. Although we focus on *A. niger*, the utility of the *hacA*-deletion strategy is also described for use in investigating the virulence of the plant pathogen *Alternaria brassicicola*.

1. Introduction

Filamentous fungi have conquered an astonishingly wide range of habitats, and individual species may be saprobic, pathogenic, or mutualistic partners (e.g., in lichenous or mycorrhizal associations). Their dispersal may be facilitated by the production and release of numerous spores (sexual or asexual), and colonization of food sources can also occur either more locally or over large areas by growth as a system of branched tubular bodies called hyphae. The expression and secretion of proteins underpin the saprobic lifestyle (e.g., by expressing hydrolase enzymes) and may also be important in virulence (e.g., by cell-surface presentation of adhesins and by secretion of lytic enzymes) and play an essential role in decomposition and recycling of organic matter in nature.

Although there are still deficits in our understanding of the fundamental mechanisms of the secretory pathway in filamentous fungi, the recent availability of the whole genome sequences of fungi (currently, several hundred yeasts and filamentous fungi, either sequenced or in progress), including *Aspergillus* species widely exploited commercially for their secreted enzymes (Machida *et al.*, 2005; Pel *et al.*, 2007), has provided a wealth of information. It is generally accepted that this pathway does not differ fundamentally from those in *S. cerevisiae* and higher eukaryotes, even though there are differences of detail (Geysens *et al.*, 2009). Key differences arise from the hyphal and branching phenotype of filamentous fungi and the

polarity of both growth and protein secretion that occurs primarily at the hyphal tips (Conesa *et al.*, 2001; Fischer *et al.*, 2008; Harris, 2008; Shoji *et al.*, 2008). As in other eukaryotes, the secretory route begins with the entry of secretory proteins into the endoplasmic reticulum (ER) either during or after translation. During transit through the ER, proteins undergo assisted folding and additional modifications such as signal peptide processing, glycosylation, disulfide bond formation, phosphorylation, and subunit assembly. Correctly folded proteins are sorted into coating-protein II vesicles (COPII) and then delivered to the Golgi (or fungal equivalent) for other modifications such as further glycosylation and peptide processing. Finally, the mature proteins are packed again into secretory vesicles and are delivered by exocytosis to the extracellular space at the hyphal tip.

This high-capacity secretory system has driven the commercial exploitation of filamentous fungi as cell factories for provision of native or heterologous enzymes that are used in a wide variety of applications. The progress in genetic manipulation and the availability of gene-transfer systems, combined with process improvements, have led to enhanced yields of native proteins and provided possibilities for improving the yields of heterologous proteins of both fungal and nonfungal origins. However, fungi often fail to secrete the heterologous proteins to the same high level as their own proteins, and this is especially so when the gene donor is not a fungus. Although factors negatively influencing the yield seem to be multiple (Gouka *et al.*, 1997), protein maturation in the ER is regarded as the major bottleneck to achieving high-secreted yields of at least some heterologous proteins from filamentous fungi. Many strategies have been applied to address those limitations and include manipulations of the secretory pathway and, in particular, the ER lumenal environment by overexpression of genes encoding foldases and chaperones or enzymes of the glycosylation machinery. Furthermore, increased gene dosage, optimized codons, use of protease-deficient host strains, expression as translational fusions with an efficiently secreted protein, and introduction of efficient secretion signals or prosequences—have all been used to some advantage (Archer and Turner, 2006; Conesa *et al.*, 2001; Lubertozzi and Keasling, 2009).

In yeast, the ER plays a pivotal role for quality control of proteins by ensuring that correctly folded proteins are delivered to subsequent cellular compartments. A variety of adverse physiological and environmental conditions can disturb ER homeostasis and lead to the accumulation of misfolded proteins. This is the case in expression systems, where there is a high flux of proteins being translocated into the ER. The folding, assembly, and secretion machinery may become saturated, leading to improperly folded structures or protein aggregates that are not secreted. To cope with ER stress, the intracellular signaling pathway, termed the unfolded protein response (UPR), is activated and triggers an extensive transcriptional response (Travers *et al.*, 2000) that increases the protein-folding capacity

within the ER. The UPR is also intimately linked to proteolytic systems that deliver misfolded proteins to vacuoles (autophagy) or to proteasomes (ER-associated degradation—ERAD) for degradation. In yeast, the basic leucine zipper (bZIP)-type transcription factor Hac1p is the transcriptional regulator of the UPR. Hac1p synthesis is dependent on the splicing of an unconventional (nonspliceosomal) intron in the *HAC1* mRNA initiated by the ER-located transmembrane kinase and endoribonuclease Ire1p (Ruegsegger *et al.*, 2001). This splicing event is activated in response to ER stress and relieves a translational block, allowing for the synthesis of Hac1p that regulates the expression of UPR target genes. Most of the key elements constituting the signal transduction pathway of the yeast UPR have been identified in filamentous fungi, and the central activation mechanism of the pathway, that is the stress-induced splicing of an unconventional intron, is conserved among filamentous fungi, yeast, and even mammalian cells (Kohno, 2010). An additional feedback mechanism that leads to selective transcriptional downregulation of some genes that encode secreted enzymes is termed repression under secretion stress (RESS) and has been reported in *Trichoderma reesei* and *Aspergillus niger* (Al-Sheikh *et al.*, 2004; Pakula *et al.*, 2003). Taken together, these ER-stress responses diminish the pool of newly synthesized proteins and provide homeostatic protection for the host cell. Thus, understanding more in detail about the process of protein maturation and secretion-related stress in filamentous fungi may hold the major key to improving their use as cell factories.

The genome sequences of filamentous fungi (including several *Aspergillus* spp.) and the availability of DNA GeneChips have provided an unprecedented resource for exploring expression profiles in response to particular environmental cues, including various secretion stresses (Breakspear and Momany, 2007). Transcriptomics to investigate the UPR in fungi was first used in *Aspergillus nidulans* and *T. reesei* (Arvas *et al.*, 2006; Sims *et al.*, 2005). In *A. nidulans*, the authors reported the effects of recombinant protein secretion by comparing a bovine chymosin-producing strain with its parental wild-type strain by using expressed sequence tag microarrays, which covered approximately one-third of the predicted open reading frames. In *T. reesei*, cultures expressing the heterologous protein tissue plasminogen activator (t-PA) and cultures treated with the reducing agent dithiothreitol (DTT) were analyzed with cDNA subtraction and cDNA-amplified fragment length polymorphism (AFLP). A genome-wide expression analysis of secretion stress responses in *A. niger* was reported by Guillemette *et al.* (2007). In that study, ER-associated stress was induced either by chemical treatment of the wild-type cells with DTT or tunicamycin, or by expressing t-PA. The predicted proteins encoded by most of the upregulated genes functioned as part of the secretory system including chaperones, foldases, glycosylation enzymes, vesicle transport proteins, and ERAD proteins. The authors also investigated translational regulation under ER stress by polysomal

fractionation. Combining proteomic and transcriptomic profiling of the events following protein secretion stress should lead to a better understanding of the molecular basis of protein secretion and provide targets for strain improvement. Indeed, further transcriptomic studies of *A. niger* in relation to hyphal development and branching, and to protein secretion, have been described and illustrate the value of transcriptomic data and, more broadly, integration of data from the transcriptome and proteome (Jacobs *et al.*, 2009; Jorgensen *et al.*, 2009; Meyer *et al.*, 2009).

We describe some of the methodologies that have been used in the genome-wide analysis of protein secretion stress in filamentous fungi, taking *A. niger* as our model organism. We give less emphasis to transcriptomic analyses because methods are well described in several papers already cited but are also evolving rapidly, for example, with the advent of deep sequencing. We therefore devote more coverage to the preparation of samples for analysis, and include cultivation and the construction and use of *hacA*-deletion strains and of constitutive *hacA* mutants. Deletion of *hacA* from *A. niger* has been reported (Carvalho *et al.*, 2010; Mulder and Nikolaev, 2009) and shown to induce a severe phenotype that emphasizes the important role for HacA in the biology of the fungus. Deletion of *HAC1* was also achieved in the dimorphic fungus *Candida albicans* (Wimalasena *et al.*, 2008), and the deletion strain was less able to produce filaments and was down-regulated for expression of some cell-surface adhesins, suggesting that Hac1p would have a role in the invasive virulence of *C. albicans*. The *hacA* gene was deleted from another fungal pathogen, *Aspergillus fumigatus*, and virulence tests showed that the knockout strain was indeed attenuated (Richie *et al.*, 2009). Because of the importance of Hac1/A in virulence, we also provide an example of deleting the gene from a plant pathogen, *Alternaria brassicicola*.

2. Conditions to Study the UPR in Filamentous Fungi

In this section, we discuss the growth conditions and ER-stress-inducing methods that can be used with *A. niger* for producing fungal material destined for transcriptomic or polysome analyses.

2.1. Culture conditions

Filamentous fungi can grow either in pellets or as a dispersed mycelium. For our transcriptomic studies on UPR, a dispersed growth of the mycelium is preferred as this reduces heterogeneity of the mycelia. Growth in pellets is caused by an early aggregation of conidial spores and, after subsequent germination of the spores, micropellets are formed. Upon further growth,

these pellets increase in size which results quickly in low oxygen concentration in the center of the pellet, which increases heterogeneity. With the protocol indicated below, reproducible dispersed growth of mycelia can be obtained.

