Effects of a defective ERAD pathway on growth and heterologous protein production in *Aspergillus niger*

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Abstract
Endoplasmic reticulum associated degradation (ERAD) is a conserved mechanism to remove misfolded proteins from the ER by targeting them to the proteasome for degradation. To assess the role of ERAD in filamentous fungi, we have examined the consequences of disrupting putative ERAD components in the filamentous fungus *Aspergillus niger*. Deletion of *derA*, *doaA*, *hrdC*, *mifA* or *mnsA* in *A. niger* yields viable strains and, with the exception of *doaA*, no significant growth phenotype is observed when compared to the parental strain. The gene deletion mutants were also made in *A. niger* strains containing single-copy or multi-copies of a glucoamylase-glucuronidase (GlaGus) gene fusion. The induction of the Unfolded Protein Response (UPR) target genes (*bipA* and *pdiA*) was dependent on the copy number of the heterologous gene and the ERAD gene deleted. The highest induction of UPR target genes was observed in ERAD mutants containing multiple copies of the GlaGus gene. Western blot analysis revealed that deletion of the *derA* gene in the multi-copy GlaGus overexpressing strain resulted in a 6-fold increase in the intracellular amount of GlaGus protein detected. Our results suggest that impairing some components of the ERAD pathway in combination with high expression levels of the heterologous protein results in higher intracellular protein levels, indicating a delay in protein degradation.
Chapter 4

Effects of a defective ERAD pathway on growth and heterologous protein production in *Aspergillus niger*

4.1. Introduction

The use of filamentous fungi with the natural property of secreting high amounts of extracellular proteins as cell factories for the production of homologous and heterologous proteins has been extensively exploited for many years. *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* are most often used in industry for the production of proteins. In the search for further improving the properties as protein producer, many attempts and strategies have been employed and optimized such as the knock-out of certain genes, the use of strong promoters, mutagenesis, among others (Jeenes et al., 1991; Archer et al., 1994; Punt et al., 1994 Gouka et al., 1997; Nemoto et al., 2009; Nakari-Setälä et al., 2009; Meyer et al., 2010). The recent sequencing of the genomes of these industrially important fungi (Machida et al., 2005; Pel et al., 2007; Martinez et al., 2008; Wortman et al., 2009) provides another starting point to understand and manipulate the outstanding secretion capacities of these fungi (Maeda et al., 2004; Arvas et al., 2006; Guillemette et al., 2007; Gasser et al., 2007; Pel et al., 2007; Jacobs et al., 2009). Several steps occurring during the secretion pathway in filamentous fungi have been pointed out as potential bottlenecks for heterologous protein production (Gouka et al., 1997; Sims et al., 2005). Proteins that enter the secretory pathway begin their journey in the ER, where they are assembled and subjected to a strict quality control (Ellgaard et al., 1999; Lederkremer, 2009). The proteins that fail proper folding usually accumulate in the ER leading to the induction of the Unfolded Protein Response (UPR) (Cox et al., 1993) and, if UPR is not sufficient to relieve stress, they are eventually targeted to destruction by the ER-associated degradation (ERAD) (Nishikawa et al., 2005). Both the UPR and the ERAD pathways are conserved from yeasts to mammalians (reviewed in Kincaid and Cooper, 2007; Anelli and Sitia, 2008; Mori, 2009); however, apart from a recent publication which studies the effect of deleting *A. niger doaA* gene (Jacobs et al., 2009), the functional analysis of other putative ERAD related genes in filamentous fungi has not been reported.

Accumulation of unfolded proteins in the ER lumen results in the dissociation of BiP from Ire1p leading to Ire1p dimerization and thereby, the activation of its kinase and endoribonuclease functions (Shamu and Walter, 1996; Sidrauski et al., 1997; Oikawa et al., 2009). In *Saccharomyces cerevisiae*, Ire1p is responsible for excising a 252 nt intron in Hac1 mRNA, enabling its translation into an active protein and migration into the nucleus where it binds to UPRE (CANCNTG, Mori et al., 1998) in target genes coding for chaperones and foldases as well as other components of the secretory pathway (Sidrauski et al., 1998; Travers et al., 2000). By homology with the *S. cerevisiae* model, it is assumed that in *A. niger*, IreAp is also responsible for the removal of a 20 nt intron in the hacA mRNA. Splicing of the intron leads to the activation of the HacA transcription factor, which in turn controls the expression of genes involved in UPR (Mulder et al., 2004 and 2006).

The ER degradation pathway in *S. cerevisiae* consists of a number of highly conserved proteins. The UPR induced BiP and PDI play important roles in ERAD by preventing misfolded proteins aggregation (Nishikawa et al., 2001) and delivering ERAD substrates to the retrotranslocation machinery (Plemper et al., 1999a). Moreover, glycosylation is an important factor in protein folding and the processing of glycans is indicative of the folding state of the protein (reviewed in Kleizen and Braakman, 2004;
Lederkremer, 2009). If the protein fails to achieve correct conformation, the removal of 1,2 α-mannose units by a specific 1,2 α-mannosidase (mns1) targets the substrate to degradation by ERAD (Gonzalez et al., 1999; Tremblay and Herscovics, 1999). When marked for degradation, proteins are retrotranslocated through the Sec61p translocon (Schäfer and Wolf, 2009) and/or through Der1p retrotranslocation channel (Goder et al., 2008; Ye et al., 2001a), although the later one only seems to be required for some substrates (Lilley and Ploegh, 2004). The Hrd1 complex is involved in the ubiquitination of substrates that contain misfolded luminal domains (Bordallo et al., 1998; Deak and Wolf, 2001). In S. cerevisiae, Hrd3p regulates the activity and stability of Hrd1p (Plemper et al., 1999b; Gardner et al., 2000). Together with Sec61p, Hrd1-Hrd3 complex mediates the transfer to the cytosol of proteins targeted for degradation (Plemper et al., 1999a). Doa1p forms a complex with Cdc48p allowing the extraction of ubiquitinated substrates via AAA-ATP Cdc48 complex (Ye et al., 2001b; Jarosch et al., 2002; Ogiso et al., 2004; Mullally et al., 2006). Ubiquitinated proteins are degraded by the 26S proteasome in an ATP-dependent manner (Fisher et al., 1994). The translocation of the 26S proteasome from the cytoplasm to the ER membrane seems to be mediated by Mif1p (van Laar et al., 2001). In mammalian systems, the response to ER stress involves four major steps: I) attenuation of protein synthesis; II) transcriptional induction of UPR target genes, including chaperones and foldases, III) transcriptional induction of ERAD components and, in case these three steps are not sufficient, IV) induction of apoptosis (reviewed in Yoshida, 2007). For a detailed description of the ERAD pathway, we refer to a recent review by Vembar and Brodsky (2008).

From yeasts to mammals, several elements involved in the recognition and targeting of misfolded proteins for destruction are conserved, allowing the cells to cope with the presence/accumulation of aberrant proteins and their harmful effects. However, not all the processes described in yeast and mammalian system have been established in filamentous fungi (reviewed in van Anken and Braakman 2005a,b).

