Functional genomics to study protein secretion stress in *Aspergillus niger*

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Aos meus queridos pais:
Domingos e Adelina

Contents

Chapter 1

General Introduction

1.1. A general introduction to *Aspergillus niger*

The Fungi Kingdom includes both yeasts and filamentous fungi and is estimated to contain 1.5 million species and *Aspergillus niger* is one of the 100.000 species that has been described in detail (Hawksworth, 2001). *A. niger* is abundantly present in rich soils, but can also be found on plants or indoor air environments and has a saprophytic lifestyle. *A. niger* is specialized in secreting extracellular enzymes which enables growth on decaying organic materials (Raper and Fennell, 1955) in a wide range of temperatures (6-47°C), pH-values (1.4-9.8), and at relatively high water activity (0.88) (Reiss, 1986). This fungus forms compact whitish or yellowish septed mycelial hyphae covered with a dense layer of conidiophores containing millions of asexual spores (Tzean *et al*., 1990). Taxonomically, it has been placed in the black *Aspergilli* group (Raper and Fennell, 1965). *A. niger* is known to cause black mold disease on certain fruits and vegetables such onions and peanuts, and is a widespread food contaminant (Samson *et al*., 2001). Unlike other *Aspergillus* species, *A. niger* rarely causes human diseases, although when large amounts of spores are inhaled and able to colonize, it might cause aspergillosis (lung disease), mostly associated to immune compromised patients (reviewed in Denning, 1998).

1.2. Biotechnological importance of *Aspergillus niger*

Products produced by *A. niger* have acquired the Generally Regarded As Safe (GRAS) status by the United States Food and Drug Administration (FDA) (Bigelis and Lasure, 1987), although the production of ochratoxin A and fumosins by this species has been described (Nielsen *et al*., 2009). The effective secretion of enzymes and metabolites (e.g. organic acids) together with the ability to use a wide variety of substrates makes this fungus very attractive for exploitation by different types of industry. Through the development and improvement of many genetic modification techniques such as DNA-mediated transformation, generation of gene knock-outs, the use of strong promoters, mutagenesis (Tilburn *et al*., 1983; Archer *et al*., 1994, Punt *et al*., 1994, Gouka *et al*., 1997a) and the establishment of a Δ*ku70* system in the first decade of this century (Ninomiya *et al*., 2004; Meyer *et al*, 2007), *A. niger* became even more appealing as a host for the (over)production of homologous (van Gorcom *et al*., 1991, van Hartingsveldt *et al*., 1993) and heterologous proteins (Jeenes *et al*., 1991.; Archer and Peberdy, 1997; Punt *et al*., 2002, Lubertozzi and Keasling, 2009). *A. niger* has a high capacity for the production and secretion of extracellular enzymes; for instance α-glucosidases and amylases (Frost and Moss, 1997), cellulose and lignin degrading enzymes (reviewed in Dashtban *et al*., 2009), invertases (Ge *et al*., 2009), beta-galactosidases (O'Connell and Walsh, 2008), oxidases and catalases (Berka *et al*., 1992), pectinases (Grassin and Fanguenbergue, 1999) and acid proteases (te Biesebeke *et al*., 2005). Production of these extracellular enzymes allows the fungus to grow on plant cell wall and complex polysaccharides such as xylan, pectin, starch and inulin (de Vries and Visser, 2001; Yuan *et al*., 2006). The high secretion capacity of *A. niger* is well illustrated by the enzyme α-amylase, which can be produced up to 30 g/L during industrial cultivation (Durand *et al*., 1988; Finkelstein *et al*., 1989). Apart from the production of proteins, *A. niger* is also able to

produce high amounts of citric acid, a weak organic acid and natural preservative used in the beverage industry and mostly commercially produced by *A. niger* during fermentation processes (Berry *et al*., 1977; Kubicek and Rohr, 1986). The genome of *A. niger* has been sequenced and the genome of 33.9 Mb in size, comprises 14,165 predicted ORFs of which about 46% are related to known functions (Pel *et al*., 2007). Additionally, Affymetrix microarrays are also available. Together, the genome and transcriptomic data availability present powerful tools for the study and further exploitation of this biotechnologically important fungus for industrial applications.

1.3. Protein secretion: bottlenecks and improvements in protein yields

The increasing knowledge about the metabolism of *A. niger* and its unique secretion capacity awards this fungus a special place among the modern-biotechnology microorganisms useful for mankind (Fleissner and Dersch, 2010). However, the mechanisms that enable such high levels of protein production and secretion seem to be rather complex and are still poorly understood. The mycelium of filamentous fungi is formed when a spore germinates and forms compartmentalized tubular shaped hyphae, which extends at the tip while also branching sub-apically (Momamy, 2002). Elongation and secretion of proteins into the extracellular environment occurs at the hyphal apices (Wösten *et al*., 1991; Lee *et al*., 1998; Gordon *et al*., 2000b) and involves the presence of a complex called Spitzenkörper (reviewed in Harris *et al*., 2005). Although the processes related to polarized growth and understanding the true nature of the Spitzenkörper are ongoing, still many questions remain to be answered (reviewed in Harris *et al*., 2009). Additionally to this complex process of polarized growth, protein secretion seems to be heterogeneous between different hyphae in the fungal colony (Wösten *et al*., 1991; Vinck *et al*., 2005). To improve protein production yields it is very important to understand the key factors, not only in protein secretion and events throughout the secretory pathway, but also controlling gene expression (reviewed in Fleissner and Dersch, 2010). Strong promoters are usually used to improve gene expression, include the promoter of glucoamylase (*glaA*) gene (Carrez *et al*., 1990; Fowler *et al*., 1993) or glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene (Punt *et al*., 1991). In other cases, the use of the catalytic subunit *glaA* gene instead of only the promoter fused to the gene of interest also proved to be successful (Ward *et al*., 1990; Gouka *et al*., 1997b). An increase copy number of the gene of interest usually correlates to an increase to the protein yield until a certain threshold, as observed for *glaA* (Verdoes *et al*., 1994a).

A major bottleneck for efficient protein secretion that is usually pointed out is at the level of protein folding in the endoplasmic reticulum (ER). ER localized chaperones are responsible for protein folding and minimizing protein aggregations (reviewed in Sharma *et al*., 2009). Therefore, attempts to modify the cellular levels of chaperones have been performed, in order to improve homologous/heterologous protein production (Conesa *et al*., 2002; Lombraña *et al*., 2004). The structure of the signal peptides present in the secretory proteins and its processing can also be a rate limiting parameter in secretion (Contreras *et al*., 1991; Spencer *et al*., 1998). Glycosylation is another important factor in protein secretion as

it has been reported that glycosylation improves the secretion of heterologous proteins (Sagt *et al*., 2000; Perlińska-Lenart *et al*., 2005; van den Brink *et al*., 2006). Apart from the ER-related bottlenecks, one of the other major limitations when using fungal hosts for protein production is the secretion of high levels of proteases. As a result, *Aspergillus sp.* strains deficient in proteases have been developed (Mattern *et al*., 1992; van den Homberg *et al*., 1995; Wang *et al*., 2008; Punt *et al*., 2008; Yoon *et al*., 2010) to increase the yield of heterologous protein production.

1.4. The secretory pathway in eukaryotes

The secretory pathway in eukaryotic cells is a coordinated network of organelles that transport membrane lipids and proteins to the cell surface. In the yeast *Saccharomyces cerevisiae* and in mammalian cells this pathway has been very well studied and is often used as reference for the study of the secretory pathway in filamentous fungi. The journey of secreted proteins begins with the transcription of DNA information contained in the genes into mRNA which is translated into proteins. Proteins destined to be secreted or destined to cellular compartments contain an N-terminal signal sequence and are delivered to the endoplasmic reticulum (ER) membrane, via one of two routes: the signal recognition particle (SRP)-dependent pathway or SRP-independent pathway. Independently of which pathway the proteins follow, they engage the translocation machinery, leading to the co- or post-translationally translocation of proteins from the cytosol into the ER (Grudnik *et al*., 2009). Folding is assisted by ER chaperones/foldases until they reach the proper 3-dimentional conformation and cleared for secretion (Harding *et al*., 1999). Proteins that fail to acquire their native conformation, due to genetic errors, cellular stresses or random incidents might be prejudicial to the cells and are, therefore, targeted for destruction (Ellgaard and Helenius, 2001). The different steps occurring throughout the secretory pathway will be discussed in more detail in the following sections.

1.4.1. Signal sequence, protein translocation, folding and maturation in the ER

The journey of secretory proteins begins with the insertion of a pre-protein into the lumen of the ER. A signal sequence present on the N-terminus of a nascent polypeptide can be recognized during translation by the signal recognition particle (SRP), and then the SRP-ribosome complex is targeted to the ER membrane via interaction with the SRP receptor. Next, the nascent chain is transferred from SRP receptor to the protein conducting channel Sec61 into the ER (translocation), through which it is co-translationally threaded (Ganoza and Williams, 1969; Schnell and Hebert, 2003; Grudnik *et al*., 2009). After translation of the polypeptide chain is finished, the signal sequence is cleaved and the polypeptide release into the ER. Alternatively to the SRP-dependent pathway, posttranslational translocation of newly synthesized polypeptide chains also occurs (SRP-independent pathway) (Ng *et al*., 1996). In this case, after being synthesized in the cytoplasm, the nascent protein forms a protein-chaperone complex with the Hsp70 chaperone which is directed by the signal sequence to the

ER membrane. At the ER, the Sec-complex acts as a membrane receptor and translocation occurs similarly to the SRP-dependent pathway (Ng *et al*., 1996). Ng and co-workers (1996) have shown that proteins usually follow specifically one of the SRP pathways, but some proteins can use both.

Protein folding can already start during translocation and continues until native protein structure is achieved (Hendershot, 2000). The efficiency of the folding process is protein dependent and in the ER, proteins are subjected to a strict quality control that assures that only properly folded proteins continue their travel along the secretory pathway (reviewed in Sayeed and Ng, 2005). The ER contains high concentrations of molecular chaperones that assist proteins in their folding and prevent aggregation of unfolded polypeptides. BiP (Binding protein) is one of the most important and well studied chaperones of the ER. It not only assists folding (Haas and Wabl, 1983) but also facilitates translocation (Matlack *et al*., 1999), regulates protein aggregation (Puig and Gilbert, 1994), plays a role in the ER calcium homeostasis (Lièvremont *et al*., 1997), contributes for the dislocation of misfolded proteins into the cytosol (Molinari *et al*., 2002; Kabani *et al*., 2003), which makes BiP an important factor in the Unfolded Protein Response (UPR, reviewed in Patil and Walter, 2001). The formation of disulfide bonds is another crucial stage in protein maturation. Protein Disulfide Isomerase (PDI) is a multifunctional enzyme that can act as a chaperone as well as an oxireductase (Gilbert, 1998; Xiao *et al*., 2004). Additionally, it can also inhibit the aggregation of misfolded proteins without disulfide bonds (Cai *et al*., 1994) and hence, increase the reliability of the folding/maturation process.

Secretory proteins often become glycosylated either at serine or threonine residues (O-glycosylation), a modification that is thought to be important for the proper folding of protein and to protect the proteins from proteolytic activity; or at selected asparagines residues (N-glycosylation) (Chen *et al*., 1994). Protein N-glycosylation plays a very important in the quality control mechanism to detect misfolded proteins in the ER. These misfolded proteins are removed from the ER by the Endoplasmic Reticulum Associate Degradation pathway (ERAD) (see next section for details). In eukaryotic cells, the targeting of substrates for degradation depends on the presence of glucose residues on the N-glycan chains (Fig. 1). The majority of proteins in the ER are modified with N-linked oligosaccharides that consist of nine mannose residues and three glucose residues (Fig. 1). Glucosidases I and II sequentially remove the outer two terminal glucoses. Monoglucosylated N-glycans are recognized by calnexin (CNX) and calreticulin (CRT). Once in the CNX-CRT cycle (Caramelo and Parodi, 2008), the protein can become proper folded and is subsequently released by a final glucose removal by Glucosidase II. If proper folding is not achieved, the protein is recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT), which transfers a glucose to the N-Glycan of the unfolded protein, allowing re-entering in the CNX-CRT cycle for further rounds of folding (Trombetta and Helenius, 2000; Ritter and Helenius, 2000; Caramelo *et al*., 2003). The de-glycosylation and re-glycosylation cycles continue until the protein is fully folded (quality control) (reviewed in Lederkreme, 2009). The modifications of the N-glycans of proteins by ER mannosidases seem to act as a timer for targeting proteins for degradation and prevent that misfolded proteins accumulate in the ER or are kept on being re-glucosylated by UGGT and trapped in the CNX-CRT cycle (Avezov *et al*., 2008; Termine *et al*., 2009).