In our UPR studies, we have used two methods of cultivation to perform transcriptomic studies. The choice of cultivation, either a batch culture, or a chemostat culture, depends on the purpose. We have used controlled batch cultivations to examine the response of *A. niger* cells to drugs that interfere with protein folding. This protocol is of course not limited to study the effect of UPR-inducing compounds, but can also be used to study the effect of other compounds that affect fungal growth (Meyer *et al.*, 2007). For these studies, spores were germinated for 5 h which resulted in the formation of a small (20 μm long) germtube, providing a more uniform population of cells than older, morphologically more heterogeneous, cells. At this stage, the antifungal compound is added.

Controlled batch cultivations can also be used to compare gene expression between different mutants. One important consideration here is that for a reliable comparison, the maximum growth rate during batch growth of both strains should be identical. Before performing transcriptomic studies on different mutants, it is important to establish that both mutants have an identical maximum specific growth rate. If not, the transcriptomic study will not only identify differences related to gene expression directly caused by the mutation, but will also identify differences related to a different growth rate. To prevent difference in growth rate to be reflected in the transcriptome, continuous cultivations should be performed in which the growth rate can be controlled (Jorgensen *et al.*, 2009). Here, we provide detailed conditions about controlled batch cultivation of *A. niger* in bioreactors.

2.1.1. Required materials

2.1.1.1. Devices of the bioreactor apparatus

Bioreactor cultivations are performed in Bioflo3000 bioreactors (New Brunswick Scientific). Using the NBS Biocommend software, the pH, temperature-dissolved oxygen tension and agitation can be controlled and monitored. The pH is measured using an autoclavable glass electrode (Mettler Toledo) and the dissolved oxygen tension is measured with an InPro-6000 series O_2 sensor (Metller Toledo). For measuring the content of CO_2 and O_2 in the exhaust gas, a Xentra 4100C Gas Purity analyzer (Servomex BV, the Netherlands) is used. To minimize wall growth in the head space of the reactor, the glass surface of the head space is cooled by tubing through which cold water is flowing.

2.1.1.2. Additional materials

- *Strains of interest*: The *A. niger* strains used in our UPR studies are all derived from the N402 strain. This strain is a UV-mutagenized derivative

of strain N400 that produces short conidiophores and is often used as a starting strain for genetic modifications (Guillemette *et al.*, 2007; van Hartingsveldt *et al.*, 1987). In our bioreactor controlled cultivations that are used for transcriptomic studies, only prototrophic strains are used to prevent effects on gene expression as a result from supplementation of any auxotrophy, for example, by uridine.

- *Growth medium*: A defined minimal medium (MM) is used to perform bioreactor cultivation. The growth-limiting component of the medium is the carbon source (0.75%, w/v). The MM contains the following: 7.5-g glucose, 4.5-g NH_4Cl, 1.5-g KH_2PO_4, 0.5-g KCl, 0.5-g $MgSO_4 \cdot 7H_2O$, and 1-ml trace metal solution per liter. The trace metal solution contains per liter, 10-g EDTA, 4.4-g $ZnSO_4 \cdot 7H_2O$, 1.01-g $MnCl_2 \cdot 4H_2O$, 0.32-g $CoCl_2 \cdot 6H_2O$, 0.315-g $CuSO_4 \cdot 5H_2O$, 0.22-g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.47-g $CaCl_2 \cdot 2H_2O$, and 1-g $FeSO_4 \cdot 7H_2O$ (modified from composition given by Vishniac and Santer (1957). The pH is adjusted to 3. The carbon source is heat-sterilized separately from the MM. Germination of conidial spores is improved by adding 0.003% (w/w) yeast extract to the culture medium.

  Complete medium plates contain per liter 10-g glucose, 6.0-g $NaNO_3$, 1.5-g KH_2PO_4, 0.5-g KCl, 0.5-g $MgSO_4 \cdot 7H_2O$, 1.0-g casamino acids, 5.0-g yeast extract, 20-g agar, and 1-ml trace metal solution.

2.1.1.3. Other reagents

- Detergent solution (Tween80 0.05%, w/v, NaCl 0.9%, w/v)

2.1.1.4. Disposables

- Petri dishes

2.1.2. Inoculation

Conidia for inoculation of bioreactor cultures are harvested from solidified complete medium plates with sterile detergent solution and sterile cotton sticks to scrape off the spores. Bioreactors containing 5 l of MM are inoculated with 1×10^9 conidia/l. During cultivation, the temperature is maintained at 30 or 37 °C and the pH is kept at 3, by computer-controlled addition of 2-*M* NaOH or 1-*M* HCl. During the first 6 h of cultivation, the culture is aerated through the head space of the reactor vessel and the stirrer speed is kept low at 250 rpm to prevent hydrophobic conidia escaping from the water. After 6 h, allowing for germination of the conidia (now hydrophilic), sterile air is sparged into the culture and mixing is intensified (750 rpm). During the growth, the dissolved oxygen tension is kept above 40% of air saturation at any time, ensuring sufficient oxygen for growth.

2.1.2.1. Short batch cultivations To examine transcriptomic responses to growth-disturbing compounds or antifungals, germlings are grown for 5 h at 37 °C before addition of the compound. Small volume sampling (10 ml) can be performed at various times after exposure, and we normally sample at 15-min intervals for microscopic analysis. Large sampling volumes (400 ml) of culture are taken after 1 or 2 h after addition of the antifungals, and mycelial samples are quickly harvested via filtration and frozen using liquid nitrogen. Sampling of a 400-ml culture yields enough biomass for RNA extraction to perform Affymetrix microarray analysis (Meyer *et al.*, 2007). DDT and tunicamycin are the most commonly used compounds to induce an UPR response. In common with other antifungals, the effect of the response is dependent on the concentration of the compound and the concentration of the cells. To study transcriptomics responses, we use sublethal concentrations of the antifungal compound and monitor the response of the cells shortly after adding the compound (within 1 h) to minimize secondary effects. Concentrations of the antifungal drug used should be empirically determined.

2.1.2.2. Prolonged batch cultivation After 6 h of cultivation and the start of sparger aeration and increasing the stirrer speed to 750 rpm, 0.01% polypropyleneglycol (PPG 2000, Fluka Chemika) is added to the medium as an antifoam agent. Acidification of the culture and the amount of NaOH used to maintain the pH at 3 is used as an indirect measure for growth. Dissolved oxygen tension is always above 40% of air saturation at any time ensuring oxygen-sufficient growth. During cultivation, samples are drawn regularly to monitor culture growth. Medium can be analyzed for residual carbon source and extracellular protein concentrations are determined to calculate protein production yields. Dry weight biomass concentration in the culture is determined by weighing lyophilized mycelia from a known weight of culture. Mycelium is separated from the culture broth by filtration through a GF/C glass microfiber filter (Whatman) and dried. The end of the batch phase (depletion of glucose) can by monitored by plotting biomass concentrations as well as monitoring the alkali addition. An example of a typical growth profile of a batch culture of *A. niger* is shown in Fig. 1.1.

2.2. Induction of ER-associated stress

The UPR is classically induced by treating mycelium cultures with two chemical agents which disrupt protein folding in the ER. DTT is a strong reducing agent that disturbs the oxidative environment of the ER and prevents disulfide bond formation. Tunicamycin is a drug produced by *Streptomyces lysosuperificus*, which inhibits N-linked glycosylation by preventing core oligosaccharide addition to nascent polypeptides and thereby blocks protein folding and transit through the ER. To assess the UPR under

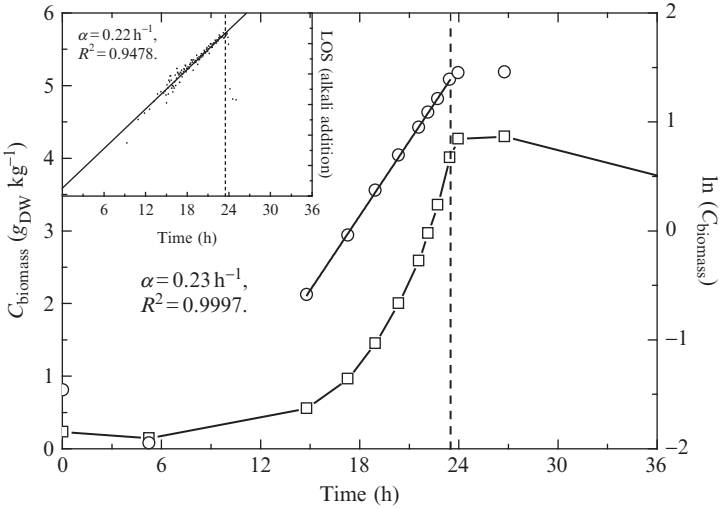


Figure 1.1 Growth profile of *A. niger* wild-type batch culture. Dry weight biomass concentration (g_{DW} kg^{-1}) as a function of time (h) illustrates the growth of the cultures. The maximum specific growth rate is determined from the slope (α) of the ln transformation of biomass ($C_{biomass}$) in the exponential growth phase as a function of time (h), as well from log transformation of alkali addition as a function of time (h) (see insert). Dash-line represents the end of the exponential growth phase (depletion of glucose).

conditions of heterologous protein production, expression profiles from a recombinant protein-producing strain and its parental wild-type strain can be compared. Thus, we have used a strain-producing recombinant t-PA, a serine protease. We recommend the use of at least two distinct UPR inducer treatments since discrepancy between responses is sometimes obtained. We know in particular that DTT has a variety of other effects on the cell and triggers a relatively large overall variation in gene expression levels compared to other treatments. The responses to each stress are then compared and the overlaps common to these conditions should lead to the identification of robust sets of UPR-regulated genes.