In this study, we have examined the role of the ERAD pathway in A. niger by disrupting genes that encode proteins suggested to be involved in different parts of ERAD pathway. We have assessed its role both during normal growth conditions, under ER stress inducing conditions by treatment with DTT or tunicamycin and under conditions when a UPR-inducing heterologous protein is produced. Our results indicate that a functional ERAD pathway is not required for normal growth, but that a defective ERAD pathway increases intracellular levels of the UPR-inducing GlaGus protein, indicating that the ERAD pathway is, at least partially, responsible for the degradation of heterologous proteins in A. niger.

4.2. Materials and methods

4.2.1. Strains, culture conditions and molecular techniques

Aspergillus niger strains used throughout this study are all derivatives of N402 (Bos et al., 1988) (See Table 1 for details).
Table 1. Strains used in this study.

| Strain         | Genotype                                      | Description                                      | Reference                      |
|----------------|-----------------------------------------------|--------------------------------------------------|--------------------------------|
| N402           | cspA1 derivative of ATCC9029                  | -                                                | Bos et al., 1988               |
| MA70.15        | ΔkasA::amdS in AB4.1 pyrG                      | -                                                | Moyer et al., 2007             |
| MA78.6         | ΔkasA::amdS in N402                           | -                                                | Carvalho et al., 2010          |
| NC5            | FAA-resistant derivative from MA78.6ΔkasA,   | -                                                | Carvalho et al., 2010          |
|                | amdS)                                         |                                                  |                                |
| MA97.2         | ΔderA::amdS in NC5                            | -                                                | this study                     |
| MA98.1         | ΔdoaA::amdS in NC5                            | -                                                | this study                     |
| MA94.3         | ΔhdcC::amdS in NC5                            | -                                                | this study                     |
| MA99.9         | Δmfa::amdS in NC5                             | -                                                | this study                     |
| MA96.6         | ΔmsnA::amdS in NC5                            | -                                                | this study                     |
| MV3.2          | pBH19-3pyrG* in MA70.15 (ΔkasA::amdS)         | pGpdA-Gla514-Gus-pyrG*                           | this study                     |
| MA99.3         | FAA-resistant derivative from MV3.2           | pGpdA-Gla514-Gus-pyrG*                           | this study                     |
| MA110.1        | ΔderA::amdS in MA99.3                         | pGpdA-Gla514-Gus-pyrG*                           | this study                     |
| MA111.3        | ΔdoaA::amdS in MA99.3                         | pGpdA-Gla514-Gus-pyrG*                           | this study                     |
| MA112.10       | ΔhdcC::amdS in MA99.3                         | pGpdA-Gla514-Gus-pyrG*                           | this study                     |
| MA113.2        | Δmfa::amdS in MA99.3                          | pGpdA-Gla514-Gus-pyrG*                           | this study                     |
| MA114.7        | ΔmsnA::amdS in MA99.3                         | pGpdA-Gla514-Gus-pyrG*                           | this study                     |
| MA115.1        | ΔderA FAA-resistant derivative from MA97.2   | -                                                | unpublished                    |
| MA116.2        | ΔhdcC FAA-resistant derivative from MA94.3   | -                                                | this study                     |
| MA117.1        | Δmfa FAA-resistant derivative from MA95.9    | -                                                | this study                     |
| MA118.2        | ΔmsnA FAA-resistant derivative from MA96.6   | -                                                | this study                     |
| MA119.1        | ΔhdcC, ΔderA::amdS (ΔderA::amdS in MA116.2)  | -                                                | this study                     |
| MA120.1        | ΔhdcC, ΔdoaA::amdS (ΔdoaA::amdS in MA116.2)  | -                                                | this study                     |
| MA122.4        | ΔhdcC, ΔmsnA::amdS (ΔmsnA::amdS in MA116.2)  | -                                                | this study                     |
| MA123.7        | Δmfa, ΔderA::amdS (ΔderA::amdS in MA117.1)   | -                                                | this study                     |
| MA124.2        | Δmfa, ΔdoaA::amdS (ΔdoaA::amdS in MA117.1)   | -                                                | this study                     |
| MA125.1        | Δmfa, ΔhdcC::amdS (ΔhdcC::amdS in MA117.1)   | -                                                | this study                     |
| MA127.3        | ΔmsnA, ΔderA::amdS (ΔderA::amdS in MA118.2)  | -                                                | this study                     |
| MA128.1        | ΔmsnA, ΔdoaA::amdS (ΔdoaA::amdS in MA118.2)  | -                                                | this study                     |
| MA130.3        | Δmfa, ΔmsnA::amdS (ΔmsnA::amdS in MA118.2)   | -                                                | this study                     |
| MA131.1        | ΔderA, ΔdoaA::amdS (ΔdoaA::amdS in MA115.1)  | -                                                | this study                     |
| AB4-1dglaA363#3| &pBH19-3pGpdA (ΔAfA::pGpdA)                     | Multicopy pGpdA-Gla514-Gus                      | Punt et al., 1998, 1998        |
| MA134.64       | ΔkasA::amdS in AB4-1dglaA363#3 (ΔAfA::pGpdA)  | Multicopy pGpdA-Gla514-Gus                      | this study                     |
| MA135.3        | FAA-resistant derivative from MA134.64        | Multicopy pGpdA-Gla514-Gus                      | this study                     |
| MA136.18       | Δdoa::amdS in MA135.3                         | Multicopy pGpdA-Gla514-Gus                      | this study                     |
| MA137.2        | Δdoa::amdS in MA135.3                         | Multicopy pGpdA-Gla514-Gus                      | this study                     |
| MA139.6        | Δmfa::amdS in MA135.3                         | Multicopy pGpdA-Gla514-Gus                      | this study                     |
| MA140.8        | ΔmsnA::amdS in MA135.3                        | Multicopy pGpdA-Gla514-Gus                      | this study                     |
| ABI1.13972     | [pIL6-3A#Bp]#72panT-7                         | IL6 (PgdA)                                      | Broekhuysen et al.             |
| ABI1.13954     | [pAN56-3hIL6#Bp]#54pAN7-1                     | GLA::IL6 (PgdA)                                 | 1993                          |
| ABI1.13958     | [pAN56-4hIL6#Bp]#58pAN7-1                     | GLA::kex::IL6 (PgdA)                            | Punt et al., 1998, 1998        |
| D15            | pGpdA-Gla::tpA::ΔAfA::pGpdA#25                | GLA::kex::PA (PgdA)                              | Wiebe et al., 2001             |
| MGG029#25      | [pGlA-MNP1]::pGpdA                            | MnP1 from Phanerochaete chrysosporium            | Conesa et al., 2000            |
| MGG029#13      | [pGlA-GlcA::MNPI]#13                          | MnP1 from P. chrysosporium expressed as GlA fusion protein | Conesa et al., 2000            |
| B36            | pAB6-10#Bp#3                                  | Contain over 80 copies of the Glucomylase gene  | Verdoes et al., 1993           |
| AR1.1          | [pGlA-GlcA::pGpdA]::GFP                       | Glucomylase-GFP                                 | Gordon et al., 2000a           |
| XW2.2.1        | [pGlA-GlcA::pGpdA-HDEL]                       | Glucomylase-GFP fusion with ER targeting sequence | Gordon et al., 2000a           |
| MA231.1.1      | [pGpd-Cpa::pGpdA]::GFP                        | CpaA - GFP fusion expressed from gpdA promoter | Weenink and Ram, unpublished   |
| NW5.1          | [pGpd-Cwp::pGpdA]::GFP                        | Cwp-A-GFP from A. niger                         | Danved and Ram, unpublished    |
| XW5.2          | [pGlA-GlcA::POX2]::pGpdA                      | Laccase from Pleurotus ostreatus                | Weenink et al., 2006           |
| XW6.1          | [pGlA-GlcA::pGpdA]::GFP                       | -                                               | Weenink et al., 2006           |