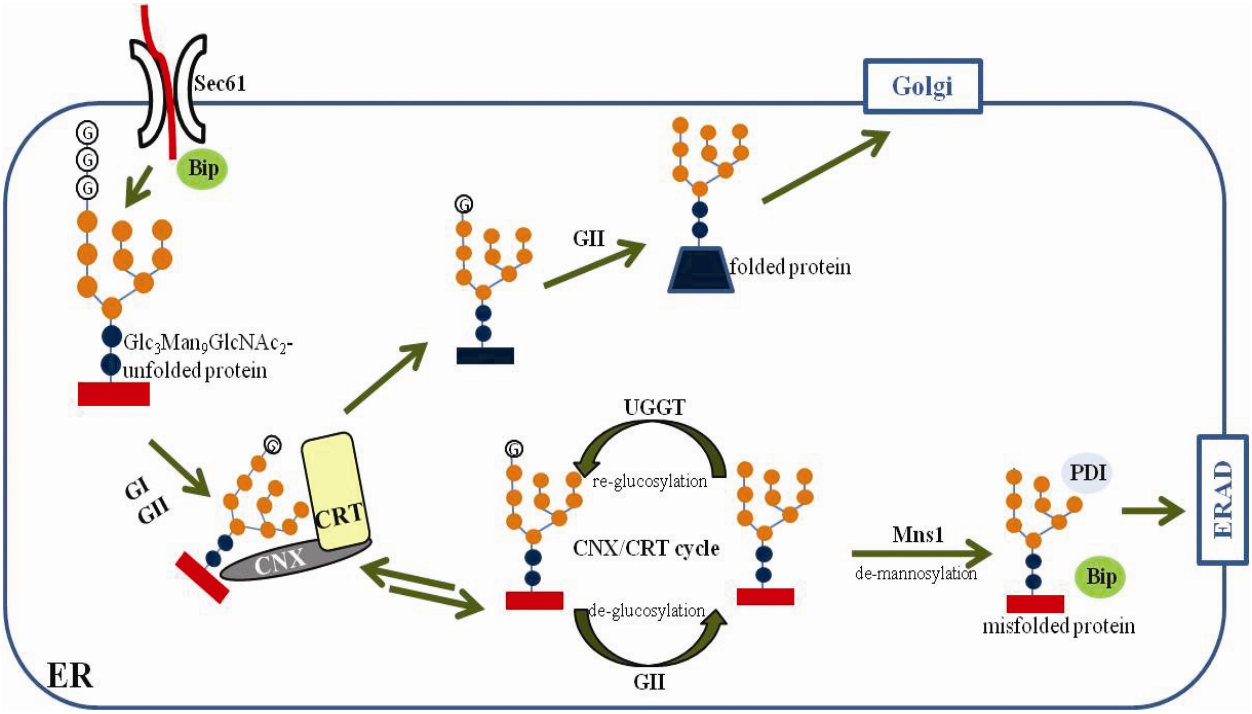

Figure 1. Folding and degradation of a glycan protein. N-linked glycans are added to newly synthesized polypeptides that enter the ER via the Sec61 complex. Two glucose residues are trimmed from the glycan chain by GI (glucosidase I) and GII (glucosidase II) before associating with calnexin (CNX) and calreticulin (CRT) and entering the CNX-CTR cycle. Then, GII removes the last glucose residue. The protein is recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT) which transfers a glucose to the N-Glycan of the unfolded protein, allowing further rounds of folding. When correctly folded, the protein is no longer recognized by UGGT, leaves the CNX-CRT cycle, again de-glucosylated and packed in vesicles heading towards the Golgi. If misfolding persists, the protein becomes a substrate for the ER mannosidase I (MnsI), which removes one (or more) mannose residue(s). De-mannosylated proteins attract chaperones and foldases that target the misfolded protein to the ERAD machinery to be degraded. (Adapted from Hebert and Molinari, 2007).

Differences between *S. cerevisiae* and mammalian cells are the absence of calreticulin and glucosyltransferase (GT) homologues in this yeast. Furthermore, in *S. cerevisiae* calnexin is required for ERAD while in mammalian cells this enzyme delays the actual protein disposal (Fernández *et al*., 1994). *S. cerevisiae* contains only one ER-mannosidase and removal of one mannose seems enough for protein disposal, whereas in mammalian cells the process and elements responsible for de-mannosylation are more complex (Hosokawa *et al*., 2001; Oda *et al*., 2003). A GT homologue can be found in *Schizosacharomyces pombe* (Fernández *et al*., 1994; Fanchiotti *et al*., 1998) and *A. niger* (Pel *et al*., 2007), although no calreticulin homologue has been reported from genomic studies in filamentous fungi.

When successfully matured, secretory proteins are ready to be packed in transport vesicles and leave the ER towards the Golgi apparatus. ER resident proteins are transported back in this organelle due to the presence of ER retention signals, such as HDEL, KDEL and HEEL in *Aspergillus* (Ngiam *et al*., 1997; Derkx and Madrid, 2001; Peng *et al*., 2006) for soluble proteins and a KK-motif for membrane proteins (Vincent *et al*., 1998). The ER retention of lumenal proteins is achieved by a process which involves binding of escaped

proteins via the ER retention signals to the ERD2 receptor in a post-ER compartment and return of the protein-receptor complex back to the ER (Lewis *et al*., 1990; Semenza *et al*., 1990). Fluorescent-tagged proteins with ER retention signals have been successfully used to visualize ER structures in *A. niger* (Gordon *et al*., 2000a,b) and *A. nidulans* (Fernández-Abalos *et al*., 1998), an organelle characterized as tubular-shaped network throughout the hyphae.

1.4.2. Intracellular transport of proteins

The transport of proteins from the ER to the Golgi is mediated via coatamer protein II complex (COPII) vesicles (reviewed in Dancourt and Barlowe, 2010). When protein-containing vesicles arrive at the Golgi, they fuse with the membrane of this organelle and release their content. Then, proteins are transported throughout the Golgi stacks where additional modifications take place, such as further glycosylation and proteolytic processing (Gouka *et al*., 1997; Kasajima *et al*., 2006). Retention of proteins in the Golgi is carried by the processing of targeting and retention/retrieval signals by proteases such as the aminopeptidase DPAP A (Nothwehr *et al*., 1993), the carboxypeptidase Kex1p (Cooper and Bussey, 1989) and endopeptidase Kex2p (KexBp/PlcA in *A. niger*) (Wilcox *et al*., 1992; Punt *et al*., 2003). The specificity of Kex2p has been exploited in heterologous protein production, where the protein of interest is fused to the coding sequence of a carrier protein including a Kex2 processing site and in this way becomes stabilized during intracellular transport. Afterwards the Kex2 protease processing allows the secretion of the heterologous protein separately from the carrier protein (Gouka *et al*., 1997; Venancio *et al*., 2002). When processing is complete, proteins are either targeted to the vacuole (Iwaki *et al*., 2006) or continue their transport to the plasma membrane. Secretory proteins are again packed into vesicles and, with the aid of microtubules (MTs) and motor proteins, transported to the membrane through interaction with actin cytoskeleton cables and towards the fungal hyphal tips (Abenza *et al*., 2008), a process driven by the Spitzenkörper (Harris *et al*., 2005). In filamentous fungi MTs are involved in the long-distance transport of secretion vesicles, whereas actin filaments localizes at the hyphal tips, where they are involved in polarity establishment and fusion to the membrane (secretion) (Horio and Oakley, 2005; Upadhyay and Shaw, 2008). Secretion related guanosine triphosphate-binding proteins (GTPases) are key elements in the regulation of several steps of protein intracellular transport such as vesicle formation, motility, docking, membrane remodelling and fusion (Segev, 2001; Fukuda, 2008). The exocyst is a complex that has been described in *S. cerevisiae* as responsible for the docking and fusion of the vesicles with the plasma membrane (TerBush *et al*., 1996). Exocyst components have been identified in fungal genomes and studied in some detail (Taheri-Talesh *et al*., 2008, Harris SD, 2009). The secretion related GTPase Sec4p is part of this complex (Guo *et al*., 1999), and although the *A. niger* homologue has been identified – *srgA* – and characterized as involved in protein secretion, hyphal polarity and sporulation, it is not able to complement the *S. cerevisiae* Sec4p mutant (Punt *et al*., 2001). Upon fusion of vesicles with the plasma membrane, the content is released into the extracellular medium.

1.4.3. Different models for intra Golgi transport

Currently, there are three models that attempt to explain the origin of the different compartments within the Golgi complex and how the cargo is transported throughout this organelle: the "classic", the "cisternal maturation" and "rapid partitioning" models. According to the classic view, cisternae move gradually across the stack from the *cis*- to the *trans*-Golgi; new cisternae are formed at the *cis*-face by the coalescence ER-derived membranes, while cisternae at the *trans*-face appeared to be fragment into secretory vesicles (Rothman and Wieland 1996; Bonfanti *et al*., 1998). In relation to the "cisternal maturation" model, the secretory cargo is carried forward by cisternal progression, while COPI vesicles travel in the retrograde direction to recycle resident Golgi proteins. In this way, the cisternae matures as it progresses through the stack by exporting "early" Golgi proteins to younger cisternae while receiving "late" Golgi proteins from older cisternae (Bonfanti *et al*., 1998; Pelham 1998; Pelham and Rothman 2000; Matsuura-Tokita *et al*., 2006; Losev *et al*., 2006). More recently, and in addition to the "cisternal maturation" model, Patterson *et al*. (2008), have shown evidence for a role of lipids in the Golgi assembly. In this "rapid partitioning" model, lipids are sorted into different domains (processing and exit) and proteins associate with their preferred lipid environment. Glycerophospholipids and sphingolipids are the major lipid classes in the Golgi. The membrane-associated cargo proteins can freely diffuse from and to processing domains; from processing domains to exit domains, and finally transported from the exit domains to the plasma membrane (Patterson *et al*., 2008). Moreover, the trafficking of enzymes and transmembrane cargo can also occur in a bidirectional way (*cis*-*trans* and vice versa) through the Golgi (Pelham and Rothman 2000; Patterson *et al*., 2008). Microscopy studies revealed that, unlike the characteristic Golgi stacks of cisternae of animals and plants, the Golgi in filamentous fungi is a dynamic network of tubules, rings and fenestrated structures, denominated Golgi Equivalents (GEs). Common Golgi markers in filamentous fungi include PH^{OSBP} and GmtA/B localized at the *cis*-Golgi and CopA and $HypB^{Sec7}$at the *trans*-Golgi (Breakspear *et al*., 2007; Jackson-Hayes *et al*., 2008; Pantazopoulou and Peñalva, 2009).

1.5. SOS: coping with ER overload and the presence of aberrant proteins

The main purpose of the Endoplasmic Reticulum Quality Control (ERQC) system is to assure that only properly folded proteins are allowed to transit through the secretory pathway (Ellgaard and Helenius, 2001). Mechanisms have evolved in the cell to ensure proper folding of proteins either by enhancing the ER folding capacity (e.g. foldases and chaperones) through a complex signalling pathway termed UPR. When these systems do not succeed in proper folding of the secretory proteins, these misfolded proteins are targeted for destruction, via a pathway denominated ERAD. Models of both the UPR and ERAD pathways are well described in *S. cerevisiae*, and together with what has been described for mammalian cells, they have been used as reference for the studies presented in this thesis. A detailed description of these pathways follows in the next sections.