Before performing transcriptomic or polysome analysis, we confirm that each of the stress conditions leads to induction of the UPR by examining the transcriptional induction (by northern hybridization or real-time PCR) of genes known to be affected by ER stress, such as the ER chaperone-encoding gene *bipA* and the foldase-encoding gene *pdiA* (data not shown). The splicing of the *hacA* transcript also needs to be examined. This could be done from RNAs derived from treated or untreated cultures by using different methods: (i) by conventional RT-PCR with primers designed across the atypical intron (Guillemette *et al.*, 2007), (ii) by northern hybridization by using a cDNA fragment comprising the region from the start

codon up to the unconventional intron as a probe (Mulder *et al.*, 2004), (iii) by real-time RT-PCR by using a specific primer designed to span the atypical 20-bp intron (Fig. 1.2). A quantitative RT-PCR method was

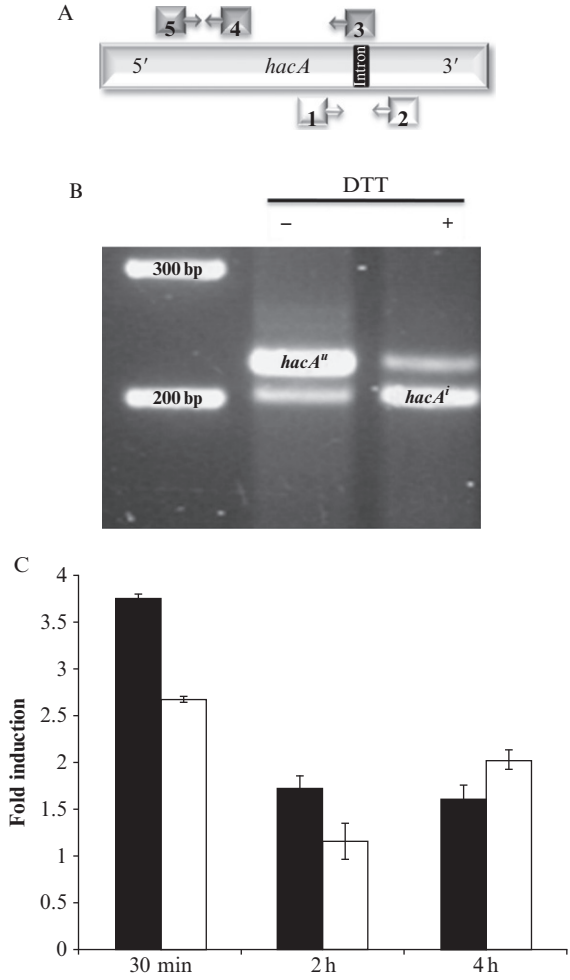


Figure 1.2 Analysis of the splicing of the *hacA* transcript in *A. niger* by PCR technology. (A) Schematic representation of the *hacA* ORF showing the positions of primers used for RT-PCR analysis. (B) Conventional RT-PCR analysis of *hacA* processing with primers 1 and 2 designed across the intron in the presence (+) or absence (−) of 20-m*M* DTT for 1 h. (B) Analysis of *hacA* expression by means of real-time RT-PCR in a wild-type strain during DTT exposure (20 m*M*) for 0.5, 2, and 4 h. The white box represents the fold induction observed with primers 4 and 5 designed for amplifying the cDNA derived from *hacA* mRNA (spliced and unspliced). The black boxes represent the fold induction observed with primers 1 and 3 designed to span the atypical 20-bp intron and are therefore specific for the spliced form, $hacA^{i}$.

initially developed to measure the splicing ratio of the mammalian *XBP1* mRNA (Hirota *et al.*, 2006) and was also recently succesfully applied to filamentous fungi (Joubert *et al.*, unpublished). The results in Fig. 1.2 clearly show an increase in the abundance of a shorter product following ER stress, indicating that treatment (DTT in this case) induces the conversion of the unspliced *hacA* mRNA ($hacA^{u}$) into the spliced form ($hacA^{i}$).

ER-stress reagents (DTT or tunicamycin) are added to fungal cultures. DTT stocks are prepared in water and the tunicamycin stock in DMSO. DTT or tunicamycin is added to the liquid medium at a final concentration of 20 m*M* or 10 μg/ml, respectively. Note that these concentrations are 10-fold higher than those routinely used with yeast. Control cultures have an equivalent volume of sterile water or DMSO added. In *A. niger*, expression analyses show that both upregulation of UPR target genes and appearance of the active *hacA* mRNA emerge within 30 min after exposure of the mycelium to DTT, and are still observed 2 h after adding the DTT (Guillemette *et al.*, 2007; Mulder *et al.*, 2004). Like DTT treatment, tunicamycin treatment and production of the recombinant t-PA lead to the activation of the UPR, although the effect appears later (1 h after induction of the ER stress) than in the DTT-treated mycelia.

3. Analysis of Expression During the ER Stress

3.1. Transcriptomic analysis

The genome sequences of two different strains of *A. niger* have underpinned the construction of two different microarrays in the Affymetrix format (Andersen *et al.*, 2008; Pel *et al.*, 2007), and one of those arrays is a trispecies array affording comparsions between *A. niger*, *A. nidulans*, and *Aspergillus oryzae* (Andersen *et al.*, 2008). The Affymetrix protocols for transcriptomics and analysis are available on-line and have proved to be very effective for transcriptomic studies with *A. niger* (Guillemette *et al.*, 2007; Jorgensen *et al.*, 2009; Vongsangnak *et al.*, 2010). Despite the availability of GeneChips for *A. niger*, other array formats have been used for *A. niger* and many other species of filamentous fungi and so, it is not feasible to present a preferred method here. Instead, we advise that the cited papers are referred to for *A. niger*, and methods for other formats and fungal species are explored through the literature. The advent of nonarray methodologies for genome-wide transcriptomics brings new opportunities and these methods are beginning to be applied to filamentous fungi. There are various platforms available for the so-called deep sequencing (Bashir *et al.*, 2010) and, while such methods have been applied in the sequencing of fungal DNA, for example, in the comparison of mutant strains (e.g., Le Crom *et al.*, 2009), it is the use of RNA sequencing that concerns us here.

Transcript sequencing holds some advantages over array approaches by providing, for example, quantitative comparisons of the abundance of different transcripts and in the detection and quantification of transcript variations, for example, in splicing (Wang *et al.*, 2009). We anticipate reports of using such approaches to investigate further the UPR in filamentous fungi.

3.2. Polysome analysis

Expression profiling data are more meaningful when mRNA samples are enriched for transcripts that are being translated (Pradet-Balade *et al.*, 2001). This can be achieved by fractionation of cytoplasmic extracts in sucrose gradients, based on the methods described for polysome analysis by Arava (2003). This method involves size separation of large cellular components and monitoring the A_{254} across the gradient. It enables the separation of free mRNPs (ribonucleoprotein particles) from mRNAs fully loaded with ribosomes (i.e., polysomes). Polysomes represent actively translated transcripts and this fraction is directly correlated with the set of *de novo* synthesized proteins in a particular cellular state, enabling the determination of the translation efficiencies that are characteristic for each transcript in a cell (Smith *et al.*, 1999). In addition, changes in the distribution of a given mRNA indicate how this translational efficiency can vary under different conditions. Because it is generally accepted that translational control predominantly occurs at the initiation step (McCarthy, 1998), the number of mRNA molecules engaged in polysomes should be a robust indicator of the synthesis rate of the corresponding protein. To examine the effect of secretion stress on net translational activity in *A. niger*, we provide a detailed description of the method to analyze the global relative distribution of ribosomes between polysomes, 80S monosomes, and dissociated 40S and 60S subunits.

3.2.1. Required materials

3.2.1.1. Devices and materials of the density gradient fractionator apparatus RNA fractionation is performed with the Foxy Jr. Fraction Collector (TELEDYNE ISCO). RNA samples are loaded onto prepared gradients and spun in a ultracentrifuge. Once the spin is completed, the system allows you to fractionate and quantitate centrifuged zones with precision. The ISCO Density Gradient System produces a continuous absorbance profile as the gradient is collected in precisely measured fractions. Fractionation is performed by introducing a dense chase solution into the bottom of the centrifuged tube, raising the gradient intact by bulk flow. Chase solution is injected by piercing the bottom of the tube. System includes tube piercing stand, peristaltic pump, UA-6 Detector with 254

and 280 nm filters, density gradient flow cell, and Foxy Jr. Fraction Collector.