*IL6-interleukine 6; tPA-tissue plasminogen activator; MnP1-manganese peroxidase; Cwp- cell wall protein; Cpy – carboxypeptidase Y.
Strains were cultivated in minimal medium (MM) (Bennett and Lasure 1991) containing 1% (w/v) of glucose as a carbon source, 7 mM KCl, 11 mM KH₂PO₄, 70 mM NaNO₃, 2 mM MgSO₄, 76 mM ZnSO₄, 178 mM H₂BO₃, 25 mM MnCl₂, 18 mM FeSO₄, 7.1 nM CoCl₂, 6.4 mM CuSO₄, 6.2 mM Na₂MoO₄, 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 using the *amdS* gene as selection marker, strains were grown in MM in which the 70 mM NaNO₃ was replaced with 10 mM acetamide and 15 mM cesium chloride (Meyer et al., 2010). All basic molecular techniques were performed according to standard procedures (Sambrook and Russell 2001). Transformation of *A. niger*, genomic DNA extraction, screening procedures, northern analysis and Southern analysis were conducted as recently described in utmost detail (Meyer et al., 2010).

### 4.2.2. Phenotypic assays

For plate growth assays, MM or CM was used (as described above) and solidified by the addition of 2% agar. Radial extension rates of the ERAD mutants were determined by inoculating 1x10⁴ spores in the centre of a CM- and MM-plate and growth at 25, 30, 37 and 42°C was followed for 3-4 days. To determine the sensitivity of the ERAD mutants towards ER- and osmotic stress, a ten-fold dilution series of spores (from 1 x 10⁵ until 1 x 10¹) were spotted on CM and MM-plates containing tunicamycin (0.1, 0.5, 1, 5 or 10 mM), DTT (1, 5, 10, or 20 µg/ml) to induce ER stress and containing 0.6 M sorbitol to induce osmotic stress. Sensitivity assays were performed at 25, 30, or 42°C and growth was monitored for 3-5 days. Growth on starch was determined by spotting ten-fold dilution series of spores on CM plates containing 2% starch as the sole carbon source.

### 4.2.3. Construction of a strain expressing a secreted form of β-glucuronidase (Gus)

Plasmid pBB19-3 was previously described (Punt et al., 1994). To generate *A. niger* strains carrying a single copy of this plasmid at a defined position, the *pyrG* gene was used (van Gorcom and van den Hondel 1988). The *pyrG* was amplified from pAF3 (Damveld et al., 2005) using primers pNC43 and pNC44 where *AscI* restriction sites were added (Table 2) to facilitate the cloning into pBB19-3. The amplified PCR fragment of 2.2 kb was ligated into pJET1.2 (pJET1.2/blunt Cloning Vector, Fermentas) to give pJetPyrG*AscI. Finally, the *pyrG* fragment was isolated with *AscI* and cloned into the unique *AscI* site in pBB19-3 to give pBB19-3pyrG*. This construct was transformed into MA70.15 (*ΔkusA, pyrG*, *amdS*) and transformants were purified by repeated streaking of conidia on media without uridine. Transformants were subjected to Southern blot analysis and MV3.2 was selected as this transformant contains a single copy of the pBB19-3 plasmid at the *pyrG* locus. The AmdS marker in this strain, which was used to delete the *kusA* gene, was looped out by selecting Fluoroacetamide resistant colonies by inoculating 2 x 10⁷ spores on MM plates containing 1% (w/v) of glucose as a carbon source, without NaNO₃ and supplemented with 0.2% 5’-Fluoroacetamide (FAA) and 10 mM urea as additional nitrogen source (for details see Meyer et al., 2010). Plates were incubated for 1-2 weeks at 30°C and FAA resistant mutants were transferred onto fresh FAA-containing plates for purification. Mutants unable to grow on
media containing acetamide as sole nitrogen source were subjected to Southern blot analysis, and strain MA99.3 in which the amdS gene was properly looped out was chosen for further studies.

4.2.4. Construction of ERAD deletion strains

The deletion constructs for the 5 selected genes involved in the ERAD pathway (derA (An15g00640), doaA (An03g04600), hrdC (An01g12720), mifA (An01g14100) and mnsA (An18g06220) were made using primers listed in Table 2.