1.5.1. UPR: dealing with the accumulation of proteins in the ER

Additionally to usual protein cargo in the ER, occasional burdens can occur due to different causes such as an increase demand for secretory proteins, misfolded proteins, genetic mutations, viral infections, unbalanced calcium levels, nutrient deprivation, or exposure to harsh chemicals (Malhotra and Kaufman, 2007; Schröder, 2008). These conditions have been shown to trigger the so-called Unfolded Protein Response pathway. The activation of the UPR pathway provides a strategy to antagonize the perturbations in the ER. In *S. cerevisiae*, ER stress leads to the dimerization and autophosphorylation of the ER transmembrane sensor inositol-requiring protein-1 (IRE1p) (Cox *et al*., 1993; Shamu and Walter, 1996), apparently as a result of BiP dissociation (Okamura *et al*., 2000). The autophosphorylation of IRE1 kinase domain activates its site specific endoribonuclease function (Shamu and Walter, 1996), resulting in the unconventional splicing of a 252-nucleotide intron from the mRNA coding for the basic leucine zipper (bZIP) transcription factor Hac1p (Sidrauski and Walter, 1997). The mRNA is cleaved at specific sites to excise the intron (Sidrauski and Walter, 1997) and the exons ligated by the tRNA ligase Rlg1p (Sidrauski *et al*., 1996). Hac1 is then translated into an active protein and migrates into the nucleus where it binds to Unfolded Protein Response Elements (UPRE) (CANCNTG, Mori *et al*., 1998) in target genes coding for chaperones and foldases as well as other components of the secretory pathway (Travers *et al*., 2000). In *A. niger*, HacA is activated by a similar mechanism as in yeast, but in this case IreA removes a 20-nt intron in the *hacA* mRNA (Mulder *et al*., 2004) and, in addition, an alternative start site reliefs the *hacA* transcript from translation attenuation, a process that has not been found in *S. cerevisiae* or mammalian cells (Mulder and Nikolaev, 2009). Once activated, HacA binds to UPR responsive genes, such as *bipA* and *pdiA*, containing the consensus sequence 5'-CAN(G/A)NTGT/GCCT-3' (Mulder *et al*., 2006).

Additionally to Hac1p/HacA, the transcription factor Gcn4p/CpcA also plays a role in the UPR. It has been characterized as the regulator of genes involved in the response to amino acid starvation (Natarajan *et al*., 2001; Wanke *et al*., 1997) and through interaction with Gcn2p/Cpc-3 (Sattlegger *et al*., 1998) it also induces UPR target genes (Patil *et al*., 2004; Arvas *et al*., 2006). Patil and co-workers have shown that is the interaction of Gcn4p and Hac1p that leads to the transcription of the UPR target genes, whereas Hac1p alone is able to bind but not activate those (Patil *et al*., 2004).

In mammalian cells, UPR is a more complex signaling pathway that comprises three main branches activated by ER-localized transmembrane signal transducers IRE1 (Yoshida *et al*., 2001), PERK (Harding *et al*., 2000a) and ATF6 (Yoshida *et al*., 2000). A schematic overview of the mammalian UPR is depicted in Fig. 2.

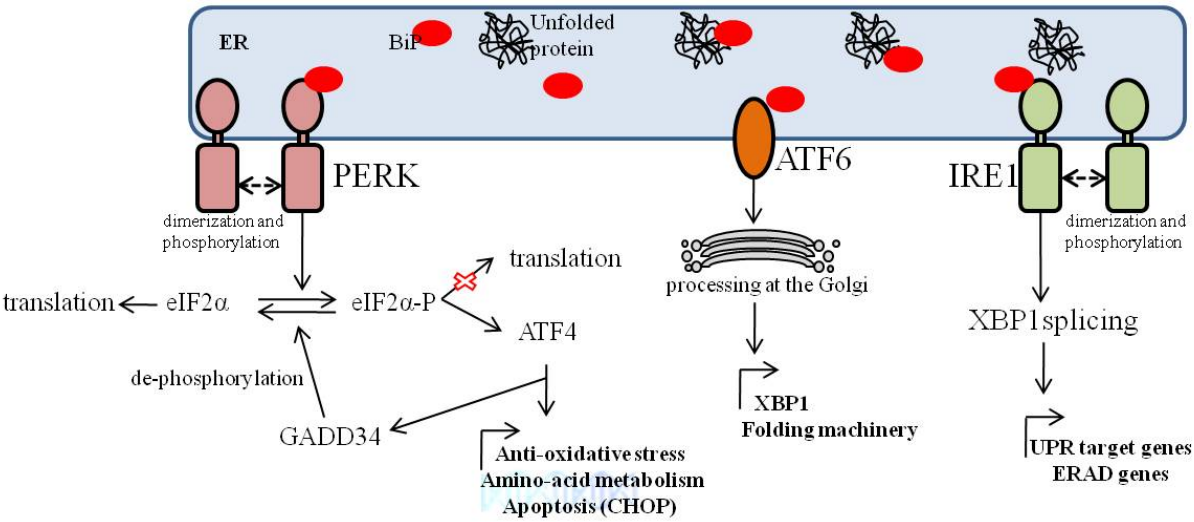

Figure 2. Schematic representation of the different branches of mammalian UPR. Three ER transmembrane stress sensors detect the accumulation of unfolded proteins by dissociation of BiP. Upon ER stress, both PERK and IRE1 oligomers are formed to become active. PERK reduces global translation, by enhancing phosphorylation of eIF2, and leads to the activation of ATF4 which also plays a role in the UPR transcription program. ATF6 is proteolytically cleaved in the Golgi, translocated into the nucleus and directs the transcription of UPR genes. IRE1 directs the splicing of XBP1 mRNA which activates UPR genes. See text for more details.

In mammalian cells, the most immediate response to ER stress is mRNA translation attenuation mediated by PERK, which prevents the influx of proteins into the ER. Like IRE1, PERK possesses a luminal stress-sensing domain with similar structure and function (Bertolotti *et al*., 2000), and a protein kinase domain that is activated by oligomerization and auto-phosphorylation (Harding *et al*., 2000a,b). Upon accumulation of unfolded proteins, BiP dissociates from PERK (Bertolotti *et al*., 2000), and the activated PERK phosphorylates the α subunit of the eukaryotic translation Initiation Factor 2 (eIF2α) (reviewed in Fels and Koumenis, 2006). PERK-mediated phosphorylation of eIF2 during ER stress results in lower levels of translation initiation, reducing the load of newly synthesized polypeptides and allowing cells more time to correct misfolded proteins (Fels and Koumenis, 2006).

Although no PERK homologue has been found in filamentous fungi, a mechanism responsible to reduce the influx of proteins to the ER through down-regulation of secretory proteins has been described and is termed RESS (Repression under Secretion Stress) and has been described both in filamentous fungi (Pakula *et al*., 2003; Al-Sheikh *et al*., 2004; this thesis) and plants (Martínez and Chrispeels, 2003). Al Sheikh and co-workers (2004) have shown that, in *A. niger*, under ER stress conditions, the transcription of glucoamylase is down-regulated. According to the authors (Al Sheikh *et al*., 2004), this process seems to represent a separate branch from the UPR (Al Sheikh *et al*., 2004), which might suggest the existence of a parallel pathway (resembling the PERK-eIF2 pathway) to deal with the accumulation of unfolded proteins.

Paradoxically, although eIF2 phosphorylation arrests protein translation, it is required for the translation of selective mRNAs such as the Activating Transcription Factor-4 (ATF4) (Vattem and Wek, 2004). In yeast (*S. cerevisiae*), phosphorylation of eIF2 by Gcn2 controls the translation of ATF4 homologue, the transcription factor Gcn4, involved in the cellular response to amino-acid starvation (Hinnebusch, 1990; Hinnebusch and Natarajan, 2002). Expression profiling studies revealed that PERK-eIF2-ATF4 core complex regulates the transcription of genes involved in UPR, amino-acid metabolism, protection against oxidative stress and regulation of apoptosis (Harding *et al*., 2003). ATF4 is also involved in the enhancement of expression of the bZIP transcription factors CHOP and ATF3 that cooperatively lead to the transcription of stress-related genes (Jiang *et al*., 2004; Marciniak *et al*., 2004). CHOP can equally promote apoptosis and/or survival by regulation of GADD34 (growth arrest and DNA damage 34) which restores the translation through a feedback control of the eIF2 pathway (Novoa *et al*., 2001).

The second pathway in mammalian UPR is mediated by the bZIP transcription factor ATF6. Mammalian cells contain two copies of ATF6, ATF6α and ATF6β that are ubiquitously expressed and activated upon dissociation from BiP (Shen *et al*., 2002). Dissociation from BiP allows ATF6 trafficking to the Golgi complex, where it is sequentially cleaved by two resident proteases (Chen *et al*., 2002; Haze *et al*., 1999). This results in the release of the cytosolic domain of AFT6, which migrates from the Golgi to the nucleus, where it binds to the promoter regions of target genes containing ATF/cAMP response elements and ERSE (Yoshida *et al*., 2000). ATF6α but not ATF6β has been shown to contribute to the up-regulation of UPR target genes (Okada *et al*., 2002). XBP1 is one of the targets of the ATF6 signaling pathway (Yoshida *et al*., 2001). During ER stress, the activation of ATF6 precedes the activation of IRE1, allowing the expression and further accumulation of unspliced XBP1 mRNAs during the initial phases of the UPR, which will then be available for splicing upon IRE1 pathway activation (Yoshida *et al*., 2003). So far, no ATF6 homologues have been reported in *S. cerevisiae* or *A. niger*.

Although most of the components of the IRE1 pathway are conserved from *S. cerevisiae* to mammalians (Niwa *et al*., 1999), there are a few differences. For instance, in *S. cerevisiae*, the intron present in *HAC1* mRNA represses translation, and relief from this repression is the key to activate UPR (Rüegsegger *et al*., 2001); but in higher eukaryotes both precursor and spliced *XBP1* (*Hac1* homologue) forms are translated. The two proteins, however, have different functions, the spliced XBP1 form is more (relatively) stable and activates UPR target genes (Calfon *et al*., 2002); on the other hand, the protein encoded by the precursor mRNA is more (relatively) unstable and represses the activation of UPR (Yoshida *et al*., 2006). In *A. niger*, an alternative site in the *hacA* mRNA relieves it from translation attenuation (Mulder and Nikolaev, 2009). Mammalian cells have two yeast IRE1 homologues: IRE1α (Tirasophon *et al*., 1998) and IRE1β (Wang *et al*., 1998), and their expression seems to be tissue specific (Niwa *et al*., 1999; Calfon *et al*., 2002). Both forms of IRE1 are responsible for the removal of a 26-nt intron in *XBP1* mRNA, which is then translated into an active transcription factor that binds to ER stress response elements (ERSE, CCAAT(N9)CCACG, Yoshida *et al*., 1999) present in UPR target genes, thereby activating expression of UPR target genes (Yoshida *et al*., 2001; Lee *et al*., 2002; Calfon *et al*., 2002). Recently, has been shown that Ire1p activation is also involved in the degradation of mRNAs

encoding membrane and secreted proteins, through a pathway called regulated Ire1-dependent decay (RIDD) (Hollien *et al*., 2009). If a similar mechanism exists in filamentous fungi has not been investigated.

In summary, UPR is a cellular process trigged by the accumulation of unfolded proteins in the ER with the aim to restore homeostasis and allow the cells to adapt to the stress events.

1.5.2. ERAD: sentencing misfolded proteins to the death row

The ER sustains a distinctive chemical environment to meet the needs of protein folding, yet this process is naturally error prone. The ER contains high concentrations of molecular chaperones, folding enzymes, and ATP, which help with the correct maturation of proteins (Zhang *et al*., 2002). It also possesses an oxidizing environment, which favours intra- and intermolecular disulfide bond formation (Sevier *et al*., 2007), and millimolar concentrations of Ca^{2+}, required for many signal-transduction pathways (Meldolesi and Pozzan, 1998). If folding is delayed or an aberrant protein conformation persists, the protein is either subjected to additional folding cycles by chaperones/foldases, or is selected for degradation by the ERAD pathway (Fig. 3). Travers and co-workers (2000) have shown that there is coordination between UPR and ERAD pathways: the UPR increases the ERAD capacity and is required for an effective ERAD.