3.2.1.2. Additional materials

- Ultracentrifugation is performed in an Optima™ MAX-XP Benchtop Ultracentrifuge or an Optima™ MAX High-Capacity Personal Micro-Ultracentrifuge (Beckman Coulter) with an MLA-80 rotor. Alternatively, you can use a Beckman SW41 Ti rotor.
- Mortar and pestle, needle

3.2.1.3. Other reagents

- Polysome extraction buffer (20-m*M* Tris–HCl, pH 8.0, 140-m*M* KCl, 1.5-m*M* $MgCl_2$, 1% Triton X-100, 0.1-mg/ml cycloheximide, 1.0-mg/ml heparin, 0.5-m*M* DTT)
- Chase solution (80% sucrose solutions prepared in 50-m*M* Tris–acetate, pH 7.0, 50-m*M* NH_4Cl, 12-m*M* $MgCl_2$, 1-m*M* DTT)
- TE (10-m*M* Tris–HCl, pH 8.0, 0,1-m*M* EDTA))

3.2.1.4. Disposables

- 8-ml centrifuge tubes, 2-ml (Eppendorf) tubes

3.2.2. Sample preparation

Ribosomal fractions are prepared according to the method described for polysome analysis (Arava, 2003) modified as follows: At the time of harvest, cycloheximide is added to a final concentration of 0.1 mg/ml to trap elongating ribosomes. The cultures are swirled rapidly and chilled on ice for 10 min. Fungal material is pelleted by centrifugation at 11,000×*g* for 10 min at 4 °C. The pellet is then resuspended in 5 ml of polysome extraction buffer and sedimented. This washing step is repeated, and cells are frozen in liquid nitrogen and stored at −80 °C. Approximately 0.25 g of cells are ground in liquid nitrogen with a mortar and pestle, and the powder is resuspended in 750 μl of ice-cold polysome extraction buffer. Excess cell debris are removed by sedimentation at 4000×*g* for 5 min at 4 °C. The supernatant is clarified by further centrifugation (12,000×*g*, 10 min, 4 °C).

3.2.3. Sucrose gradient preparation

We prepare 15–70% sucrose density gradients for ultracentrifugation without using a gradient maker. Sucrose solutions are prepared in 50-m*M* Tris–acetate (pH 7.0), 50-m*M* NH_4Cl, 12-m*M* $MgCl_2$, and 1-m*M* DTT. 1.3 ml of the lighter sucrose solution (15%) is laid on the bottom of the centrifuge tubes using a long needle. 2.6 ml each of 30% and 50% sucrose,

and finally, 1.3 ml of 70% sucrose are then carefully underlaid without disturbing the interfaces. The tubes are closed with parafilm and stored at 4 °C for at least 12 h before use to allow the gradients to thaw and diffuse to create a continuous gradient.

3.2.4. RNA fractionation

Each sample is loaded on an 8-ml 15–70% (w/v) sucrose gradient and sedimented at 150,000×*g* (55,000 rpm) and 4 °C in a Beckman MLA-80 rotor for 135 min. The gradient is fractionated with the density gradient fractionator while monitoring the absorbance at 254 nm. 0.5 ml fractions are collected from the top of the gradient directly into a 1-ml volume of 6-*M* guanidine hydrochloride. RNA is precipitated by adding an equal volume of 100% ethanol and resuspended in TE. The RNA is again precipitated by the addition of 50 µl of 3-*M* sodium acetate (pH 5.2) and 1 ml of 100% ethanol, and resuspended in TE. For RNAs destined for microarray analyses, the polysomal fractions and nonpolysomal fractions are pooled, respectively. Each fraction is treated with RQ1 RNase-Free DNase (Promega) and is again cleaned up by applying the samples to an RNeasy mini column (Qiagen). An aliquot of the RNA solution is extracted from each fraction and subjected to electrophoresis through a formaldehyde gel (Fig. 1.3).

4. Modifying the UPR Signaling by Targeted Gene Replacement

4.1. Construction of UPR-deficient strains

As described in Section 1 in more detail, key proteins in UPR signaling are conserved in eukaryotic cells. They include the ER-localized transmembrane protein Ire1/IreA and transcription factor Hac1/HacA. Genes homologous to *IRE1* and *HAC1* are usually recognized by bidirectional Blast searches. Gene disruptions are often used to study the UPR, and genes encoding Hac1/HacA and Ire1/IreA are common targets for disruption. The most commonly used selection markers for making gene disruptions in *A. niger* include the counter selectable markers *pyrG* and *amdS*, and the hygromycin resistance marker (the *hph* gene from *Escherichia coli*). The use of hygromycin as a selection strategy is widely used for transformation of filamentous fungi as it does not require the isolation of an auxotrophic mutant (e.g., the isolation of a uridine/uracil requiring *pyrG* mutant).

Deletion of the *hacA* gene with the hygromycin resistance marker has been reported in both *A. niger* and *A. fumigatus* (Mulder and Nikolaev, 2009; Richie *et al.*, 2009) and in *A. niger* using the *pyrG* gene (Carvalho *et al.*, 2010). Several approaches are available for constructing gene deletion cassettes which include; (i) traditional PCR amplification and cloning of

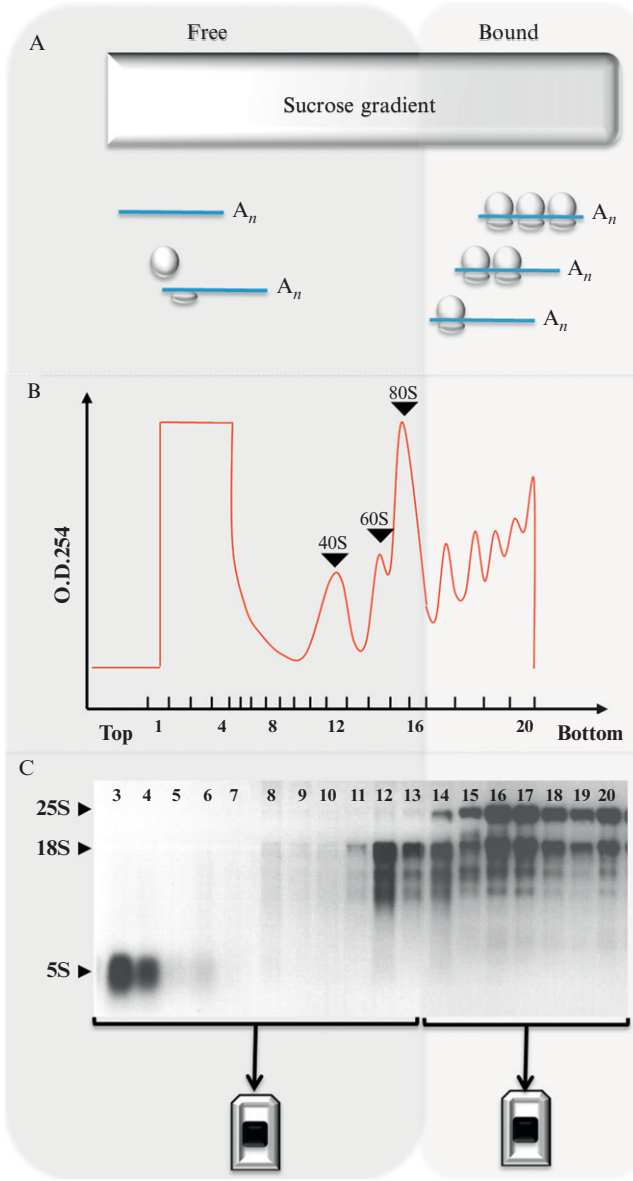


Figure 1.3 Schematic steps for polysome analysis. (A) Cytoplasmic extracts from *A. niger* cells are fractionated in 15–70% sucrose gradients. It enables the separation between free mRNAs and those loaded with ribosomes and engaged in translation. (B) Representative absorbance profile for RNA separated by velocity sedimentation through the sucrose gradient. For each fraction, absorbance at 254 nm is monitored using a UA-6 UV detector. The positions of the 40S, 60S, 80S, and polysomal peaks are indicated. The expected peaks for the tRNAs and other small RNAs in fractions 1–4 are

flanking regions on both sites of the selection markers, (ii) fusion PCR approaches to generate the entire deletion cassette by PCR, (iii) the use of Multisite Gateway recombination (Invitrogen) for the *in vitro* recombination of flanking regions and the selection markers or, (iv) the use of *S. cerevisiae* for *in vivo* recombination. It is beyond the scope of this review to discuss the different approaches to generate disruption cassettes, but in principle, all approaches are suitable. In this chapter, we include a Fusion PCR protocol which is routinely used by the authors to construct gene deletion cassettes and has been used to disrupt the *hacA* gene in *A. brassicicola* (Joubert *et al.* unpublished).

4.1.1. Required materials

4.1.1.1. Materials

- Thermocycler

4.1.1.2. Other reagents

- High-Fidelity DNA Polymerase
- Plasmid pCB1636 (Sweigard *et al.*, 1995)

4.1.1.3. Disposables

- 0.7-ml (Eppendorf) tubes

4.1.2. Construction of a gene replacement cassette for use in *A. brassicicola*

Deletion of *hacA* in *A. brassicicola* was accomplished by replacing the *hacA* ORF with a hygromycin B resistance cassette. Gene replacement occurs via homologous double crossover between a linear construct and the target genomic locus. The linear construct flanks the resistance cassette (that contains the *hph* gene under control of the *trpC* fungal promoter) with two fragments representing 5′ and 3′ regions of the target gene, and is constructed by using a PCR-assisted DNA assembly called double-joint PCR (Fig. 1.4; Yu *et al.*, 2004). For all the PCR steps, we highly

obscured by the high absorbance, presumably from proteins and detergents used in the preparation. (C) RNA is then extracted from each fraction and an aliquot is subjected to electrophoresis through a formaldehyde gel. As expected, 25-S and 18-S ribosomal RNAs were the prominent species. Before processing mRNA to cDNA, labeling, hybridization to *A. niger* Affymetrix GeneChips (Affymetrix, Inc., Santa Clara, CA), the polysomal fractions and nonpolysomal fractions can be pooled, respectively. Each pooled fraction is then treated with RQ1 RNase-Free DNase (Promega) and is again cleaned up by applying the samples to an RNeasy mini column (Qiagen).