| Primer name   | Sequence (5’ to 3’)                     | Amplification of | Restriction Enzyme |
|---------------|----------------------------------------|------------------|-------------------|
| pDER9Eco      | gcagatctgccaaacctgggacctattactgc       | derA 3’ flank    | EcoRI             |
| pDER10Hin     | gcagatctttccggacaaggaatacc             | derA 3’ flank    | HindIII           |
| pFERDMB       | gcagatcgtgaagctgtagagctgaatgcctgc     | derA 3’ flank    | BamHI             |
| pRDERKpn      | tggcagctgcagcaggttagagtgaagatgaatgctc | derA 3’ flank    | KpnI              |
| pDER3Not      | tggcagctgcagcaggttagagtgaagatgaatgctc | derA 5’ flank    | NotI              |
| pDER4Bam      | gcagatcgtgaagctgtagagctgaatgcctgc     | derA 5’ flank    | BamHI             |
| pdoaHinb      | ctagctgcagcaggttagagtgaagatgaatgctc   | doaA 3’ flank    | HindIII           |
| pdoaAsc       | gcagatcgtgaagctgtagagctgaatgctgataagc | doaA 3’ flank    | AscI              |
| pdoaEco       | ctagctgcagcaggttagagtgaagatgaatgctc   | doaA 5’ flank    | EcoRI             |
| pdoaHina      | ctagctgcagcaggttagagtgaagatgaatgctc   | doaA 5’ flank    | HindIII           |
| pdoaNot       | ctagctgcagcaggttagagtgaagatgaatgctc   | doaA 5’ flank    | NotI              |
| pdoaMlu       | ctagctgcagcaggttagagtgaagatgaatgctc   | doaA 5’ flank    | Mlu               |
| pHRD7Not      | tggcagctgcagcaggttagagtgaagatgaatgctc | hrdC 5’ flank    | NotI              |
| pHRD8Mlu      | tggcagctgcagcaggttagagtgaagatgaatgctc | hrdC 5’ flank    | Mlu               |
| phrdhin       | ctagctgcagcaggttagagtgaagatgaatgctc   | hrdC 3’ flank    | HindIII           |
| phrdasc       | cggcagctgcagcaggttagagtgaagatgaatgctc | hrdC 3’ flank    | AscI              |
| phrdMlu       | ctagctgcagcaggttagagtgaagatgaatgctc   | hrdC 3’ flank    | Mlu               |
| phrdKpn       | ctagctgcagcaggttagagtgaagatgaatgctc   | hrdC 3’ flank    | KpnI              |
| pmifNot       | ctagctgcagcaggttagagtgaagatgaatgctc   | mifA 5’ flank    | NotI              |
| pmifXmaa      | ctagctgcagcaggttagagtgaagatgaatgctc   | mifA 5’ flank    | Xmal              |
| pmifEco       | ctagctgcagcaggttagagtgaagatgaatgctc   | mifA 3’ flank    | EcoRI             |
| pmifAsa       | ctagctgcagcaggttagagtgaagatgaatgctc   | mifA 3’ flank    | Asci              |
| pmifXmab      | ctagctgcagcaggttagagtgaagatgaatgctc   | mifA 3’ flank    | Xmal              |
| pmifKpn       | ctagctgcagcaggttagagtgaagatgaatgctc   | mifA 3’ flank    | KpnI              |
| pmnsNot       | ctagctgcagcaggttagagtgaagatgaatgctc   | mnsA 5’ flank    | NotI              |
| pmnsXmaa      | ctagctgcagcaggttagagtgaagatgaatgctc   | mnsA 5’ flank    | Xmal              |
| pmnsEco       | ctagctgcagcaggttagagtgaagatgaatgctc   | mnsA 3’ flank    | EcoRI             |
| pmnsAsc       | ctagctgcagcaggttagagtgaagatgaatgctc   | mnsA 3’ flank    | Asci              |
| pmnsXmab      | ctagctgcagcaggttagagtgaagatgaatgctc   | mnsA 3’ flank    | Xmal              |
| pmnsKpn       | ctagctgcagcaggttagagtgaagatgaatgctc   | mnsA 3’ flank    | KpnI              |
| pNC43         | ctagctgcagcaggttagagtgaagatgaatgctc   | pyrG*            | AscI              |
| pNC44         | ctagctgcagcaggttagagtgaagatgaatgctc   | pyrG*            | AscI              |
Briefly, the cloning strategy was as follows: for each individual gene, respective 5’ and 3’ flanking regions and an additional 5’ or 3’ repeat (construct dependent, see Table 2 for details) were amplified using primers where specific restriction enzymes were added and cloned into pGBPEP23 (Jacobs et al., 2009). This vector uses the amdS gene behind the PgpdA promoter as a dominant selection marker. Only in the presence of the amdS gene Aspergillus is able to grow on medium containing acetamide as sole nitrogen source. In general, approximately 1 kb of the 5’- and 3’-sequences flanking the coding regions has been used and about 500-700 bp repeat of one of the flanks, was included to facilitate removal of the amdS marker by homologous recombination forced by growth on FAA. The A. niger doaA deletion strain has been previously described (Jacobs et al., 2009). Deletion constructs were linearized by digestion with NotI and AscI before transformation. To obtain high homologous recombination frequencies to construct ERAD deletion mutants in the multicopy GlaGus strain (AB4-1dglaA36[pBB19-3]#3), the ku70 gene was also deleted in this background using a kusA::amdS deletion construct as previously described (Meyer et al., 2007). Southern blot analysis identified strain MA134.64 as a strain in which the kusA gene was deleted (data not shown). Subsequently, the amdS gene was removed through the FAA loop-out technique, and yielded MA135.3 in which the kusA deletion and amdS looped out was confirmed by Southern blot analysis (data not shown). Each ERAD deletion construct was transformed into strains NC5, MA99.3 and MA135.3. Strains MA99.3 and MA135.3 will be referred to as single-copy scGlaGus and multi-copy mcGlaGus strains, respectively in the following sections. All ERAD deletion mutants in the three strain backgrounds were confirmed by Southern analysis (data not shown). All mutants were obtained except for the hrdC deletion strain in the mcGlaGus strain.

4.2.5. Western blot analysis

To analyze the extracellular and intracellular levels of Gus protein, deletion strains and control strains were grown in duplicate for 24 h in 50 ml CM containing 1% glucose as carbon source. All cultures were inoculated with 1x10^6 spores/ml. Mycelium was collected through a myracloth filter and the supernatant was stored at -20°C prior to further analysis. Total protein content was extracted by grinding approximately 200 mg frozen mycelium using mortar and pestle in liquid nitrogen. Proteins were extracted using 1ml extraction buffer (10 mM sodium phosphate buffer, pH 6.0, 2% SDS. 10 mM EDTA and 1 mM PMSF) and centrifuged twice, collecting the supernatant each time. Protein concentrations of the samples were determined with Bradford assay using BSA as standard. For each sample, 10 µg of total protein was mixed with 2x loading buffer (0.5 M HCl, 25% glycerol, 10% SDS, 0.5% bromophenol blue, 5% β-mercaptoethanol) and boiled for 5 min at 95°C. Protein samples were loaded on a pre-cast SDS-Page gel (BioRad) and blotted to a nitrocellulose membrane through semi-dry electrotransfer. The membrane was blocked for 1h with 5% low-fat milk in TTBS (TBS, 0.05% Tween20) and Gus protein was detected using a Gus-specific antibody (1/5000) over-night, followed by a goat-anti-rabbit-HRP secondary antibody (1/20000) for 1h. Detection was performed using a chemiluminessence kit (Bio-Rad), according to manufacturer’s instructions. The Gus-antibody was kindly provided by Prof. P. Punt (TNO, 71
Chapter 4  Effects of a defective ERAD pathway on growth and heterologous protein production in *Aspergillus niger*

The Netherlands. Analysis and quantification of band intensities were performed using QuantityOne 1-D Analysis Software (BioRad) and 18S rRNA as loading control.

4.3. Results

4.3.1. The level of induction of the Unfolded Protein Response Pathway by heterologous protein expression is protein specific

In order to study the effects of deleting ERAD components on heterologous protein production in *A. niger*, we started our research by choosing a suitable heterologous protein reporter through an inventory of *A. niger* strains expressing/overexpressing different heterologous proteins (Table 1). Each strain was cultured under identical conditions and UPR- and ERAD-responses were determined by examining the expression of UPR and ERAD marker genes in these strains. As markers for UPR induction we have chosen *bipA* and *pdiA* as an increase of the expression levels of these genes has been observed in strains expressing heterologous proteins (Punt *et al.*, 1998; Kauffman *et al.*, 2002; Guillemette *et al.*, 2007). The ERAD markers (*derA* and *hrdC*) were chosen based on *S. cerevisiae* studies in which induction of these genes was observed after protein folding stress (Knop *et al.*, 1996; Travers *et al.*, 2000). To confirm that *derA* and *hrdC* in *A. niger* were also induced under ER stress conditions, we grew N402 in the presence of increasing concentrations of DTT and tunicamycin to induce ER stress (Fig. 1).