The first step in the ERAD pathway is related to substrate recognition. The differences between *S. cerevisiae* and higher eukaryotes start here. In *S. cerevisiae*, ERAD substrates include proteins with errors in the cytoplasmic protein (C), luminal proteins (L) and ER-transmembrane proteins (M) and are then designated to the ERAD-C, ERAD-L and ERAD-M pathways (Fig. 3), respectively (Vashist *et al*., 2004; Carvalho *et al*., 2006; Denic *et al*., 2006). The Hrd1 and Doa10 E3 ligases are core components of the *S. cerevisiae* ubiquitination machinery. The Hrd1 (HrdA) complex mediates the turnover ERAD-L substrates, recognized by the well known Kar2 and Pdi1 ER-resident enzymes, and its ubiquitin ligase activity is stimulated by the luminal domain of Hrd3 (HrdC) (Kikkert *et al*., 2004; Huyer *et al*., 2004; Gauss *et al*., 2006a). In the ERAD-C pathway, the substrates are recognized by cytoplasmic chaperones (Fig. 3) and directed to the Doa10 complex (Hassink *et al*., 2005; Carvalho *et al*., 2006). Ubx2p recruits the AAA-ATPase Cdc48 complex to the Hrd1p and Doa10p ubiquitin ligases (Schuberth and Buchberger, 2005), which then binds to the proteasome and releases the ERAD-L and ERAD-C substrates from the ER (Ye *et al*., 2001; Jarosch *et al*., 2002). Doa1p is known to play an important role in the ubiquitin-dependent protein degradation by a direct interaction with Cdc48p (Ye *et al*., 2001; Ogiso *et al*., 2004; Mullally *et al*., 2006). Although little is known about the ERAD-M pathway, a recent study suggests that ERAD-M substrates are recognized and ubiquitylated by the Hrd1 complex (Sato *et al*., 2009).

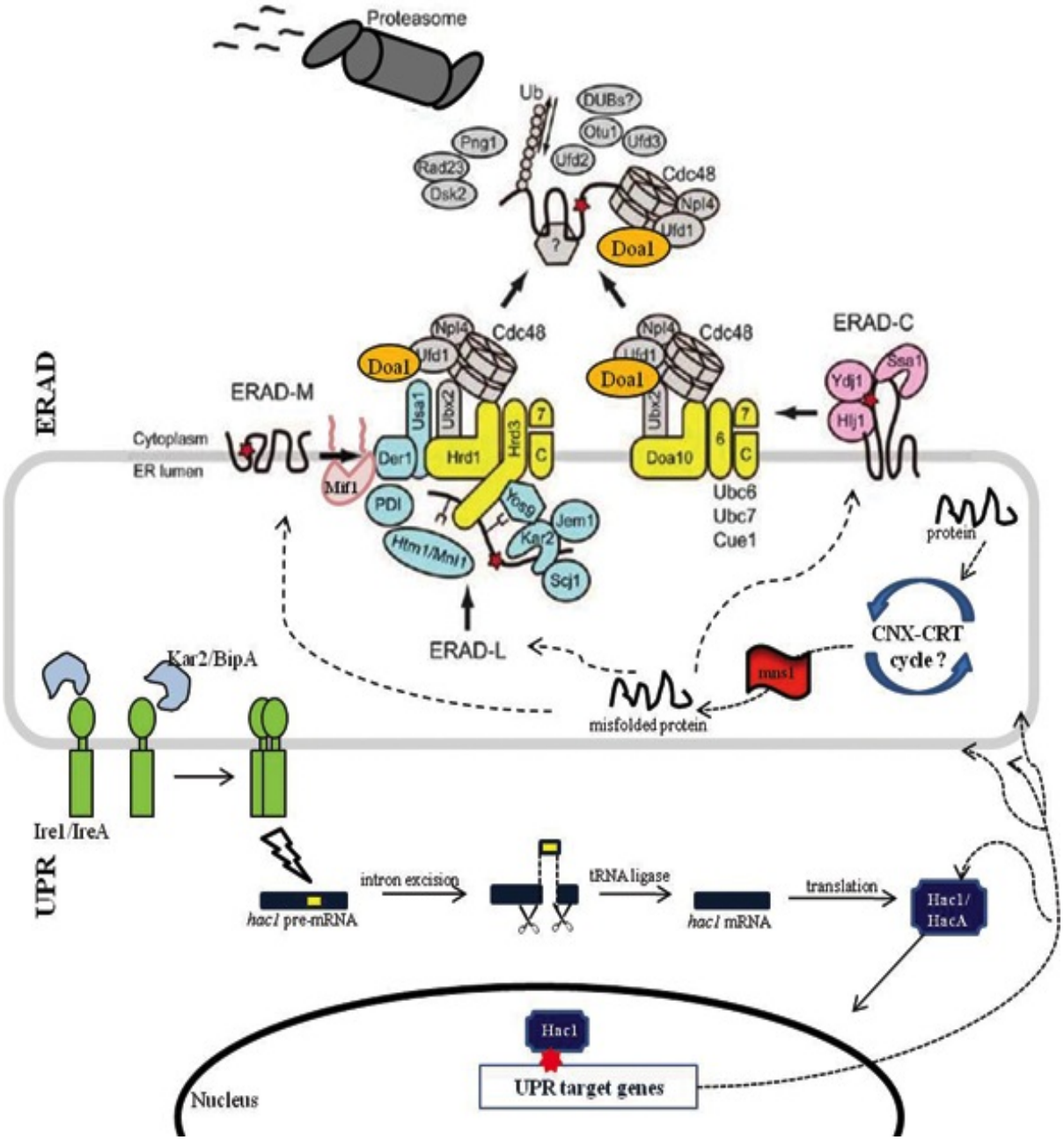

Figure 3. Schematic representation of the components of the ERAD and UPR pathways in *A. niger* based on current models of *S. cerevisiae* and mammalian systems. Newly synthesized glycoproteins are folded and subjected to a strict quality control. *A. niger* seems to lack a true CNX-CRT cycle, present in the mammalian system, since no calreticulin homologue has been found so far. Mns1 (MnsA) is responsible for trimming mannose residues, an event that marks misfolded protein to be degraded by the ERAD pathway. The location of a misfolded lesion (cytoplasmic, luminal or membrane domains) targets the protein to be degraded via the ERAD-C, ERAD-L or ERAD-M pathways. These pathways have only been defined in yeast. ERAD-M substrates seem to be directly recognized by the Hdr1(HrdA) complex, which is stabilized by Hrd3 (HrdC). Mif1 (MifA) recruits ubiquilins which in turn escort the proteasome and ubiquitinated substrates to be degraded via the Mif1/HrdA complex. Substrates targeted for the ERAD-L pathway are recognized by Kar2 (BipA) and Pdi1 (PdiA) and are also directed to the Hrd1 complex. Substrates of the ERAD-C are identified by the chaperones Hlj1, Ydj1 and Ssa1 and use the Doa10 ubiquitin ligase complex. The Doa1 (DoaA) protein forms a

complex with Cdc48p and is required for ubiquitin-mediated protein degradation of all types of ERAD substrates. The retrotranslocon pore is either formed by the Sec61 complex (not shown) or Der1 (DerA). Once in the cytosol, the substrate is degraded by the 26S proteasome. If unfolded proteins accumulate in the ER, the UPR pathway will be triggered. The chaperone Kar2 (BipA) dissociates from the ER transmembrane protein Ire1 (IreA). Ire1 dimerization and autophosphorylation of its kinase domain activates its site specific endoribonuclease function, resulting in the unconventional splicing of a 20-nucleotide intron from the mRNA coding for the basic leucine zipper (bZIP) transcription factor HacA. HacA is then translated into an active protein and migrates into the nucleus, leading to the transcription of UPR target genes that include, among others, the *hac1*itself, chaperones, foldases and some ERAD genes. Adapted from Nakatsukasa and Brodsky (2008) and Vembar and Brodsky (2008).

Once a protein is targeted for destruction by the proteasome, it must be removed from the ER. This process, both in *S. cerevisiae* and mammalian cells, appears to be mediated by the retrotranslocation channel Sec61 complex (Schäfer and Wolf, 2009). In *S. cerevisiae*, BiP interacts with the Sec61 channel and seems to mediate the delivery of the ERAD substrates for retrotranslocation (Deshaies *et al*., 1991; Nishikawa *et al*., 2001). ER-mannosidases are also thought to deliver ERAD substrates to Sec61p (Kanehara *et al*., 2007). In mammalian cells, recent studies have pointed out other alternatives for a retrotranslocation channel, such as derlin-1 (Wahlman *et al*., 2007). Homologues of derlin-1 have been found in both *S. cerevisiae* (Der1p) (Goder *et al*., 2008) and *A. niger* (DerA) (Pel *et al*., 2007).

A homoCys-responsive ER-resident protein (HERP), which is also called MIF1 (MifA in *A. niger*), has been pointed out as the factor, together with derlin-1, to deliver ERAD targets to the proteasome (van Laar *et al*., 2001; Schulze *et al*., 2005; Okuda-Shimizu and Hendershot, 2007). According to van Laar and co-workers (2001), MIF1 is an UPR target and is responsible for recruiting the proteasome(s) to the ER in response to ER stress. More recently it has been shown that MIF1 recruits ubiquilins, and these are the shuttle vectors responsible to bring the proteasome and ubiquitinated substrates to MIF1 and to other components of the ERAD machinery (Kim *et al*., 2008). In yeast, no significant sequence similarity to MIF1 has been found, however Carvalho *et al*. (2006) suggest that Usa1p may be a functional homologue.

Most of ERAD targets must be ubiquitinated before proteasome targeting. This process of ubiquitination requires the action of an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3), like Doa10 and Hrd1 (Pierce *et al*., 2009). After being polyubiquitinated, the protein is targeted to the proteasome and degraded (Mayer *et al*., 1998). De-ubiquitination is also required for the substrate to enter the proteasome (Amerik and Hochstrasse, 2004). A high number of proteasomes are located at the ER membrane surface (Rivett, 1993), which allows them to receive and degrade the ERAD substrates.

1.6. Studying protein secretion, UPR and ERAD processes in the "Genomics" Era

Genome sequencing of industrially important *Aspergilli* (Machida *et al*., 2005; Pel *et al*., 2007; Wortman *et al*., 2008) has provided the opportunity to broaden fundamental knowledge on biology of filamentous fungi and explore it for the optimization and industrial

production of proteins of interest. The secretion pathway is a primary target of investigation and several studies have been carried out to determine the effects of (over)producing homologous/heterologous with particular interest in optimizing protein production and overcoming protein secretion bottlenecks. In general, the expression of different heterologous proteins, such as chymosin and tPA (tissue plasminogen activator) or the exposure to chemicals that induce ER stress, such as DTT or tunicamycin, has shown to induce genes involved in the UPR, protein trafficking, degradation, lipid metabolism, secretion and cell wall biogenesis (Travers *et al*., 2000; Sims *et al*., 2005; Arvas *et al*., 2006; Gasser *et al*., 2007; Guillemette *et al*., 2007). These results point to the induction of the UPR pathway to counteract secretion stress, justifying the great interest of studying this signalling pathway extensively. For instance, on a transcriptomic study, Sims and co-workers (2005) have observed in an *A. nidulans* strain producing chymosin, the up-regulation of genes involved in the UPR pathway. Comparison of transcriptomic data has shown the overlapping gene expression of a large number of secretion related genes between *S. cerevisiae* and *T. reesei* (Arvas *et al*., 2006). Overexpression of HAC1 in *P. pastoris*, the most direct control for UPR genes, resulted in significant new understanding of this important regulatory pathway (Graf *et al*., 2008), allowing the establishment of similarities and differences between yeast and filamentous fungi UPR.