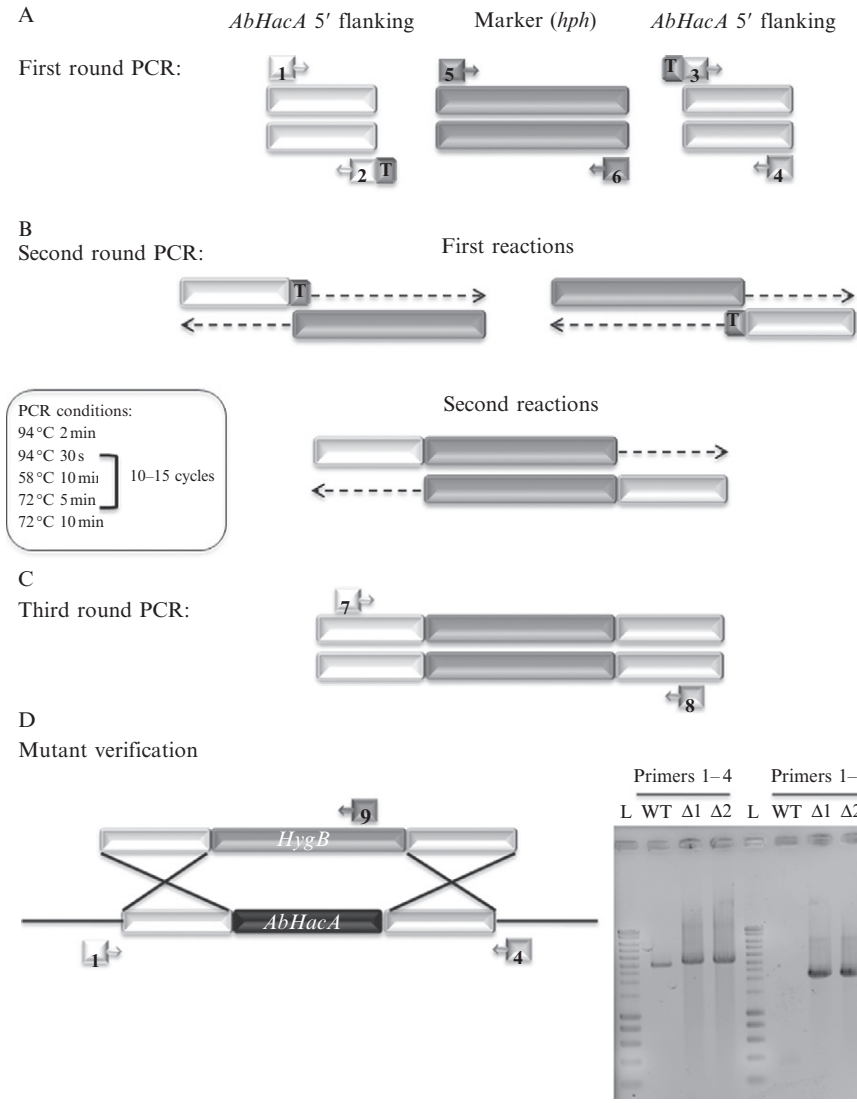


Figure 1.4 Schematic representation of the construction of a gene replacement cassette and verification of the mutants. The arrows numbered from 1 to 9 represent primers for the PCRs and primers 2 and 3 are 45–60 bases-long chimeric primers. (A) First-round PCR: amplification of the components using the specific and chimeric primers. A typical reaction will fuse DNA fragments of a 5′ flanking sequence, a 3′ flanking sequence, and a selectable marker (*hph* gene). Primers 2 and 3 carry 24 bases of homologous sequence overlapping with the ends of the selectable marker of choice. (B) Second-round PCR: the assembly reaction is carried out without using any specific primers, as the overhanging chimeric extensions act as primers. The first two cycles are shown in detail. (C) Third-round PCR: amplification of the final product using nested

recommend the use of a High-Fidelity DNA Polymerase. A typical reaction fuses DNA fragments of a 5′ flanking sequence (amplified with the primers 1 and 2), a 3′ flanking sequence (amplified with the primers 3 and 4), and the selectable marker. Here, the marker corresponds to the hygromycin resistance cassette (1436 bp) that is amplified with the primer M13F and M13R from the plasmid pCB1636 (Sweigard *et al.*, 1995). Primers 2 and 3 carry 24 bases complementary to M13F and M13R sequences, respectively, overlapping with the ends of the resistance cassette. During the first round of PCR, amplifications of 5′ flanking region, marker, and 3′ flanking region are separately carried out. The amplicons are then purified using a commercially available PCR cleanup kit. The three amplicons are mixed in 1:3:1 molar ratio, the total DNA amount of the three components being between 100 and 1000 ng. These three DNA fragments are specifically joined together during the second-round PCR. The assembly reaction is carried out without adding any specific primers, the overhanging chimeric extensions acting as primers. In the third round of PCR, the final PCR construct is amplified with a nested primer pair (primers 5 and 6). We can also use the first-round primer pair (primers 1 and 4) but the yield of amplicons is often lower and we also get PCR artifacts more easily. For round 3 fusion PCR, we generally use 1–2 μl of the purified round 1 products as template. The final amplicon is purified and further concentrated in a volume of 10 μl to a concentration of at least 1 μg/μl.

4.2. Constitutive activation of the UPR

Activation of the HacA transcription factor includes the unconventional splicing of an intron from the *hacA*^{u} mRNA, creating a transcriptionally active form of HacA. This mechanism allows a straightforward way to construct strains with a constitutively activated form of HacA and has been used in several *Aspergillus* species to generate strains with a constitutively activated UPR. Valkonen *et al.* (2003) reported the construction of an *Aspergillus awamori* strain which expressed the *hacA*^{i} cDNA lacking the 20 nt unconventional intron and including a 150-bp truncation at the 5′ end of the mRNA. In that study, the active form of *hacA* was expressed under

primers 7 and 8. (D) Confirmation of gene replacement: transformants are randomly picked and examined for double crossover-mediated gene replacement pattern by PCR amplification of the *hacA* locus using the primer pairs 1/4 or 1/9. The primer 9 is designed inside the *hph* sequence. As shown, amplicons of wild-type and deletion alleles of *hacA* obtained with primer pairs 1/4 differ in size. No amplicon is obtained from wild-type matrix when using the primer 9. The results are shown for two mutants called Δ1 and Δ2. Molecular sizes (kb) were estimated based on a 1-kb ladder (lane L, Eurogentec, Seraing, Belgium).

control of the highly expressed and inducible glucoamylase promoter. Mulder and Nikolaev (2009) constructed a constitutive HacA strain by expressing *hacA*^{i}, lacking the 20 nucleotide intron, in a *hacA* deletion background. The site of integration of the introduced *hacA*^{i} was controlled by using the *pyrG*⋆approach (van Gorcom and van den Hondel, 1988). Finally, we recently used an approach to replace the wild-type *hacA* gene with the active *hacA*^{i} form that lacks the 20 nucleotide intron at the *hacA* locus (Carvalho and Ram, unpublished). In all the three studies, the expression of the active form of HacA resulted in constitutive activation of the UPR pathway. Activation of the UPR pathway is often monitored by examining the expression level of UPR target genes such as *bipA* and *pdiA*. The latter approach in which the wild-type gene (*hacA*^{u}) is replaced by a mutated and constitutively active form (*hacA*^{i}) of the gene at the endogenous locus is an approach of general interest and is therefore described in this chapter.

4.2.1. Required materials

4.2.1.1. Materials

- Thermocycler

4.2.1.2. Additional materials

- *Strain of interest*: Use of *Ku70* mutant dramatically increases the frequency of homologous recombination and reduces the number of transformants to be analyzed in order to find a transformant in which the cassette is integrated at the endogenous locus.