![Graph](image)

*Figure 1.* Induction of two genes involved in the ERAD pathway (*derA* and *hrdC*) by the presence of increasing concentrations of DTT or tunicamycin stress agents. Samples for Northern analysis were collect after 16h growth on liquid CM (1% glucose) at 30°C. On the Y-axis is the relative expression of *derA* and *hrdC* in arbitrary units, normalized for loading differences by comparison with 18S ribosomal RNA probe.
Northern analysis and blot quantification revealed a high induction of both genes in the presence of DTT. In the case of growing in the presence of tunicamycin, an increase in derA and hrdC was observed at the higher concentrations tested (5 and 10 µg/ml).

Having established good marker genes for UPR- and ERAD-responses, we then studied the induction of these pathways in strains expressing different heterologous proteins (Fig. 2). Results in Fig. 2 visibly show different gene expression levels depending on the heterologous protein expressed. Although we see an increase in expression of UPR target genes in most of the strains bearing heterologous proteins in relation to N402, both UPR and ERAD responses were more boosted when A. niger strains expressed tPA (D15) and GlaGus (AB4-1dglaA36#3) heterologous proteins. In S. cerevisiae, a link between UPR and ERAD pathways has been established (Travers et al., 2000; Friedlander et al., 2000) and the co-induction of both UPR genes (bipA, pdiA) and ERAD genes (derA and hrdC) in A. niger in response to the expression of the heterologous GlaGus protein as observed in Fig. 2, suggests a similar link between these two pathways in A. niger. For reasons of availability of activity assays and antibodies against β-glucuronidase, the heterologous fusion protein GlaGus was then chosen as a reporter to study the fate of heterologous proteins under ERAD deficient conditions.

![Figure 2](image)

**Figure 2.** Relative expression (arbitrary units) of ERAD (derA and hrdC) and UPR (bipA and pdiA) reporter genes in strains expressing different heterologous proteins (see Table 1 for details). Samples for Northern blot analysis were collected from these strains grown for 16h at 30°C in liquid CM. Values were normalized for loading differences. The gene expression levels were normalized using the N402 values as reference.
4.3.2. The level of GlaGus expression affects UPR induction

To express a secreted form of the bacterial β-glucuronidase in *A. niger*, plasmid pBB19-3 was used (Punt *et al.*, 1994). This plasmid contains the bacterial *uidA* gene (encoding β-glucuronidase) which is fused to the glucoamylase gene. Plasmid (pBB19-3pyrG*) was constructed to generate strain MV3.2, which contains a single-copy integration of the GlaGus construct at the *pyrG* locus (data not shown). We will refer to this strain as the single-copy GlaGus (scGlaGus) strain in the remaining of the paper. Strain AB4.1Δgla#A36#3 has been reported to contain multiple copies of the pBB19-3 plasmid (Punt *et al.*, 1994; 1998).

To determine the number of copies of GlaGus gene present in the AB4.1Δgla36#3 strain, to which we will refer to as the multi-copy GlaGus (mcGlaGus) strain, we performed Southern blot analysis (Fig. 3A). After correcting for loading differences, we determined about 8 copies of the *glaA* gene in the mcGlaGus strain. Additionally, western blot analysis using a Gus specific antibody was performed on a total protein extract on these two strains and N402, where we observe that the difference in the number of copies between them relates to the amounts of Gus protein detected (or absence in the case of N402), as band intensity in mcGlaGus is higher than in scGlaGus (Fig. 3B).

![Figure 3](image.png)

**Figure 3.** (A) Southern blot analysis of the GlaGus copy number in mcGlaGus strain. Genomic DNA was digested with *Nco*I and probed with a probe annealing within the glucoamylase ORF. Expected band size for endogenous glucoamylase is 4.8 kb; for the scGlaGus strain 7.1 kb and 4.8 kb bands are expected. Ectopic integration of pBB19-3 in the mcGlaGus strain does not allow band size predictions, however the band(s) observed at 9.4 kb indicate that the plasmids have been tandemly integrated. Loading differences were corrected using a gel stained with ethidium bromide. (B) Western analysis of GlaGus amounts on total protein of mycelium samples of scGlaGus and mcGlaGus strains; N402 was used as a control for Gus antibody specificity. Samples were grown in CM for 24h at 30°C. The protein content was extracted, 10µg of total protein were separated by gel electrophoresis and immunodetected with an anti-Gus antibody. Detection was carried out through a chemiluminescence reaction for 5min.
Western analysis of medium samples from both the scGlaGus or mcGlaGus strains failed to detect the GlaGus protein in the medium, using Gus antiserum. To determine UPR induction in these strains, we examined the mRNA expression levels of bipA, pdiA and hacA (Fig. 4A). By comparison with N402, quantification of the mRNA levels show an induction of bipA and pdiA in the mcGlaGus strain but not in the scGlaGus strain (Fig. 4B), demonstrating that the copy number of this heterologous protein affects the UPR response.

**Figure 4.** (A) Northern blot analysis of mRNA levels of UPR target genes on strains containing either a single copy or multi copy GlaGus genes in comparison to N402. Total RNA was extracted from mycelia grown for 24h at 30°C in CM. (B) The UPR target genes expression levels were normalized using N402 as reference.