The first genome-wide studies on secretion stress in *A. niger* came from the work of Guillemette *et al*. (2007). In this study, ER stress was induced either chemically (DTT or tunicamycin) or by the expression of the heterologous protein t-PA. The results revealed that, in all the condition tested, the induction of UPR genes, including folding machinery, glycosylation enzymes, intracellular transport proteins and ERAD genes. Furthermore, this study also gives the first indication of the existence of a feed-back mechanism at the translational level, as well as revealing more clues on the RESS system (Al-Sheikh *et al*., 2004). More recently, a transcriptomic study by Jørgensen *et al.* (2009) has revealed the induction of UPR related genes under conditions of a high requirement for carbohydrate degrading enzymes. These results suggest that UPR pathway is triggered not only under secretion stress, but also under "normal" growth conditions which require a larger secretion capacity of the cell. With molecular techniques well establish for fungal manipulation (Chapter 2) and the data output it becomes easier to identify and modify genes in their expression (deleting, overexpressing, tagging) and make significant contributions in the fields of industrial biotechnology.

Hence, the results from genomic and transcriptomic approaches are the starting point to identify, select and modify genes and networks for optimization of industrial production of homologous and heterologous proteins (Jacobs *et al*., 2009, Chapter 5 of this thesis).

Aim and Thesis Outline

The notorious capacity of *Aspergillus niger* for secreting enzymes needed to degrade complex substrates, such as plant cell wall polysaccharides and proteins, has been exploited by the industry. However, the exploitation of *A. niger* for the production of heterologous proteins is limited by the observation that the production yields of heterologous proteins are often very low comparatively to the successful production of several native enzymes by this fungus. Low yields are often attributed to problems occurring in the protein secretory pathway, i.e., for example the recognition of the proteins as foreign, folding, overflow capacity of the ER, glycosylation patterns, secretion to the extracellular environment and the action of proteases. Secretion stress is often triggered by the expression of heterologous proteins, which in turn leads to the activation of the Unfolded Protein Response (UPR) and Endoplasmic Reticulum Associated Degradation (ERAD) pathways. This thesis aims to analyze and understand events in the secretory pathway that might act as bottlenecks for heterologous protein production, anticipating that the outcome results will serve as a starting point for the development and optimization of strategies for increasing protein yields.

Chapter 1 reviews the secretory pathway, the UPR and ERAD pathways of eukaryotes, giving insights into differences and similarities from *Saccharomyces cerevisiae*, filamentous fungi and mammalian models, as well as identified bottlenecks on protein production and recent developments on the different "omics" fields.

Chapter 2 illustrates the development of molecular tools to study secretion, UPR and ERAD. We describe the generation of a set of isogenic *kusA* deletion strains for *A. niger* in addition to the development of two efficient strategies for the complementation of knock-out mutants on a *ΔkusA* background involving autonomously replicating plasmids and a transiently disrupted *kusA* strain. To study the UPR we deleted two key players in this pathway: the ER-localized transmembrane protein IreA, which in *A. niger* revealed to be essential; and the transcription factor HacA, that although not essential, has shown to be crucial for a normal growth/development of the fungus.

Delivery of secretory proteins to the membrane and extracellular environment requires vesicle transport to and from the Golgi, an organelle that had not yet been visualized in *A.niger*. **Chapter 3** describes the identification and functional characterization of *gmtA*, the putative GDP-mannose transporter in *A. niger*. Fluorescence studies localized GmtA as punctate dots throughout the hyphal cytoplasm, representing Golgi equivalents. Furthermore, we analyzed the deletion of the small secretion related GTPase *srgC* on Golgi formation and overall fungal morphology. The development and use of GmtA as a Golgi marker is a promising biological tool to study the dynamics of Golgi bodies in *A. niger*.

The effects of disrupting genes involved in the ERAD pathway is described in **Chapter 4**. Based on the *A. niger* genome sequence, candidate ERAD related genes were identified and disruption strains were constructed: Δ*derA*, Δ*doaA*, Δ*hrdC*, Δ*mifA* and Δ*mnsA*. None of the genes in study showed to be essential to *A. niger*. Growth and morphology phenotypes were analyzed, as well as the effect of each deletion on heterologous protein production. In general, deletion of these ERAD components led to the detection of higher levels of intracellular protein in comparison to the wild-type, indicating that impairment of

this pathway might be an important point on increasing protein yields. The potential relation between the UPR and ERAD pathways is also discussed.

The UPR pathway is mediated by the HacA transcription factor. In **Chapter 5** we have investigated the transcriptomic profile of a constitutive active *hacA* form in *A. niger*. We show that under these conditions lipid biosynthesis and secretory pathway processes are enhanced while energy-related metabolic pathways are largely affected (down-regulated). Furthermore, the decreasing on extracellular proteins expression, namely by the down-regulation of the AmyR-regulon reveals a coordinated response to counteract ER secretion stress. This study provides and discusses new evidences on the role of HacA in the cells and new insights into the RESS mechanism.

Chapter 2

Expanding the *ku70* toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses

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Abstract

Mutants with a defective non-homologous-end-joining (NHEJ) pathway have boosted functional genomics in filamentous fungi as they are very efficient recipient strains for gene targeting approaches, achieving homologous recombination frequencies up to 100%. For example, deletion of the *ku70* homologous gene *kusA* in *Aspergillus niger* resulted in a recipient strain in which deletions of essential or non-essential genes can efficiently be obtained. To verify that the mutant phenotype observed is the result of a gene deletion, a complementation approach has to be performed. Here, an intact copy of the gene is transformed back to the mutant, where it should integrate ectopically into the genome. However, ectopic complementation is difficult in NHEJ deficient strains and the gene will preferably integrate via homologous recombination at its endogenous locus. To circumvent that problem, we have constructed autonomously replicating vectors useful for many filamentous fungi which contain either the *pyrG* allele or a hygromycin resistance gene as selectable markers. Under selective conditions, the plasmids are maintained, allowing complementation analyses; once the selective pressure is removed, the plasmid becomes lost and the mutant phenotype prevails. Another disadvantage of NHEJ-defective strains is their increased sensitivity towards DNA damaging conditions such as radiation. Thus, mutant analyses in these genetic backgrounds are limited and can even be obscured by pleiotropic effects. The use of sexual crossings for the restoration of the NHEJ pathway is, however, impossible in imperfect filamentous fungi such as *A. niger*. We have therefore established a transiently disrupted *kusA* strain as recipient strain for gene targeting approaches.

2.1 Introduction

Integration of DNA sequences into a genome by homologous recombination is a very useful and widely used functional genomics tool for generating gene knock-out mutants. In filamentous fungi such as *Aspergillus niger*, homologous recombination frequencies are extremely low when compared to the yeast *Saccharomyces cerevisiae*, which makes the generation of homologous transformants time-consuming. When Ninomiya *et al*. (2004) reported that inactivation of components of the non-homologousend- joining (NHEJ) pathway in *Neurospora crassa* results in strains with homologous recombination frequencies up to 100%, this system was rapidly established in other filamentous fungi (for reviews see (Meyer 2008, Kück and Hoff 2010)). The NHEJ pathway is a conserved mechanism in eukaryotes that is essential for the repair of chromosomal DNA double-strand breaks (DSBs) and competes with another conserved repair mechanism, the homologous recombination (HR) pathway (Shrivastav *et al*. 2008). The HR pathway depends on the Rad52 epistasis group and mediates interaction between homologous DNA sequences leading to targeted integration. In contrast, the NHEJ pathway ligates DSBs without the requirement of any homology and is accomplished by the activities of the Ku heterodimer (Ku70/Ku80–protein complex) and the DNA ligase IV–Xrcc4 complex (Dudasova *et al*., 2004; Krogh and Symington 2004). By deleting either *ku70*, *ku80* or *lig4* genes, non-homologous recombination is diminished or prevented, favouring the frequencies of homologous recombination events mediated by the Rad52 complex (Shrivastav *et al*., 2008). Despite the fact that NHEJ-defective mutants are powerful recipient strains for fungal gene-targeting approaches, a problem arises when one wants to complement the phenotype of a gene deletion mutant. Usually, this is performed by retransferring the respective gene back into the deletion mutant. The gene will ectopically integrate into the genome and the resulting phenotype is assessed. However, in a NHEJ-deficient background, ectopic integration is not favoured and the complementing gene construct will preferably integrate at its endogenous (deleted) locus. One possibility to bypass this disadvantage is the use of autonomously replicating vectors, where the complementing gene construct is maintained extrachromosomally. For *Aspergilli*, the use of the AMA1 sequence has been shown to promote extrachromosomal replication of a plasmid. The autonomous maintenance in *Aspergillus* (AMA1) sequence, isolated from a genomic library of *Aspergillus nidulans*, is based on two inverted sequences surrounding a unique central core sequence and displays properties similar to the autonomous replicating sequences in *S. cerevisiae* (Gems *et al*., 1991; Verdoes *et al*., 1994b; Aleksenko and Clutterbuck 1996, 1997; Khalaj *et al*., 2007). We thus established in this work AMA1-based complementing vectors conferring either uracil prototrophy (*pyrG*) or hygromycin resistance for general use in filamentous fungi. In addition, we generated a set of isogenic Δku70 deletions strains for *A. niger*, which can be used for a variety of selection markers. To test the usefulness of the AMA1-based complementation approach, two genes (*hacA* and *ireA*) have been selected which are linked to our research interest on secretion-related phenomena in *A. niger*. HacA is a transcription factor important for the unfolded protein response (UPR) and activates transcription of various chaperones and foldases (Mulder *et al*., 2004; Mulder *et al*., 2006). IreA is the predicted homologue of the *S. cerevisiae* Ire1p, which is a conserved transmembrane protein

located in the endoplasmic reticulum (ER) membrane (Cox *et al*., 1993). ER stress in yeast and mammals is sensed by Ire1p which in turn stimulates the Hac1p-UPR machinery (Patil and Walter 2001). We report here that deletion of *hacA* or *ireA* causes dramatic consequences for *A. niger* and demonstrate that the AMA1-based plasmids are exceptional useful tools for complementation analyses of essential genes.

A second drawback of NHEJ inactivation is that the consequences of deleting *ku70*, *ku80* or *lig4* in relation to DNA repair and genome stability are less studied in fungal strains. However, as several reports have shown that NHEJ deficiency makes fungal strains vulnerable to DNA damaging conditions (Malik *et al*., 2006; Meyer *et al*., 2007; Kito *et al*., 2008; Snoek *et al*., 2009), it can be assumed that an intact NHEJ pathway secures cellular fitness of filamentous fungi as shown for higher eukaryotes (Pardo *et al*., 2009). To avoid any limitations provoked by a non-functional NHEJ pathway, Nielsen *et al*. (2008) used a strategy to transiently silence the NHEJ pathway in A. nidulans. We have adapted that strategy to *A. niger* and report here the establishment of a transiently disrupted *ku70* (*kusA*) strain. This strain shows similar homologous recombination frequencies compared to a Δ*kusA* strain as exemplarily shown for two genes of our interest — *srgA* and *racA*. Both are GTPase-encoding genes and are important for secretion and morphology of *A. niger* ((Punt *et al*., 2001) and own unpublished data).

2.2. Materials and methods

2.2.1. Strains, culture conditions and molecular techniques

A. niger strains used in this study are listed in Table 1. Strains were cultivated in minimal medium (MM; (Bennett and Lasure 1991)) containing 55 mM glucose, 7 mM KCl, 11 mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO^3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4, 174 nM EDTA; or in complete medium (CM) containing, in addition to MM, 0.1% (w/v) casamino acids and 0.5% (w/v) yeast extract. When required, 10 mM uridine or/and 100 µg/ml of hygromycin was added. When using the *amdS* as selection marker, strains were grown in MM without $NaNO_3$ and supplemented with 10 mM acetamide and 15 mM cesium chloride.

To obtain *pyrG*$^-$ strains, $2x10^7$ spores were inoculated on MM agar plates supplemented with 0.75 mg/ml 5'-fluoroorotic acid (FOA), 10 mM uridine, 10 mM proline as nitrogen source. Plates were incubated for 1-2 weeks at 30°C. FOA-resistant mutants were isolated, purified and tested for uridine auxotrophy on MM with and without uridine (mutants should not grow on medium lacking uridine). To obtain *amdS*$^-$ strains, 2×10^7 spores were inoculated on MM agar plates supplemented with 0.2% 5'-fluoroacetamide (FAA) and 10 mM urea as nitrogen source. After 1-2 weeks incubation at 30°C, FAA-resistant mutants were isolated, purified and tested for growth on acetamide medium (mutants should not grow on medium containing acetamide as sole nitrogen source).