4.2.1.3. Other reagents

- High-fidelity DNA polymerase
- Plasmid PBluescript-SK
- Restriction enzymes

4.2.1.4. Disposables

- 0.7-ml (Eppendorf) tubes

4.2.2. Replacement of *hacA*^{u} by a constitutive *hacA*^{i} at the endogeneous locus in *A. niger*

To construct the replacement cassette, primers are designed to amplify various fragments, as depicted in Fig. 1.5. Three fragments, consisting of the *hacA*^{u} gene, encoding promoter and terminator regions, the *Aspergillus oryzae pyrG* selection marker, and the *hacA* terminator region are amplified

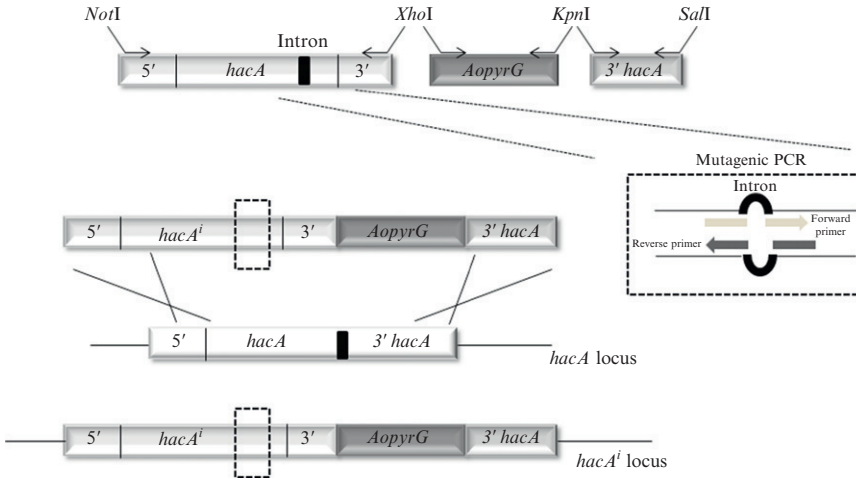


Figure 1.5 Schematic representation of the construction of a cassette for obtaining a constitutive UPR strain. Three fragments containing the *hacA* locus, the *Aspergillus oryzae pyrG* gene, and the *hacA* terminator region are amplified by PCR and ligated into a standard cloning vector (pBluescript-SK) using appropiate restriction enzymes. This plasmid is used as a template for site-directed mutagenesis using two complementary primers lacking the intron sequence. Transformants containing the construct lacking the intron ($hacA^i$) at the *hacA* locus can be selected by Southern blot analysis and the lacking of the intron can then be confirmed by PCR analysis.

by PCR and cloned into pBluescript-SK using appropriate restriction enzymes. The *A. oryzae pyrG* gene and flanking regions (de Ruiter-Jacobs *et al.*, 1989) are used to prevent homologous recombination of the final construct at the *A. niger pyrG* locus. The plasmid containing the $hacA^u$ ORF is used as a template to introduce the $hacA^i$ gene by site-directed mutagenesis according to the Quick Change II site-directed mutagenesis protocol (Stratagene). Overlapping complementary primers lacking the intron sequence are used to obtain the $hacA^i$ gene in which the intron is deleted. To prevent single crossover of the construct, the construct should be linearized before transformation into an *A. niger ku70* mutant (Meyer *et al.*, 2007). Southern blot analysis should be performed to confirm integration of $hacA^i$ at the endogenous locus. As depicted in Fig. 1.5, the *A. oryzae pyrG* marker is flanked by repeats of the *hacA* terminator region. The direct repeat flanking the *AopyrG* gene allows efficient looping out of the *pyrG* marker after subjecting the strain to counter-selection using 5-fluoro-orotic acid (5-FOA). A detailed protocol for 5-FOA counter-selection has been published recently(Meyer *et al.*, 2010). The removal of the *pyrG* gene from the *hacA* locus completely restores the wild-type context of the *hacA* locus, and the *pyrG* auxotrophy can be used for transform additional constructs into this strain.

4.3. PEG-mediated transformation of protoplasts

The establishment of an effective transformation method for the filamentous fungi is of crucial importance to examine gene functions. At the moment, protoplast-mediated transformation and *Agrobacterium*-mediated transformation are the most commonly used techniques. As a detailed protocol for *Agrobacterium*-mediated transformation of *Aspergillus* sp. has been published recently (Michielse *et al.*, 2008), we describe here a protocol that we routinely use for the transformation of *A. brassicicola* protoplasts. This protocol can be used for many other filamentous fungi, providing that the enzyme solution is adapted to the targeted fungus. Indeed, the yield of protoplasts in fungi depends mainly of the composition of the lytic enzyme batch used. *Alternaria* spp. are members of the *Dothideomycetes* and they commonly present a highly melanized cell wall that requires specific enzyme mixture.

4.3.1. Required materials

4.3.1.1. Materials

- Thoma counting chamber
- 250-ml flasks

4.3.1.2. Other reagents

- Enzyme-osmoticum is prepared in 0.7-*M* NaCl and contains kitalase (Wako Chemicals, Richmond, VA, USA) at 10 mg/ml and driselase (Interspex) at 20 mg/ml.
- STC buffer (1.2-*M* sorbitol, 10-m*M* Tris–HCl, pH 7.5, and 50-m*M* $CaCl_2$)
- PEG solution (MW 3350–4000) 60%, 10-m*M* Tris–HCl, pH 7.5, and 50-m*M* $CaCl_2$)
- *Regeneration medium*: you need to prepare the flasks A, B, C, and autoclave them separately. Combine them after autoclaving and hold at 55 °C.
  - *Flask A*: 1-g yeast extract, 1-g casein hydrolysate, water to 50 ml
  - *Flask B*: 342 g sucrose, water to 500 ml
  - *Flask C*: 16-g agar, water to 450 ml

4.3.1.3. Disposables

- Petri dishes
- 50-ml tubes and 10-ml tubes

4.3.2. Protoplast-mediated transformation of *A. brassicicola*

Transformation is carried out with linear PCR products based on the transformation protocol for *A. alternata* (Akamatsu *et al.*, 1997), with modifications. Approximately 5×10^6 fungal conidia are harvested from a potato dextrose (PD) agar culture plate and introduced into 50 ml of PD broth media. They are cultured for 16 h at 25 °C with shaking at 100 rpm. The fungal material is harvested by centrifugation at 3500–4000×*g* for 10 min, washed twice with 0.7-*M* NaCl, followed by centrifugation again under the same conditions as before. During centrifugation, start making the enzyme-osmoticum. The enzyme-osmoticum is added to the mycelia in Falcon tubes and the cell walls of the germlings are digested for 3–4 h at 32 °C with gentle shaking every 30 min during incubation. The protoplasts are separated from undigested mycelia and cell-wall debris by filtering the suspension through 2–4 layers of cheesecloth. The protoplasts are then collected by centrifugation at 2500×*g* for 10 min. The enzyme mixture can be reused by collecting the supernatant in other tubes and storing at −80 °C. The pellet is washed twice with 10 ml of 0.7 *M* NaCl and then with 10 ml of STC buffer. The protoplasts are gently resuspended in 500-µl–1-ml STC to reach a concentration of 10^6–10^7 protoplasts per ml. The counting is performed by using a Thoma chamber.

At least 10 µg of PCR products in 10 µl of ddH_2O is added to the protoplast suspension in 12-ml Greiner tubes and gently mixed. The transformation mix is incubated on ice for 20 min. PEG solution is then added in three aliquots of 200, 200, and 800 µl each. The tubes are warmed by hand before each addition and drops must be added slowly (drop by drop). The tubes are also mixed after each addition by rolling. Incubations for 5 min on ice are required between each addition. Finally, the transformation mix is diluted with 1-ml STC. Then, 200 µl of the transformation mixture is added to 25 ml of molten regeneration medium in a 50-ml tube and subsequently poured into a 100 × 15-mm Petri dish. Allow the medium to solidify and then incubate the plates at 32 °C. After 24 h, the plates are overlaid with 10 ml of 1% agar containing hygromycin B (Sigma-Aldrich, St. Louis, USA). The final concentration of hygromycin B should be 15 µg/ml. Please note that sensitivity to hygromycin B varies according to strain, so strain sensitivity must be assessed in advance. Individual hygB-resistant transformants are transferred to a fresh hygB-containing plate between 5 and 15 days after each transformation. Each transformant is purified further by picking isolated conidia under the microscope and transferring them to a fresh hygB-containing plate. Three successive rounds of single-spore isolation are performed.

4.4. Mutant verification

Two approaches, diagnostic PCR and Southern analysis of genomic DNA, are used to analyze mutants at the gene level and to verify that linear DNA products have inserted correctly. For both approaches, we routinely use high-throughput methods for isolation of genomic DNA from different fungal species. Detailed protocols have been reported by Meyer *et al.* (2010). We carry out a PCR screen by using primers homologous to the selection marker and genomic sequence outside the flanking regions, to confirm that integration of the replacement constructs occurred by homologous recombination at the targeted loci (Fig. 1.4D). The complete deletion of the coding region in mutants is further confirmed using two internal primers. Moreover, genomic Southern blot genotyping is performed to check single-copy deletion of the target gene in mutants that will be used for further phenotypic analysis (data not shown).

4.5. Phenotypic analysis

Detailed analysis of the phenotypes of both the wild-type and mutants affected in the process under investigation is essential to determine gene function or investigate the importance of a pathway in a cell. We summarize in Table 1.1 the main phenotypic criteria that have been investigated in Δ*hacA* UPR-deficient strains obtained in *A. niger* (Mulder and Nikolaev, 2009), *A. fumigatus* (Richie *et al.*, 2009), and in *A. brassicicola* (Joubert *et al.*, unpublished).

Among all the criteria that could be considered for establishing the phenotypic pattern of null mutants in filamentous fungi, growth characteristics and susceptibility to different drugs are key elements that we routinely monitor on agar plates as well as in liquid medium using laser nephelometry (NEPHELOstar Galaxy, BMG Labtech, Offenburg, Germany). By contrast with photometry (i.e., the measurement of light transmitted through a particle suspension), nephelometry is a direct method of measuring light scattered by particles in suspension. As the scattered light intensity is directly proportional to the suspended particle concentration, nephelometry is a powerful method for recording microbial growth and especially for studying filamentous fungi, which cannot be efficiently investigated through spectrophotometric assays. The advantages of nephelometry compared to analysis of colony expansion rates on solid media or spectrophotometric assays are discussed in a recent paper (Joubert *et al.*, 2010), in which we described a filamentous fungi-tailored procedure based on microscale liquid cultivation and automated nephelometric recording of growth.