### 4.3.3. Construction and analysis of ERAD deletion strains

Misfolded proteins that become destined to be degraded are taken by the ERAD pathway, which involves many components that recognize aberrant proteins and activate their retrotranslocation to the cytosol for proteasome-mediated degradation. Among these many components, we have selected five genes indicated to be involved in different parts of the ERAD system to assess the effects of having a compromised ERAD in different A. niger backgrounds. We have deleted derA, doaA, hrdC, mifA and mnsA in the control strain (NC5; ΔkusA, amdS), the scGlaGus strain (MA99.3; ΔkusA, amdS, scpBB19-3pyrG*) and the mcGlaGus strain (MA135.3 (ΔkusA, amdS, mcpBB19-3). Transformants for each strain were purified on media containing acetamide and further examined by Southern blot analysis (data not shown). All five ERAD genes were successfully deleted in both NC5 and scGlaGus (MA99.3) backgrounds. In the mcGlaGus background, four ERAD genes were successfully disrupted, but obtaining a deletion mutant of the hrdC gene was unsuccessful, although over 140 putative transformants were screened. It should be noted that the inability to obtain this
disruptant was not caused because the disruption was lethal as also no heterokaryons were obtained on primary transformation plates. For unknown reasons the frequency of getting a homologous recombination in the hrdC locus is very low, even in the ku70 mutant background. During the process of making deletion mutants in hrdC in the other two strain backgrounds we also noticed a low homologous recombination frequency to obtain the knockout strains. Thus, in total 14 disruptions strains have been generated (Table 1). To further confirm the deletions and to examine whether the deletion of any of the ERAD related genes has an effect on the expression of ERAD itself, northern blot analysis was performed. Figure 5 depicts an example of one of the northern analysis and shows the effect of deleting ERAD related genes on the expression of the other ERAD genes in the scGlaGus strain background. First, the northern blot analysis confirmed the Southern blot data and no mRNA was detected when using probes corresponding to the respective gene deletion mutant. In addition, the hybridization (Fig. 5) and subsequent blot quantifications (data not shown) revealed no apparent increase or decrease in expression of any of the ERAD genes tested among the different strains, suggesting that deletion of a single component of the ERAD pathway does not affect the expression of other components of this pathway. Furthermore, probes against glaA and gus were used as an indication of the transcription of the fusion gene in the scGlaGus and mcGlaGus background, or its absence in the case of N402 (Fig. 5).

![Figure 5. Expression analysis of different genes in the five ERAD deletion strains on the scGlaGus background, scGlaGus parental strain, mcGlaGus and N402. Total RNA was extracted from mycelia grown for 24h at 30°C in CM. RNA (5 µg) was separated by agarose gel electrophoresis, blotted and hybridized with 32P-labeled probes specific for the genes indicated. 18S rRNA was used as loading control.](image-url)
The morphological and growth effects of the disruption of these ERAD genes in the 14 \textit{A. niger} strains were analyzed on CM and MM agar plates and compared to the growth phenotype of its corresponding wild type. We performed a drop dilution test on solid MM and monitored growth at 25, 30 and 42°C (Fig. 6). At 25 and 30°C, strains are able to grow and only Δ\textit{doaA} revealed a different phenotype. This mutant strain showed irregular colony morphology, slower growth and reduced sporulation. At 42°C and at the lower spore concentrations, the mcGlaGus and respective ERAD deletions are no longer able to form colonies, unlike N402 and the other strains tested. As the sensitivity towards high temperature is already observed in the mcGlaGus parental strain we can conclude that is the expression of a high copy number of this heterologous protein that confers this growth defect and not a defective ERAD. Also, at this temperature Δ\textit{derA} shows a more apparent growth defect than the other deletions.

\textbf{Figure 6.} Growth assay at different temperatures of parental strains NC5, scGlaGus and mcGlaGus and respective ERAD deletions. Spore serial dilutions were spotted onto solid MM and incubated under the given conditions. Growth was monitored for 3 days.
Subsequently, the sensitivity of the ∆ERAD strains towards a chemical that disturbs the ER homeostasis was tested by spotting $10^4$ spores per 10 µl on solid MM containing increasing concentrations of DTT and incubating at 25, 30 and 42°C for 3 days (Fig. 7). In general, at the temperatures 25 and 30°C and either absence or in the presence of increasing concentrations of the DTT, deletions strains grew like their parental strain. The growth of the ∆doaA mutant was affected in the absence of DTT, but the ∆doaA did not seem to be more sensitive towards DTT in this spot assay in comparison to the other ERAD deletions. As the growth phenotype of the doaA deletion strain was observed in all the different backgrounds, we attribute this growth phenotype to the absence of the doaA gene and not to the expression of the heterologous protein. On the other hand, at 42°C, the mcGlaGus strain not only shows a reduction on colony size compared to NC5 and scGlaGus, but also reveals an increased sensitivity towards 5 mM DTT (Fig. 7). At the concentration of 10 mM DTT, the growth of both scGlaGus and mcGlaGus strains is almost completely abolished, whereas the NC5 wild-type and ERAD deletion mutants are able to grow. A 20 mM concentration of DTT abolishes growth of all the strains and indicated that none of the ERAD ∆strains becomes more resistant towards DTT. As also observed in Fig. 6, the mcGlaGus∆derA strain displays a reduced growth and sporulation phenotype at 42°C (Fig. 7). Thus, the strains expressing the GlaGus protein are more sensitive to DTT compared to the respective parental strain that does not express the GlaGus protein, and high levels of GlaGus expression is correlated with a higher sensitivity to DTT.

**Figure 7.** Comparison of colony morphology of parental strains and respective ERAD deletion mutants incubated at different temperatures (indicated on the right) and in the presence of increasing concentrations of the stress agent DTT (indicated at the bottom). $10^4$ spores per 10 µl of each strain were spotted on solid MM and growth was monitored for 3 days. NA= not available.
We further conclude that the disruption of ERAD component had no further effect on the growth and/or the sensitivity towards DTT. Additional growth tests such as on simple/complex carbon sources (glucose vs starch) or under osmotic stress conditions (1 M NaCl, 0.6 M sorbitol) with those deletion mutants (besides ΔdoaA) resulted in no significant differences (data not shown). As none of the ERAD genes described revealed to be essential to A. niger, we decided to test for synthetic lethality by the combination of deleting any two of the five genes in study. The double ERAD knockout mutants were made by deleting an additional gene in the existing single knockouts strains in the NC5 background (data not shown) after looping out the andS marker used to disrupt the first ERAD gene. Then, the ERAD deletion constructs were transformed into these new 5 ERAD deletions (andS) strains obtained in order to get the 10 possible double deletion combinations. All the double KO transformants were purified on acetamide media and confirmed by Southern blot (data not shown) (see Table 1). All the double KO mutants were subjected to the phenotypic tests as described above but no additional differences or effects on morphology and growth were found by having any of the double ERAD deletions compared to the single mutants. The combination of doaA deletion with any of the other deletions did not give extra phenotypic features then the ones observed for the single doaA KO in any of the background strains (data not shown).

4.3.4. Activation of the UPR by strains expressing/overexpressing the GlaGus protein in combination with a defective ERAD pathway

To investigate whether deletion of ERAD components in combination with expression or overexpression of GlaGus has an effect on the UPR, the ERAD mutants were analyzed for the expression of UPR target genes (hacA, bipA and pdiA) and their expression was compared to their corresponding parental strain (Fig. 8).