All basic molecular techniques were performed according to standard procedures (Sambrook and Russel 2001). Transformation of *A. niger*, genomic DNA extraction, screening procedures, diagnostic PCR and Southern analysis were conducted as recently described in detail (Meyer *et al*., 2010).

Table 1. *Aspergillus niger* strains used in this study

Name	Genotype	Reference
N402	*csp, amdS*$^-$	(Bos *et al*., 1988)
AB4.1	*pyrG*$^-$, *amdS*$^-$	(van Hartingsveldt *et al*., 1987)
MA70.15*	*ΔkusA, pyrG*$^-$, *amdS*$^+$	(Meyer *et al*., 2007)
MA78.6*	*ΔkusA, amdS*$^+$	This study
NC4.1*	*ΔkusA, pyrG*$^-$, *amdS*$^-$	This study
NC5.1*	*ΔkusA, pyrG*$^+$, *amdS*$^-$	This study
NC6.2	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔhacA*	This study
NC7.1	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔireA/ireA*	This study
NC8.1	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔhacA*, pAMA-hacA	This study
NC9.1	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔireA,* pAMA-ireA	This study
MA169.4*	*kusA::*DR-*amdS*-DR, *pyrG*$^-$	This study
MA171.1	*kusA::*DR-*amdS*-DR, *pyrG*$^+$, *ΔracA*	This study
MA172.1	*kusA*$^+$, *pyrG*$^+$, *ΔracA*	This study
MK15.A	*Δkus*, *pyrG*$^+$, *ΔsrgA*	This study
MK18.A	*kusA::*DR-*amdS*-DR, *pyrG*$^+$, *ΔsrgA*	This study

*: Strains have been deposited at the Fungal Genetics Stock Center (www.fgsc.net)

2.2.2. Construction of AMA1-based complementation vectors

The pBlueScriptII SK (Stratagene) was used as a backbone for the construction of the autonomously replicating plasmids. The *hph* expression cassette was obtained by digesting pAN7.1 (Punt *et al*., 1987) with *Xho*I and *Hind*III. The *AopyrG* gene was obtained by PCR using pAO4-13 (de Ruiter-Jacobs *et al*., 1989) as template DNA and primers pAO-XhoI-Rev and pAO-HindIII-For which introduced the restrictions sites *Xho*I and *Hind*III, respectively, into the *AopyrG* fragment (Table 2). The 3 kb *hph* cassette and the 1.7 kb *AopyrG* fragment were independently cloned into *Xho*I/*Hind*III digested pBlueScriptII SK, giving rise to vectors pBS-hyg and pBS-pyrG, respectively. The 6-kb AMA1 fragment was obtained by digesting pAOpyrGcosArp1 (Gems *et al*., 1991) with *Hind*III. The fragment was then cloned into the unique *Hind*III site in pBS-hyg and pBS-pyrG vectors, giving plasmids pBS-hyg-AMA (pMA171) and pBS-pyrG-AMA (pMA172), respectively.

2.2.3. Generation of Δ*hacA* and Δ*ireA* deletion strains

The *A. niger hacA* gene (An01g00160) was deleted in MA70.15 by replacing its open reading frame (ORF) with a DNA fragment containing the *pyrG* marker form *A. oryzae* (van Hartingsveldt *et al*., 1987). The cassette used for *hacA* deletion was produced by Fusion-PCR in two steps: first, independent amplification of the *hacA* promoter and terminator regions (each ≅ 550 bp) and the *AopyrG* gene, respectively, using primers summarised in Table 2. Genomic DNA of strain N402 and pAB4-1 (van Hartingsveldt *et al*., 1987) served as template DNA. Second, Fusion-PCR using the three fragments as template DNAs and NC16hacA5F / NC19hacA3R as outward primers (Table 2). Deletion of the *A. niger ireA* ORF (An01g06550) followed the same approach as described for *hacA*. Respective primers are listed in Table 2. The deletion cassettes were transformed into MA70.15 and uridine prototrophic transformants were selected and analyzed by Southern hybridisation.

Table 2. Primers used in this study.

Primer name	Sequence (5' to 3')	Targeted sequence
pAO-HindIII-For	CCCAAGCTTGTTGCTCGGTAGCTGATTA	*AopyrG*
pAO-XhoI-Rev	CCGCTCGAGCGATGGATAATTGTGCCG	*AopyrG*
NC14 pyrGfor	GGATCTCAGAACAATATACCAG	*AopyrG*
NC15 pyrGrev	CCGCTGTCGGATCAGGATTA	*AopyrG*
NC16 hacA5F	CATATTCACCCAACCGGACG	*hacA* 5'deletion flank
NC17 hacA5R	CTGGTATATTGTTCTGAGATCCAACAATGGCAACTCAGGCGT	*hacA* 5'deletion flank
NC18 hacA3F	TAATCCTGATCCGACAGCGGTCGCCAGACTTCTAGCGTGC	*hacA* 3'deletion flank
NC19 hacA3R	GGTAGTAAAGTCTCACCGCTG	*hacA* 3'deletion flank
NC20 ireA 5F	CGTCCGTGCATCTGGCTTA	*ireA* 5'deletion flank
NC21ireA5R	CTGGTATATTGTTCTGAGATCCCCTTCGCTGATCGCTGTCTCT	*ireA* 5'deletion flank
NC22 ireA 3F	TAATCCTGATCCGACAGCGGTGAGCTGCCACTCCGTCAT	*ireA* 3'deletion flank
NC23 ireA3R	GGGGATTGGTGTACTTACGG	*ireA* 3'deletion flank
NC58 hacA R	ATAAGAATGCGGCCGCCATCCCGATTGCCGTATCC	*hacA* ORF
NC8 PHACF2	ATAAGAATGCGGCCGCCTCCATACCACTTTGTGCTAG	*hacA* ORF
NC60 ireA R	ATAAGAATGCGGCCGCGGGGATTGGTGTACTTACGG	*ireA* ORF
NC61 ireA F	ATAAGAATGCGGCCGCCGTCCGTGCATCTGGCTTA	*ireA* ORF
P1- NC46 5'ku70 F	GGTGCGAGAAGCCGGTCGCA	*kusA* – diagnostic PCR
P2 - NC48 amdS Rev	AGAGAGGACGTTGGCGATTG	*kusA* – diagnostic PCR
P3 - NC47 3' ku70 R	TTACGGCGAATCTGGGTGG	*kusA* – diagnostic PCR
P4 - NC49 ku70 orf R	AAATGAGTGCGACGCGGA	*kusA* – diagnostic PCR
ku70P1Not	AAGGAAAAAAGCGGCCGCCAGAACGGCTTGATGACGG	*kusA* 5'disruption flank
ku70P2EcoRI	CACAGAATTCGACCTCATGAGCCGAAGGAA	*kusA* 5'disruption flank
ku70P3Kpn	GGGGTACCACCAGTCAAAGATGCGGTCC	*kusA* 3'disruption flank
ku70P4Kpn	GGGGTACCGGCGCTTGCCTTCGTAAGA	*kusA* 3'disruption flank

Restriction sites added are underlined.

2.2.4. Analysis of Δ*hacA* and *ΔireA* deletion strains

Spores from primary transformants were carefully removed to prevent transfer of mycelia and conidiophores using a sterile cotton stick moistened in 0.9% NaCl and suspended in 10 ml 0.9% NaCl. Spores were plated out on MM (selective) and MM + uridine (non-selective) agar plates and incubated at 30°C for 5 days. In the case that an essential gene has been deleted, no colonies will be formed under selective conditions, as both the deletion strain and the parental strain are not able to grow. These heterokaryons can only be propagated by transferring mycelium. The heterokaryon rescue technique (Osmani *et al*., 2006) was used to purify the poor growing *hacA* deletion mutant. No viable *ireA* deletion mutants could be obtained after purification of the primary transformants containing the *ireA* deletion. Propagation and maintenance of *ireA* heterokaryotic strains was done by transfer of mycelia from the primary transformants onto MM. Putative Δ*hacA* and Δ*ireA* heterokaryotic mutants were further analyzed by Southern hybridisation. For complementation studies using pMA171, the ORFs of *hacA* and *ireA*, including approximately 0.6 kb promoter and 0.6 kb terminator regions, were PCR amplified using N402 genomic DNA as template and respective primers containing *Not*I overhangs (Table 2). The fragments were cloned into

pJET (Fermentas), sequenced, released from pJET via *Not*I restriction and cloned into *Not*I-linearised pMA171. Respective plasmids (pMA171-hacA and pMA171-ireA) were then transformed into the *hacA* and *ireA* deletion mutants. Primary transformants containing the complementation plasmid were isolated on MM containing 100µg/ml of hygromycin and further analyzed by Southern blot. To provoke plasmid loss, spores were streaked for several rounds on non-selective medium (MM without hygromycin).

2.2.5. Generation of isogenic *kusA* deletion strains

A *kusA* deletion construct, consisting of the *amdS* selection marker flanked by each 1.5 kb of 5' and 3' regions of *kusA* and localised on plasmid pGBKUS-5 (Meyer *et al*., 2007), was used to transform the *A. niger* wild-type strain N402 (Bos *et al*., 1988). Transformants in which the *kusA* gene was replaced by *amdS* were selected on acetamide agar plates and via Southern blot analysis as described (Meyer *et al*., 2007). The resulting strain MA78.6 (Δ*kusA*, *amdS+, pyrG*$^{+}$) was used for further studies. In order to loop-out the *amdS* marker, strains MA78.6 and MA70.15 (Δ*kusA*, *amdS+, pyrG*$^{-}$, (Meyer *et al*., 2007)) were plated on MM agar plates containing FAA. FAA resistant strains were selected, subjected to Southern analysis and analyzed by diagnostic PCR using primers P1-P4 (Table 2). Strains NC4.1(Δ*kusA*, *amdS*$^{-}$, *pyrG*$^{-}$) and NC5.1 (Δ*kusA*, *amdS*$^{-}$, *pyrG*$^{+}$) were selected.

2.2.6. Generation of a *kusA* disruption strain and restoration of the *kusA* locus

A *kusA* disruption construct (*kusA::*DR-*amdS*-DR) was made based on the *kusA* deletion construct pGBKUS-5 (Meyer *et al*., 2007). PCR 1 using primers ku70P1Not and ku70P2Eco was used to amplify a 700 bp region covering part of the 5' untranslated region of *kusA* and its ORF. The primer pair ku70P3Kpn and ku70P4Kpn was used in PCR 2 to amplify a 900 bp region from the *kusA* ORF. Both PCR products contained the same 300 bp sequence from the *kusA* ORF (later on named direct repeat, DR). Using a two step ligation, PCR product 1 (restricted with *Not*I and *Eco*RI), the *amdS* gene cassette (released via *Eco*RI and *Kpn*I restriction from pGBKUS-5) and PCR product 2 (restricted with *Kpn*I) were cloned into pBlueScriptII SK giving plasmid pMA183. The *kusA::*DR-*amdS*-DR cassette was amplified from pMA183 using primers ku70P1Not and ku70P4Kpn and transformed into strain AB4.1 (van Hartingsveldt *et al*., 1987). Transformants, in which the *kusA* locus was disrupted by the DR-flanked *amdS* marker gene, were screened for growth on acetamide and via Southern blot analysis. Strain MA169.4 was selected (*kusA::*DR-*amdS*-DR) and subsequently used as recipient strain for deleting *srgA* and *racA*. The *A. niger srgA* gene (An14g00010) was deleted in MA169.4 using the 5.3 kb *Eco*RI–*Bam*HI fragment from plasmid pΔsrgA as described earlier (Punt *et al*., 2001). The deletion construct contained the *Trichoderma reesei pyr4* gene as selection marker (Gruber *et al*., 1990). The gene deletion approach followed for *racA* will be described elsewhere (Kwon, Meyer, Ram *et al*.; manuscript in preparation).