Table 1.1 Main phenotypic criteria investigated in fungal Δ*hacA* strains

Phenotypic analysis	Fungal organism	Methods
Growth rate	*A. niger* *A. fumigatus* *A. brassicicola*	– Measure of colony diameter on different agar media (minimal or complete media, IMA, PDA, skim milk)
Sporulation	*A. niger* *A. brassicicola*	– Microscopic observation of conidia (using stereomicroscope or scanning electron microscopy and quantification of sporulation using a Thoma counting chamber
Hyphal morphology	*A. niger* *A. brassicicola*	– Microscopic observations of mycelia in liquid culture or grown between glass slides
Susceptibility to ER stress	*A. niger* *A. fumigatus* *A. brassicicola*	– Incubation in the presence of DTT, tunicamycin, or brefeldin A – Measure of colony diameter (from solid media) or growth monitoring using nephelometry (from liquid media)
Susceptibility to thermal stress	*A. fumigatus*	– Measure of colony diameter from solid media incubated at 37 and 45 °C – Measure of the percentage of surviving CFUs
Susceptibility to antifungal drugs	*A. fumigatus*	– Use of Etest strips impregnated with caspofungin, fluconazole, amphotericin B, and itraconazole
Expression of UPR target genes	*A. niger* *A. fumigatus* *A. brassicicola*	– Northern hybridization, real-time RT-PCR
Susceptibility to host defense metabolites	*A. brassicicola*	– Growth monitoring using nephelometry in the presence of cruciferous phytoalexins
Cell wall structure and composition	*A. fumigatus* *A. brassicicola*	– Treatment with calcofluor white and Congo red – Biochemical analysis of the cell wall – Observations with transmission electron microscopy
Protein secretion	*A. fumigatus* *A. brassicicola*	– SDS-PAGE analysis of culture supernatants – Quantification of secreted proteolytic activity with the Azocoll assay

Table 1.1 (*continued*)

Phenotypic analysis	Fungal organism	Methods
		– Quantification of esterase activity with the artificial substrate *p*-nitrophenyl butyrate (PNB)
Virulence	*A. fumigatus* *A. brassicicola*	– Use of an outbred and inbred mouse model of invasive aspergillosis – Inoculation of spore suspension on intact and prewounded leaves of *Arabidopsis thaliana*
Visualization of infection structures	*A. brassicicola*	– Staining of fungal material with Solophenyl Flavine 7GFE 500 followed by microscopic observations – Observations with scanning electron microscopy

We recommend this technique for the evaluation of antifungal activity and for large-scale phenotypic profiling.

Figure 1.6A shows a representative growth curve for *A. brassicicola* wild-type strain and Δ*hacA* mutant plotted from the nephelometric assays. Nephelometric monitoring confirms that, at least for early steps of the growth kinetics, the Δ*hacA* mutant is growth-impaired in PD broth. In another example (Fig. 1.6B), disruption of ER homeostasis is triggered by exposure to brefeldin A, which is an ER stress-inducing agent. As expected, we observe that the *hacA* null strain is growth-impaired in the presence of concentrations of brefeldin A that could be tolerated by the wild-type strain, indicating that *hacA* inactivation increased the sensitivity of *A. brassicicola* cells to these treatments.

ACKNOWLEDGMENTS

We thank Thomas. R. Jørgensen for his guidance in bioreactor cultivations of *A. niger* and preparing Fig. 1.1. This work was partly supported by the French Région Pays de la Loire (QUALISEM research program), the UK Biotechnology and Biological Sciences Research Council (BioResearch Industry Club), and the research program of the Kluyver Centre for Genomics of Industrial Fermentation which is part of the Netherlands Genomics Initiative/ Netherlands Organization for Scientific Research.

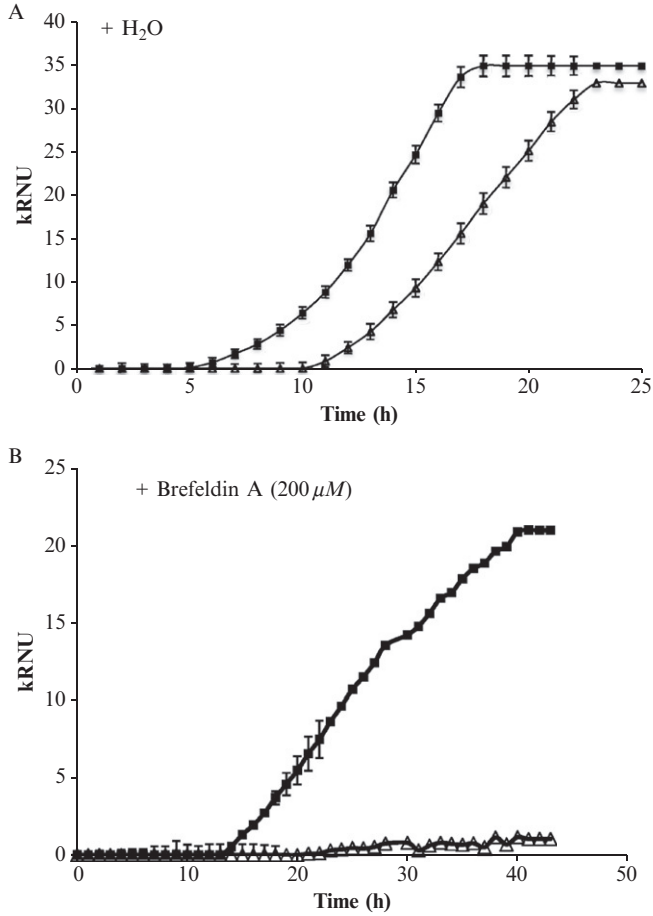


Figure 1.6 Nephelometric monitoring of growth of wild-type and Δ*hacA* strain. Conidial suspensions (10^5 spores/ml, final concentration) of wild-type (black squares) and Δ*hacA* (open triangles) are used to inoculate microplate wells containing a standard PDB medium that is supplemented (B) or not (A) with 200-μ*M* brefeldin A. Microplates are placed in a laser-based microplate nephelometer (NEPHELOstar Galaxy, BMG Labtech) and growth is monitored automatically over a 30-h period. Each genotype is analyzed in triplicate and the experiments are repeated three times per growth condition.

REFERENCES

Akamatsu, H., Itoh, Y., Kodama, M., Otani, H., and Kohmoto, K. (1997). AAL-toxin-deficient mutants of alternaria alternata tomato pathotype by restriction enzyme-mediated integration. *Phytopathology* **87,** 967–972.

Al-Sheikh, H., Watson, A. J., Lacey, G. A., Punt, P. J., MacKenzie, D. A., Jeenes, D. J., Pakula, T., Penttila, M., Alcocer, M. J., and Archer, D. B. (2004). Endoplasmic

reticulum stress leads to the selective transcriptional downregulation of the glucoamylase gene in *Aspergillus niger*. *Mol. Microbiol.* **53,** 1731–1742.

Andersen, M. R., Vongsangnak, W., Panagiotou, G., Salazar, M. P., Lehmann, L., and Nielsen, J. (2008). A trispecies *Aspergillus* microarray: Comparative transcriptomics of three *Aspergillus* species. *Proc. Natl. Acad. Sci. USA* **105,** 4387–4392.

Arava, Y. (2003). Isolation of polysomal RNA for microarray analysis. *Methods Mol. Biol.* **224,** 79–87.

Archer, D. B., and Turner, G. (2006). Genomics of protein secretion and hyphal growth in *Aspergillus*. *In* "The Mycota XIII, Fungal Genomics," (A. Brown, ed.), pp. 75–96. Springer, New york.

Arvas, M., Pakula, T., Lanthaler, K., Saloheimo, M., Valkonen, M., Suortti, T., Robson, G., and Penttila, M. (2006). Common features and interesting differences in transcriptional responses to secretion stress in the fungi *Trichoderma reesei* and *Saccharomyces cerevisiae*. *BMC Genomics* **7,** 32.

Bashir, A., Bansal, V., and Bafna, V. (2010). Designing deep sequencing experiments: Detecting structural variation and estimating transcript abundance. *BMC Genomics* **11,** 385.

Breakspear, A., and Momany, M. (2007). The first fifty microarray studies in filamentous fungi. *Microbiology* **153,** 7–15.

Carvalho, N. D., Arentshorst, M., Jin Kwon, M., Meyer, V., and Ram, A. F. (2010). Expanding the ku70 toolbox for filamentous fungi: Establishment of complementation vectors and recipient strains for advanced gene analyses. *Appl. Microbiol. Biotechnol.* **87,** 1463–1473.

Conesa, A., Punt, P. J., van Luijk, N., and van den Hondel, C. A. (2001). The secretion pathway in filamentous fungi: A biotechnological view. *Fungal Genet. Biol.* **33,** 155–171.

de Ruiter-Jacobs, Y. M., Broekhuijsen, M., Unkles, S. E., Campbell, E. I., Kinghorn, J. R., Contreras, R., Pouwels, P. H., and van den Hondel, C. A. (1989). A gene transfer system based on the homologous pyrG gene and efficient expression of bacterial genes in *Aspergillus oryzae*. *Curr. Genet.* **16,** 159–163.