Figure 8 shows the northern blot results and quantified mRNA levels of UPR target genes in the ΔERAD strains not expressing the GlaGus protein (NC5 background; 8A and B), the ΔERAD strains in the scGlaGus background (8C and D) and the ΔERAD strains in the mcGlaGus background (Fig. 8E and F). In the case of having a deficient ERAD pathway but no expression of heterologous protein (NC5 background), the UPR pathway is not induced (Fig. 8A and B). In the ΔdoaA, ΔmifA and ΔmnsA strains in the scGlaGus background, no increase in the expression levels of the UPR target genes is observed (Fig. 8C and D). However, the bipA expression level in derA and hrdC deletion strains are 1.8 and 2-fold higher, respectively, in comparison to the scGlaGus parental strain. Hence, there seems to be specific induction of bipA expression upon deletion of derA or hrdC. As depicted in Fig. 8E and F, the combination of overexpression of GlaGus with the deletion of any of ERAD components tested further induces the transcription of the UPR reporter genes.
4.3.5. Effects of the deletion of ERAD genes on protein production

To examine the effects of an impaired ERAD pathway on the GlaGus protein production or accumulation, we performed western blot analysis on medium samples and intracellular protein samples collected from scGlaGus and mcGlaGus strains. In the medium samples, no Gus activity was detected and no GlaGus protein could be detected using a Gus-specific antibody (data not shown), indicating that secretion levels are low. At this stage we cannot exclude the possibility that some secreted GlaGus protein is degraded by extracellular proteases. To examine the effect of the ERAD deletion mutant on the intracellular pool of
GlaGus protein, total protein content was extracted from fungal biomass as described in Material and Methods. For each set of experiments, two gels were run in parallel, one of them was immunoblotted and probed with an antibody against Gus (Fig. 9A and D) and the other gel was stained with Coomassie blue to be used as loading control (Fig. 9B and E). The relative amount of protein present in each deletion strain was determined in relation to the amount of protein detected in the parental strain (Fig. 9C and F). Using the Gus-antibody, we were able to detect a band corresponding to the GlaGus fusion protein (around 140 kDa), as well as smaller bands (Fig. 9A and D), which might represent truncated versions of the protein most likely caused by proteolytic activity. The amount of fusion protein detected in the scGlaGus background strains (Fig. 9C) is the highest in the ΔderA and ΔhrdC strains. Subsequently, we determined the amounts of fusion protein present in ERAD deletion strains in the mcGlaGus background by western blot analysis (Fig. 9F). Both the western blot and the quantified data clearly indicate higher amounts of fusion protein for all the deletions when compared to mcGlaGus parental strain. The deletion of derA had the most significant effect as a 6-fold increase in the GlaGus protein levels was detected. The results indicate that a defective ERAD leads to the accumulation of intracellular GlaGus, but this does not result in detectable production in the culture medium.

Figure 9. Effect of deletion of ERAD components on the amount of GlaGus fusion protein in total protein extracts. Western analysis of GlaGus amounts in total protein of mycelium samples of scGlaGus (A) and mcGlaGus (D) ERAD deletion strains. Samples were grown in CM for 24h at 30°C. 10μg of total protein was separated by gel electrophoresis and immunodetected with an anti-Gus antibody. Detection was carried out through a chemiluminescence reaction for 5 min. As a positive and negative control, 50 ng of purified Gus and a total protein extract from N402 were loaded. The arrow indicates the band corresponding to the GlaGus fusion protein (≈140kDa). The relative amounts of protein were normalized for loading differences by comparison with a “twin” gel stained with Coomassie blue (B, E). (C, F) Relative amount of GlaGus fusion protein detected in total protein extracts of strains with impaired ERAD and respective parental strain. Bars indicate standard deviations from two independent experiments.
4.4. Discussion

4.4.1. Different heterologous proteins, distinctive bottlenecks?

In filamentous fungi, the levels of heterologous protein production are often low (Gouka et al., 1997). Possible processes and mechanisms involved in protein degradation, especially those related to the ERAD pathway, are poorly described or understood in these fungi. As *A. niger* has such an outstanding capacity as a cell factory, the understanding of these mechanisms becomes crucial to improve heterologous protein production. We started out our study by comparing the effect of expressing different heterologous proteins in *A. niger*. In our analysis we have included proteins from bacterial origin, β-glucuronidase, which has successfully used as a reporter in gene expression in innumerable cell systems (Punt et al., 1994, 1998; Gilissen et al., 1998; Ayra-Pardo et al., 1999); the metazoan GFP, widely used as a fluorescent marker; the human proteins tPA and IL6 with valuable medical applicability (Upshall et al., 1987; Broekhuysen et al., 1993; Punt et al., 1998; Wiebe et al., 2001); and basidiomycetes enzymes with wide biotechnological applications Manganese Peroxidase (MnP) and laccase (Conesa et al., 2000; Weenink et al., 2006; Elisashvili and Kachlishvili, 2009). Expression of all the heterologous proteins result in relative low production levels compared to the production of glucoamylase expressed form the same promoters (Archer et al., 1994; Gouka et al., 1997). Several potential bottlenecks for the production of proteins have been evaluated and discussed over the last decade and a potential bottleneck for efficient secretion in folding of the heterologous proteins in the ER has been considered as a major issue. BipA and PdiA, encoding a chaperone and a foldase respectively, have been identified as reliable reporter genes as indicated for ER stress in filamentous fungi (Punt et al., 1998; Kauffman et al., 2002; Guillemette et al., 2007). Comparison of the different *bipA* and *pdiA* mRNA levels in the strains expressing the different heterologous proteins revealed that not all heterologous proteins induce a strong UPR response (Fig. 2) despite the fact that the production levels of e.g. the Human IL6 protein are low. Clearly, the lack of a strong UPR response in some strains producing low levels of heterologous proteins strongly suggests that also non-UPR mediated bottlenecks exist in *A. niger* that hamper efficient secretion. Two heterologous proteins, human tissue plasminogen activator (t-PA) and the bacterial glucuronidase (Gus) displayed a strong induction of the *bipA* and *pdiA* reporters, indicating that these two proteins induce a strong UPR response (Fig. 2). Interestingly, the expression of two genes involved in ER associated degradation pathway (*derA* and *hrdC*) was also induced, suggesting that t-PA and Gus might be targeted for proteolytic degradation via the ERAD system. Besides the protein specific issues, we also show that the induction of the UPR pathway is dependent on the level of expression (Fig. 4). The UPR inducing property of the glucuronidase is only observed when the protein is highly expressed. Apparently, under relative low-expressing conditions, *A. niger* is capable of dealing with the protein in such a way that the protein does not induce the UPR. Our results suggest that in the case of having a single-copy of GlaGus, the basal protein folding and quality control machinery is able to cope with the heterologous protein and only high levels of GlaGus protein in the ER induces ER stress.
4.4.2. Expression of the bacterial glucuronidase results in increased thermo and DTT sensitivity

The growth of transformants containing single-copy or multi-copy insertions of the GlaGus construct at different temperatures was compared to the parental strains (Fig. 6). Growth of the mcGlaGus strain was severely impaired at 42°C suggesting that the temperature stress (42°C is above the optimal growth temperature of A. niger) in combination with the presence of misfolded GlaGus protein in the ER also affects the processing of endogenous cargo resulting in a growth retardation. Environmental factors have an influence on cells productivity, and it has been recently shown in Pichia pastoris that cultivating this fungus below its optimal growth temperature results in a more efficient secretion of heterologous proteins due to a general decrease of folding stress at lower temperatures (Dragosits et al., 2009). Furthermore, we reasoned that an additional ER stress inducing condition might further aggravate this phenotype and therefore the strains were also growth impaired in the presence of increasing concentrations of DTT (Fig. 7). The results clearly indicate that high temperature, the presence of DTT and the expression of the GlaGus protein acts additionally and interferes with growth. In the case of expressing high levels of GlaGus, a concentration of 5 mM DTT was enough to prevent growth at 42°C, whereas growth of the single-copy GlaGus strain was inhibited at 10 mM DTT, a condition that still allowed growth of the strain lacking this heterologous protein.