2. 3. Results

2.3.1. Establishment of AMA1-based vectors as molecular tools for complementation analyses

We have constructed autonomously replicating vectors containing either the auxotrophic selection marker pyrG of A. oryzae encoding an orotidine-5′-monophosphate decarboxylase or harbouring the hygromycin resistance cassette as dominant selection marker. Both markers have already successfully been used for a variety of filamentous fungi. A schematic drawing of both plasmids pMA171 (hygromycin- based) and pMA172 (AopyrG-based) is given in Fig. 1. Common to both shuttle vectors is the pBluescript backbone and the 6-kb AMA1 sequence, allowing autonomous maintenance in Escherichia coli and filamentous fungi. We further ensured that a unique rare-cutting restriction site is present in both plasmids (NotI) which should facilitate easy insertion of complementing genes.

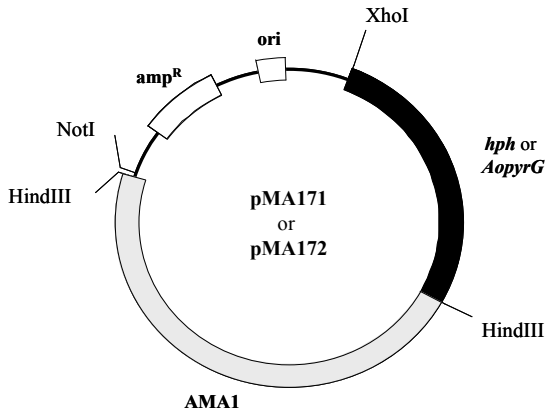

Figure 1. Schematic representation of the autonomously replicating plasmids containing either hygromycin (pMA171; 11239 bp) or *AopyrG* (pAM172; 9943 bp) as selection markers. Abbreviations: *hph*: hygromycin resistance cassette containing the *hph* gene from *E. coli* under control of the *gpdA* promoter and the *trpC* terminator of *A. nidulans*. *AopyrG: A. oryzae pyrG* gene flanked by its own promoter and terminator sequences. ori: origin of replication in *E. coli*; amp^R: gene conferring ampicillin resistance in *E. coli*. Sites for restriction enzymes used for cloning are indicated. Both plasmids have been deposited at FGSC.

2.3.2. Analysis of the *hacA* and *ireA* deletion strains and heterokaryon rescue

In order to judge the usefulness of the AMA1-based complementation tool, we deleted two genes of our research interest on protein secretion in *A. niger* - *hacA* and *ireA*. In doing so, the uridine-requiring strain MA70.15 (Δ*kusA*, *pyrG*$^-$, *amdS*$^+$) was selected as a recipient strain (Fig. 2D). The respective deletion cassettes were made using the *AopyrG* gene as a selection marker (for details see "Materials and methods"). After transforming strain MA70.15 with the deletion cassettes, no obvious phenotypes were observed for the primary transformants obtained in both gene deletion approaches (data not shown), and each four primary transformants were randomly selected for purification. Hereby, only conidiospores from the primary transformants were transferred onto new selective medium (MM without uridine; note that conidia of *A. niger* are uninucleate). Remarkably, the phenotype of the four putative Δ*hacA* transformants did no longer resemble the wild-type's phenotype, but instead

all strains displayed reduced growth and formed compact colonies (Fig. 2B). In the case of Δ*ireA* transformants, none of the four primary transformants formed colonies after transfer (Fig. 2A). These results indicated that the primary transformants of both deletion approaches were heterokaryons, containing nuclei with the genotype *hacA*/*pyrG*$^{-}$ (*ireA*/*pyrG*$^{-}$) and nuclei with the genotype Δ*hacA*/*pyrG*$^{+}$ (Δ*ireA*/*pyrG*$^{+}$). We were only able to obtain pure, homokaryotic transformants in the case of the Δ*hacA* transformants (compact growing colonies), whereas propagation of the ΔireA transformants was only possible when substrate mycelium was transferred (data not shown). This finding suggested that Δ*ireA* strains are only viable as heterokaryons and that *ireA* is an essential gene. To confirm the homokaryotic genotype of the purified Δ*hacA* transformants, mycelium from a Δ*hacA* colony were transferred on MM plates containing uridine. If still wild-type nuclei would have been present (*hacA*/*pyrG*$^{-}$), vigorous growth would have been observable on MM+uridine plates. As shown in Fig. 2C, the phenotype of the Δ*hacA* transformant is stable on MM+uridine plates, indicating the absence of wild-type nuclei and proves that the strain is homokaryotic.

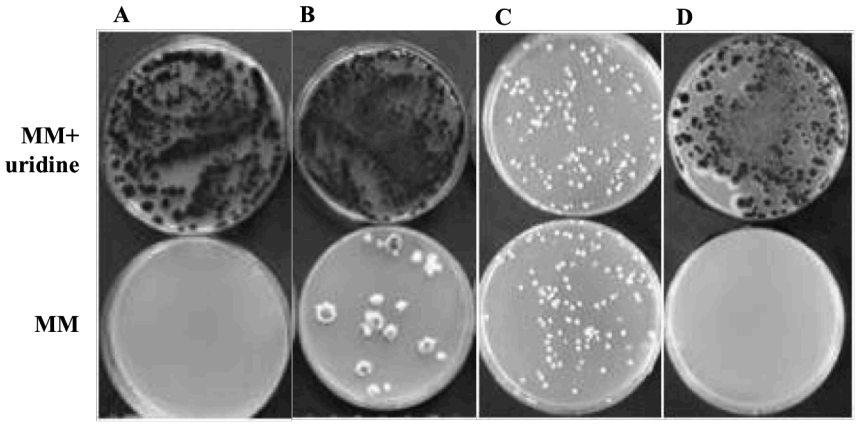

Figure 2. Heterokaryon tests of primary transformants from putative *ireA* (A), *hacA* (B) deletion strains on MM agar plates supplemented or lacking uridine. To prove purity of the Δ*hacA* strain, spores from the putative deletion strain were grown in the presence of uridine (C). As a control, growth of the parental strain MA70.15 (Δ*kusA*, *pyrG*$^{-}$, *amdS*$^{+}$) is shown (D).

In order to verify deletion of both genes on molecular level, Southern analyses were conducted. Due to reduced sporulation of the putative *hacA* deletion strain and the heterokaryotic nature of the putative Δ*ireA* primary transformants, the isolation of genomic DNA was done by inoculating pieces of mycelium in MM lacking uridine. Southern blot analysis was performed on one Δ*hacA* mutant (NC6.2) and two Δ*ireA* primary transformants (NC7.1 and NC7.2) and confirmed deletion of both genes (Fig. 3). For strains NC7.1 and NC7.2, it also confirmed their heterokaryotic nature (signals corresponding to both the presence and absence of the ireA allele were observed) and suggested an unbalanced proportion of both nuclei (Fig. 3B, lanes 1 and 2).

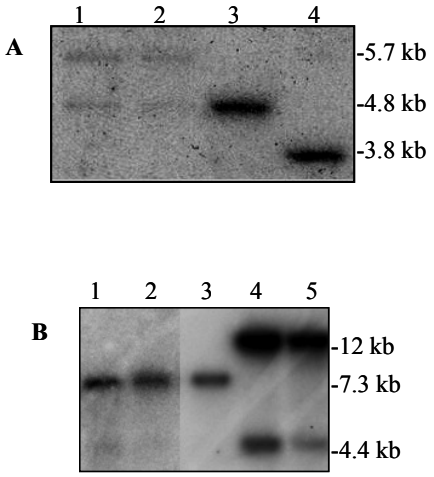

Figure 3. Southern analysis of *ΔhacA* (A) and *ΔireA* (B) strains. (A) Genomic DNA was digested with *Eco*RI and probed with a 560 bp fragment corresponding to the *hacA* 3'-untranslated region. Lanes 1 and 2: two strains (NC8.1 and NC8.2) derived from NC6.2 transformed with pMA171-hacA; lane 3: NC6.2 (*ΔhacA*); lane 4: N402 (*hacA*). Predicted sizes of the hybridizing fragments are as predicted and indicated on the left. (B) Genomic DNA was digested with *Kpn*I and probed with a 600 bp fragment of *ireA* 5'-untranslated region. Lanes 1 and 2: Heterokaryotic NC7.1 and NC7.2 strains (*ΔireA/ireA*). The two signals correspond to the wild-type locus (7.3 kb) and the *ireA* deleted locus (4.4 kb); lane 3: N402 (*ireA*); lanes 4 and 5: NC9.1 and NC9.2 correspond to two different *ΔireA* complemented strains derived from NC7.1 transformed with pAM171-ireA. The two signals correspond to the deleted locus (4.4 kb) and the vector (12 kb).

2.3.3. Complementation of Δ*hacA* and Δ*ireA* with AMA1-based vectors

As the *hacA* and *ireA* deletion strains were established by using *AopyrG* as a selection marker, the AMA1-based vector pMA171 conferring hygromycin resistance was used for complementation experiments. Two vectors, pMA171-*hacA* and pMA171-*ireA*, were constructed as described under "Materials and methods" and transformed into NC6.2 (Δ*hacA*) and NC7.1 (Δ*ireA*/*ireA*), giving strains NC8 and NC9, respectively. Transformants were selected and purified on MM lacking uridine but containing hygromycin. Complementing strains were analysed by Southern hybridisation which confirmed, in the case of Δ*hacA*, the presence of the disrupted gene as well as the complementing plasmid (Fig. 3A, lanes 1 and 2). For the Δ*ireA*-complemented strains, we observed a band pattern corresponding to the presence of the AMA1-plasmid and a deleted ireA locus (Fig. 3B, lanes 4 and 5). The difference in band intensities between the complementing plasmid and *ireA* deletion could suggest that more than one plasmid copy is present per nucleus as previously proposed for *A. niger* (Verdoes *et al*., 1994b). All NC8 transformants obtained grew like the wild-type, indicating that a plasmid-based *hacA* gene can fully restore the severe growth defect provoked by a *hacA* deletion (Fig. 4). In the case of NC9 strains, the lethal *ireA* deletion phenotype was almost fully rescued by a plasmid-based *ireA* gene (Fig. 4). However, the wild-type phenotype was not completely restored, suggesting that the cellular amount of IreA is strongly controlled. Any overexpression, as might have resulted from plasmid-based expression, could have caused a mild stress phenotype.

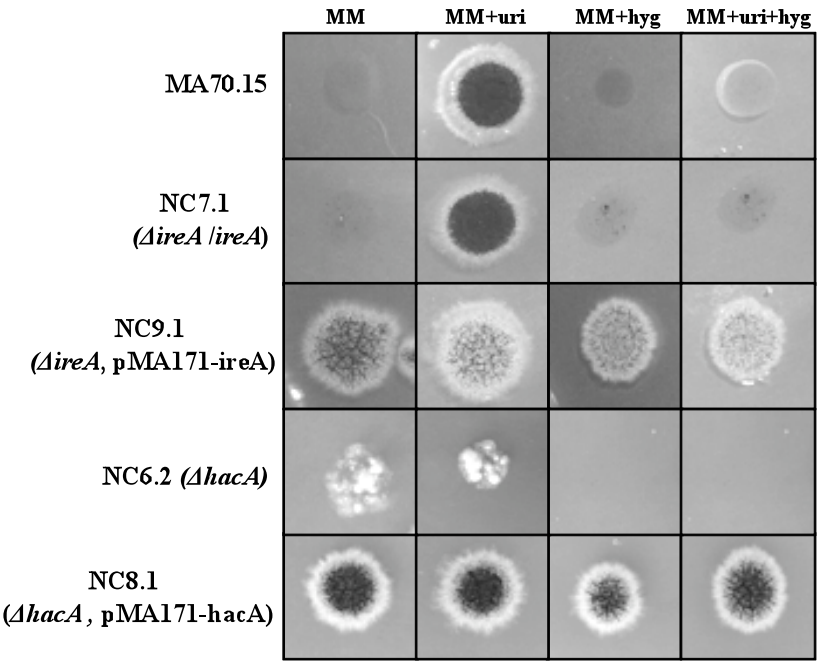

Figure 4. Phenotypic analysis of *ΔhacA* mutants and *ΔireA/ireA* heterokaryons and respective complemented strains. Spores (10^4) were spotted on the different types of media indicated and incubated at 30°C for 3 days, except for the *ΔhacA* mutant which was cultivated for 6 days. Note that the presence of 100 µg/ml hygromycin allows a slight background growth of *A. niger* (e.g. as seen for MA70.15). To fully repress any growth, 150 µg/ml hygromycin are preferable. *uri* uridine, *hyg* hygromycin.