Fischer, R., Zekert, N., and Takeshita, N. (2008). Polarized growth in fungi—Interplay between the cytoskeleton, positional markers and membrane domains. *Mol. Microbiol.* **68,** 813–826.

Geysens, S., Whyteside, G., and Archer, D. B. (2009). Genomics of protein folding in the endoplasmic reticulum, secretion stress and glycosylation in the aspergilli. *Fungal Genet. Biol.* **46,** S121–S140.

Gouka, R. J., Punt, P. J., and van den Hondel, C. A. (1997). Efficient production of secreted proteins by Aspergillus: Progress, limitations and prospects. *Appl. Microbiol. Biotechnol.* **47,** 1–11.

Guillemette, T., van Peij, N. N., Goosen, T., Lanthaler, K., Robson, G. D., van den Hondel, C. A., Stam, H., and Archer, D. B. (2007). Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*. *BMC Genomics* **8,** 158.

Harris, S. D. (2008). Branching of fungal hyphae: Regulation, mechanisms and comparison with other branching systems. *Mycologia* **100,** 823–832.

Hirota, M., Kitagaki, M., Itagaki, H., and Aiba, S. (2006). Quantitative measurement of spliced XBP1 mRNA as an indicator of endoplasmic reticulum stress. *J. Toxicol. Sci.* **31,** 149–156.

Jacobs, D. I., Olsthoorn, M. M., Maillet, I., Akeroyd, M., Breestraat, S., Donkers, S., van der Hoeven, R. A., van den Hondel, C. A., Kooistra, R., Lapointe, T., Menke, H., Meulenberg, R., *et al.* (2009). Effective lead selection for improved protein production in *Aspergillus niger* based on integrated genomics. *Fungal Genet. Biol.* **46**(Suppl. 1), S141–S152.

Jorgensen, T. R., Goosen, T., Hondel, C. A., Ram, A. F., and Iversen, J. J. (2009). Transcriptomic comparison of *Aspergillus niger* growing on two different sugars reveals coordinated regulation of the secretory pathway. *BMC Genomics* **10,** 44.

Joubert, A., Calmes, B., Berruyer, R., Pihet, M., Bouchara, J. P., Simoneau, P., and Guillemette, T. (2010). Laser nephelometry applied in an automated microplate system to study filamentous fungus growth. *Biotechniques* **48,** 399–404.

Kohno, K. (2010). Stress-sensing mechanisms in the unfolded protein response: Similarities and differences between yeast and mammals. *J. Biochem.* **147,** 27–33.

Le Crom, S., Schackwitz, W., Pennacchio, L., Magnuson, J. K., Culley, D. E., Collett, J. R., Martin, J., Druzhinina, I. S., Mathis, H., Monot, F., Seiboth, B., Cherry, B., *et al.* (2009). Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. *Proc. Natl. Acad. Sci. USA* **106,** 16151–16156.

Lubertozzi, D., and Keasling, J. D. (2009). Developing Aspergillus as a host for heterologous expression. *Biotechnol. Adv.* **27,** 53–75.

Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., Abe, K., Gomi, K., *et al.* (2005). Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **438,** 1157–1161.

McCarthy, J. E. (1998). Posttranscriptional control of gene expression in yeast. *Microbiol. Mol. Biol. Rev.* **62,** 1492–1553.

Meyer, V., Damveld, R. A., Arentshorst, M., Stahl, U., van den Hondel, C. A., and Ram, A. F. (2007). Survival in the presence of antifungals: Genome-wide expression profiling of *Aspergillus niger* in response to sublethal concentrations of caspofungin and fenpropimorph. *J. Biol. Chem.* **282,** 32935–32948.

Meyer, V., Arentshorst, M., Flitter, S. J., Nitsche, B. M., Kwon, M. J., Reynaga-Pena, C. G., Bartnicki-Garcia, S., van den Hondel, C. A., and Ram, A. F. (2009). Reconstruction of signaling networks regulating fungal morphogenesis by transcriptomics. *Eukaryot. Cell* **8,** 1677–1691.

Meyer, V., Ram, A., and Punt, P. J. (2010). Genetics, genetic manipulation, and approaches to strain improvement of filamentous fungi. *In* "Manual of Industrial Microbiology and Biotechnology," (A. L. Demain and J. L. Davies, eds.), 3rd edn., pp. 318–329. Wiley, New York.

Michielse, C. B., Hooykaas, P. J., van den Hondel, C. A., and Ram, A. F. (2008). Agrobacterium-mediated transformation of the filamentous fungus *Aspergillus awamori*. *Nat. Protoc.* **3,** 1671–1678.

Mulder, H. J., and Nikolaev, I. (2009). HacA-dependent transcriptional switch releases hacA mRNA from a translational block upon endoplasmic reticulum stress. *Eukaryot. Cell* **8,** 665–675.

Mulder, H. J., Saloheimo, M., Penttila, M., and Madrid, S. M. (2004). The transcription factor HACA mediates the unfolded protein response in *Aspergillus niger*, and up-regulates its own transcription. *Mol. Genet. Genomics* **271,** 130–140.

Pakula, T. M., Laxell, M., Huuskonen, A., Uusitalo, J., Saloheimo, M., and Penttila, M. (2003). The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei*. Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. *J. Biol. Chem.* **278,** 45011–45020.

Pel, H. J., de Winde, J. H., Archer, D. B., Dyer, P. S., Hofmann, G., Schaap, P. J., Turner, G., de Vries, R. P., Albang, R., Albermann, K., Andersen, M. R., Bendtsen, J. D., *et al.* (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* **25,** 221–231.

Pradet-Balade, B., Boulme, F., Beug, H., Mullner, E. W., and Garcia-Sanz, J. A. (2001). Translation control: Bridging the gap between genomics and proteomics? *Trends Biochem. Sci.* **26,** 225–229.

Richie, D. L., Hartl, L., Aimanianda, V., Winters, M. S., Fuller, K. K., Miley, M. D., White, S., McCarthy, J. W., Latge, J. P., Feldmesser, M., Rhodes, J. C., and Askew, D. S. (2009). A role for the unfolded protein response (UPR) in virulence and antifungal susceptibility in *Aspergillus fumigatus*. *PLoS Pathog*. **5,** e1000258.

Ruegsegger, U., Leber, J. H., and Walter, P. (2001). Block of HAC1 mRNA translation by long-range base pairing is released by cytoplasmic splicing upon induction of the unfolded protein response. *Cell* **107,** 103–114.

Shoji, J. Y., Arioka, M., and Kitamoto, K. (2008). Dissecting cellular components of the secretory pathway in filamentous fungi: Insights into their application for protein production. *Biotechnol. Lett*. **30,** 7–14.

Sims, A. H., Gent, M. E., Lanthaler, K., Dunn-Coleman, N. S., Oliver, S. G., and Robson, G. D. (2005). Transcriptome analysis of recombinant protein secretion by *Aspergillus nidulans* and the unfolded-protein response in vivo. *Appl. Environ. Microbiol*. **71,** 2737–2747.

Smith, C. W., Klaasmeyer, J. G., Edeal, J. B., Woods, T. L., and Jones, S. J. (1999). Effects of serum deprivation, insulin and dexamethasone on polysome percentages in C2C12 myoblasts and differentiating myoblasts. *Tissue Cell* **31,** 451–458.

Sweigard, J. A., Carroll, A. M., Kang, S., Farrall, L., Chumley, F. G., and Valent, B. (1995). Identification, cloning, and characterization of PWL2, a gene for host species specificity in the rice blast fungus. *Plant Cell* **7,** 1221–1233.

Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P.\ (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101,** 249–258.

Valkonen, M., Ward, M., Wang, H., Penttila, M., and Saloheimo, M. (2003). Improvement of foreign-protein production in *Aspergillus niger* var. awamori by constitutive induction of the unfolded-protein response. *Appl. Environ. Microbiol*. **69,** 6979–6986.

van Gorcom, R. F., and van den Hondel, C. A. (1988). Expression analysis vectors for *Aspergillus niger*. *Nucleic Acids Res*. **16,** 9052.

van Hartingsveldt, W., Mattern, I. E., van Zeijl, C. M., Pouwels, P. H., and van den Hondel, C. A. (1987). Development of a homologous transformation system for *Aspergillus niger* based on the pyrG gene. *Mol. Gen. Genet*. **206,** 71–75.

Vishniac, W., and Santer, M. (1957). The thiobacilli. *Bacteriol. Rev*. **21,** 195–213.

Vongsangnak, W., Nookaew, I., Salazar, M., and Nielsen, J. (2010). Analysis of genome-wide coexpression and coevolution of *Aspergillus oryzae* and *Aspergillus niger*. *OMICS* **14,** 165–175.

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: A revolutionary tool for transcriptomics. *Nat. Rev. Genet*. **10,** 57–63.

Wimalasena, T. T., Enjalbert, B., Guillemette, T., Plumridge, A., Budge, S., Yin, Z., Brown, A. J., and Archer, D. B. (2008). Impact of the unfolded protein response upon genome-wide expression patterns, and the role of Hac1 in the polarized growth, of *Candida albicans*. *Fungal Genet. Biol*. **45,** 1235–1247.

Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Reyes-Dominguez, Y., and Scazzocchio, C. (2004). Double-joint PCR: A PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol*. **41,** 973–981.