4.4.3. ERADication of misfolded proteins in A. niger

The function of the ERAD pathway during normal vegetative growth and its possible involvement in the degradation of misfolded proteins in the ER was analyzed by disrupting putative ERAD components in a wild-type background and in backgrounds expressing the glucoamylase-β-glucuronidase (GlaGus) fusion protein as a reporter. Five genes (derA, doaA, hrdC, mifA and mnsA) involved in different aspects in the ERAD pathway were selected and identified in the A. niger genome to establish whether this pathway has an important role during the degradation of the GlaGus protein. The systematic analysis of these five genes either as single deletions or as double mutants clearly showed that the effect of the gene deletion on growth as well as on the faith of the heterologous protein was limited. Phenotypic assays performed on the ERAD deletion strains showed that, except for ΔdoaA, the deletion of ERAD components does not result in an apparent phenotype (Fig. 6). Moreover, deletion of the ERAD genes did not increase the sensitivity of the ERAD mutants in comparison to the respective parental strains towards tunicamycin (data not shown) or DTT (Fig. 7). It has been reported that the deletion of DER1 and HRD3 in the yeast S. cerevisiae does not lead to a detectable growth phenotype although the ERAD pathway is strongly affected (Knop et al., 1996; Travers et al., 2000). However, this lack of phenotype has been explained as a result of compensatory effects of the UPR induction (Travers et al., 2000), as deletion of DER1 only becomes lethal when combined with the deletion of IRE1 and at the restrictive temperature of 37°C (Mori et al., 1993; Travers et al., 2000). The deletion of the DER1 and HDR3 homologues in A. niger does not result in a phenotype different from the wild-type strain, but contrary to Travers and co-workers results (2000), under normal growth conditions there is
Chapter 4

Effects of a defective ERAD pathway on growth and heterologous protein production in *Aspergillus niger*

no evidence for activation of UPR in the *A. niger* strains lacking *derA* or *hrdC* (Fig. 8A and B). The *doaA* deletion was the only mutant showing a growth defect in all the strains tested, translated into an irregular morphology and reduced sporulation (Figs. 6 and 7). In *S. cerevisiae*, Doa1p is known to play an important role in the ubiquitin-dependent protein degradation by a direct interaction with Cdc48p (a member of the AAA-ATPase family of molecular chaperones) (Ye *et al.*, 2001b; Ogiso *et al.*, 2004; Mullally *et al.*, 2006). In fission yeast, the deletion of the *doa1* homologue (*lub1*) results in a defective ubiquitin/proteasome-dependent proteolysis, causing increased cell sensitivity to several stress conditions (Ogiso *et al.*, 2004). Although there is no evidence for protein accumulation either in scGlaGus or mcGlaGus Δ*doaA* background strains (Fig. 9), we might hypothesize that in *A. niger*, the lack of ubiquitination fails to target proteins to ERAD-mediated destruction, inducing another degradation pathway that could impair the fungal growth.

4.4.4. Induced BipA levels correlate with increased levels of intracellular GlaGus

To examine possible UPR induction in the strains with an impaired ERAD pathway in combination with the GlaGus protein expressed, we analyzed mRNA expression levels of *hacA*, *bipA* and *pdiA*. Results in Fig. 8A and 8B clearly show that under normal growth conditions, in the wild type background, the absence of any of the ERAD genes in study does not lead to induction of the UPR pathway. Overall, in the scGlaGus background (Fig. 8C and 8D), the deletion of ERAD components does not seem to trigger the UPR as values of *hacA*, *bipA* and *pdiA* are maintained relatively constant. Only in the *derA* and *hrdC* deletion strains an increase of about 2-fold of *bipA* mRNA is observed. In parallel, we observe in scGlaGus *derA* and *hrdC* deletions the highest accumulation of GlaGus intracellularly, indicating that high levels of *bipA* mRNA are correlated with higher levels of GlaGus protein (Fig. 9C). Our results suggest that in the absence of ERAD proteins DerA and HrdC, GlaGus might be retained in the ER longer which might be responsible for triggering the induction of *bipA* levels. In the mcGlaGus background, an increase in the levels *bipA* and *pdiA* mRNA levels was observed for all the ERAD gene disruptions (Fig. 8F). Hence, not only the high levels of GlaGus produced triggers a UPR (Fig. 4A), but the combination with the deletion of any of the ERAD genes and consequent accumulation of intracellular GlaGus might stimulate it even further (Fig. 8F). Again, the highest level of induction of *bipA* mRNA is correlated with the highest levels of GlaGus protein. In the mcGlaGus background, the deletion of *derA* had the most significant effect on the amount of intracellular protein detected and resulted in a 6-fold increase in GlaGus levels.

A general observation concerning the UPR induction throughout our study, is the constant values of *hacA* mRNA itself. Mulder and co-workers (2004) have shown that upon UPR induction, *hacA* is able to up-regulate its own transcription via HacA binding sites in the HacA promoter region (Mulder *et al.*, 2004, 2009). Examining the *hacA* expression levels in our studies showed that the levels of *hacA* mRNA were not induced in response to the expression of the GlaGus protein (Figs. 8, 9). In the studies of Mulder *et al.*, in which the induced expression levels of HacA are reported, the cells were suddenly exposed to ER-stress inducing chemicals, whereas in our case the strains might have been adapted to the conditions
of expressing/overexpressing the heterologous protein and therefore the hacA induction is not evident.

4.4.5. Alternative mechanisms of degradation

Our research revealed a surprisingly modest effect on the deletion of ERAD functions in A. niger, even under ER stress conditions. It was anticipated that the inability to remove misfolded proteins from the ER by deleting ERAD components would result in severe ER stress situation and, by analogy to metazoans, might induce apoptosis-like phenotypes (see for review Rasheva and Domingos, 2009). Therefore, other mechanisms besides ERAD might be of importance in the clearance of misfolded proteins and help the cells cope with the stress. The lack of proteins in the medium together with the observed degradation in our western analysis (Fig. 9) suggests alternative pathways to remove misfolded proteins, such as the presence of proteases directly in the ER, as it has been shown for mammalian systems (Evnouchidou et al., 2009); Sec61p-DerA/HrdC independent transport to the cytosol; or mechanisms of direct targeting of misfolded proteins to the vacuole. The GlaGus reporter strains used in this study allow non-biased genetic screens to identify mutants involved in these alternative protein degradation pathways.
Effects of a defective ERAD pathway on growth and heterologous protein production in *Aspergillus niger*