Interestingly, our results also suggested that the AMA1- based complementation plasmids are rather stably maintained in *A. niger*, as under non-selective conditions they did not easily become lost. However, after multiple rounds of cultivation under non-selective conditions, we observed that the wild-type phenotype (provided by the presence of pMA171 or pMA172) reverted back into the mutant phenotype, i.e., the plasmids became lost (data not shown).

2.3.4. Construction of a set of isogenic Δ*kusA* strains for *A. niger*

In order to expand the repertory of Δ*kusA* strains for *A. niger* offering different choices of selection markers, we established two new isogenic Δ*kusA* strains. The deletion of *A. niger kusA* gene in strain AB4.1 (*pyrG*$^-$, *amdS*$^-$) has previously been reported (Meyer *et al*., 2007), using a construct consisting of 1.5 kb of 5′- and 3′-flanking regions of the *kusA*, the *amdS* selection marker and a repeat of the 5′ flanking region to facilitate *amdS* removal by recombination. The resulting strain was named MA70.15 (Δ*kusA*, *amdS*$^+$, *pyrG*$^-$; (Meyer *et al*., 2007)). The same deletion construct was used in the present work to transform the *A. niger* wild-type strain N402 (*amdS*$^-$). Transformants with a deleted *kusA* gene were identified via Southern blot analysis (data not shown), and MA78.6 (Δ*kusA*, *amdS*$^+$) was selected for further studies. This strain and MA70.15 were subjected to counter-selection using the antimetabolite 5-fluoroacetamide (FAA). The direct repeat of the 5′- flanking region of the *kusA* gene flanking the *amdS* marker allowed efficient loop-out of the amdS marker. The correct loop-out of the *amdS* cassette was confirmed by Southern blot (data not shown), and

two strains were used for further analysis: NC4.1 (MA70.15 derivative) and NC5.1 (MA78.6 derivative). For rapid strain identification, a diagnostic PCR approach was designed to detect the presence and/or absence of *kusA* and *amdS*, respectively (Fig. 5).

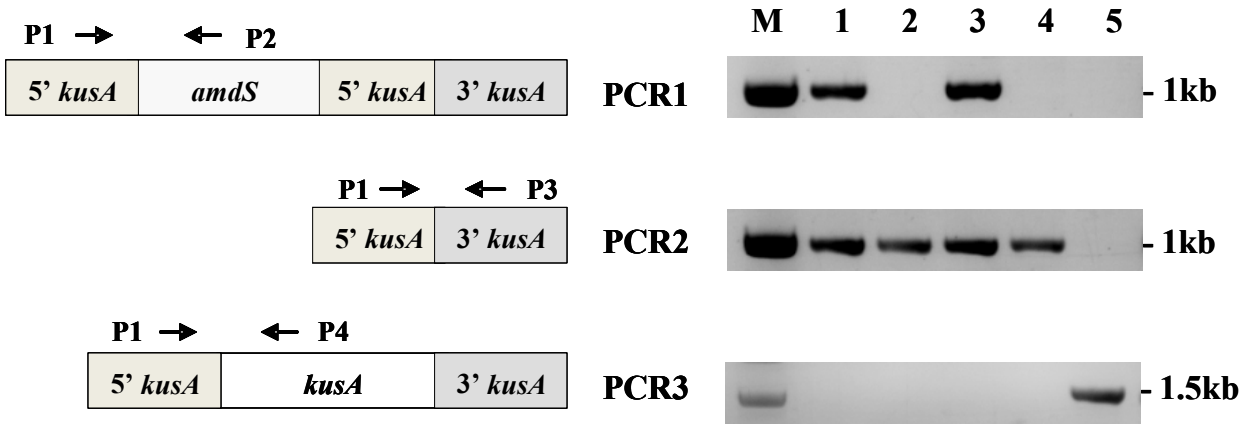

Figure 5. Diagnostic PCR approach to analyse different genetic backgrounds with respect to the *kusA* locus. Three PCR reactions using different primer combinations were performed using genomic DNA obtained from the following strains (relevant genotypes are given in brackets): lane 1, MA70.15 (Δ*kusA*, *amdS*$^{+}$); lane 2, NC4.1 (Δ*kusA*, *amdS*$^{-}$); lane 3, MA78.6 (Δ*kusA*, *amdS*$^{+}$), lane 4, NC5.1 (Δ*kusA*, *amdS*$^{-}$); lane 5, N402 (*amdS*$^{-}$). In PCR1, only those strains which harbour the *amdS* gene are expected to generate a PCR product (primer combination P1 and P2). In PCR2, a 1 kb amplicon is expected only for Δ*kusA* strains. Under the conditions used (primer combination P1 and P3, short elongation time), the generation of the 1-kb amplicon is favoured over the generation of a 3-kb fragment which would result in MA70.15 and MA78.6 (contain the *amdS* gene between the 5'-*kusA* and 3'-*kusA* flanking regions) and N402 (contains the *kusA* gene between the 5'-*kusA* and 3'-kusA flanking regions). In the case of PCR3 (primer combination P1 and P4), only N402 bearing an intact kusA locus is expected to generate a 1.5 kb band. The predicted sizes of PCR fragment are indicated. *M* molecular weight marker.

By performing three different PCR reactions with selected combinations of four primers, strains can be identified carrying the wild-type *kusA* locus, the Δ*kusA*(*kusA::amdS*) locus or the Δ*kusA* locus, where *amdS* has been looped out (Fig. 5). As described previously for the Δ*kusA* mutant MA70.15 (Meyer *et al*., 2007), no obvious differences among AB4.1, MA78.6, NC4.1 and NC5.1 were observed with respect to growth, morphology and biomass accumulation (data not shown).

2.3.5. Design and performance of a transient *kusA* disruption strain

To establish a transiently disrupted *kusA* allele in *A. niger*, we adapted a strategy, followed recently for *A. nidulans* (Nielsen *et al*., 2008). As described in detail in Fig. 6 and in the "Materials and methods" section, a construct was made in which the *amdS* marker is flanked by *kusA* sequences which have in common a direct repeat of 300 bp (DR) from the kusA ORF.

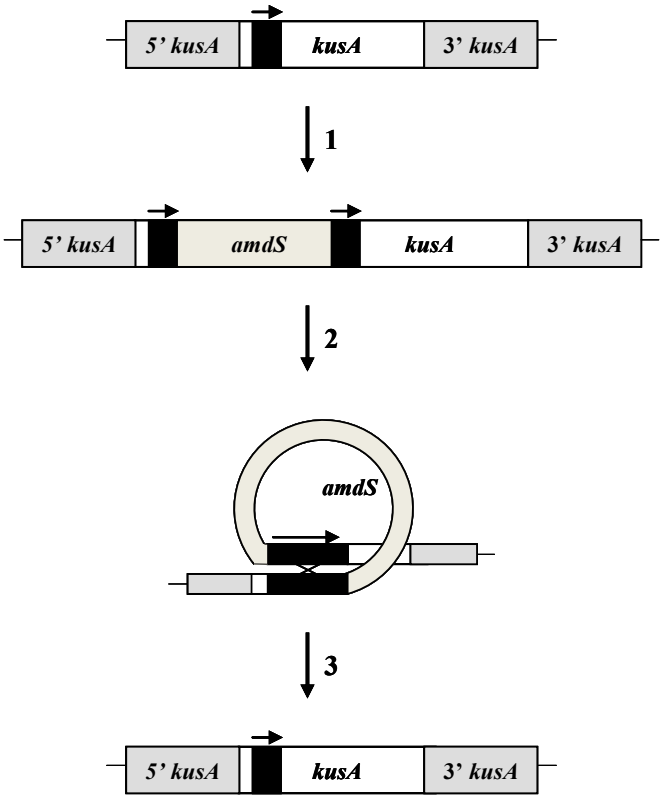

Figure 6. Strategy for transient disruption of *kusA*. The counterselectable marker *amdS*, flanked on each site with a 300-bp sequence of the *kusA* ORF (DR, marked by a black box and an arrow) was integrated into the *kusA* gene (1). In the resulting strain MA169.4, carrying the disrupted *kusA* allele, any gene-targeting approach can be performed with high efficiency. After counter-selection on FAA medium (2), the *amdS* gene loops out via single crossover between the DR (3), and a functional *kusA* allele becomes restored.

That construct was transformed into *A. niger* strain AB4.1 (*pyrG*$^-$), and one strain was isolated (out of ten analysed) that carried a disrupted allele of *kusA* (Figs. 6, 7 and data not shown). The strain selected, MA169.4 (*kusA*$^-$, *pyrG*$^-$, *amdS*$^+$), as well as MA70.15 (Δ*kusA*, *pyrG*$^-$, *amdS*$^+$) were subsequently used as recipient strains for targeted deletion of a GTPase-encoding gene *srgA*. Its deletion phenotype is easy to score because *srgA* null strains display clear defects in growth and are hyperbranching (Punt *et al*., 2001). About 60 transformants were obtained for each of the transformations, ~95% of which clearly showed the deletion phenotype (data not shown). Hence, both recipient strains ensure similar HR frequencies. A similar conclusion we could draw after performing a gene deletion approach targeting another GTPase-encoding gene, *racA*. Here, about ~55% of the transformants showed the deletion phenotype in both the *kusA* deletion and *kusA* disruption background strains (n>90; data not shown). Three of the Δ*racA* strains (MA171.1–MA171.3), where deletion of *racA* was verified by Southern hybridisation (Fig. 7 and data not shown), were selected and subjected to FAA counter-selection. From the three MA171 strains, each two *amdS*$^-$ colonies were randomly selected (MA172.1– MA172.6) and their *kusA* locus analysed by Southern hybridisation (Fig. 7 and data not shown), PCR-amplified and sequenced. No deviations from the *kusA* wild-type sequence were encountered in all six sequencing reactions (data not

shown), demonstrating that the *kusA* gene can accurately and fully be restored by looping out the *amdS* marker via the 300-bp DRs (Fig. 6).

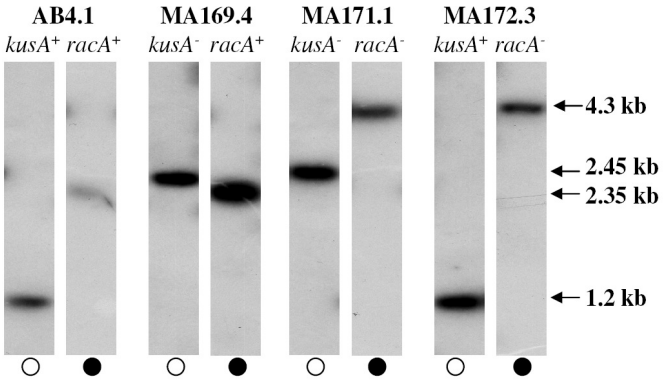

Figure 7. Southern analysis for strains in which *kusA* has transiently been disrupted. Genomic DNAs of the four strains indicated were restricted with *Nco*I (marked with open circle) or *Bgl*II (marked with closed circle). *Nco*I restricted DNAs were hybridised with a *kusA* probe targeting the 5' untranslated region of *kusA*. Those strains which harbour an intact *kusA* allele show a signal at 1.2 kb, whereas strains in which *kusA* has been disrupted with the *amdS* marker display a 2.45 kb signal. *Bgl*II-restricted genomic DNAs were hybridised with a *racA* probe targeting the 5' untranslated region of *racA*. In case of an intact *racA* allele, a 2.35 kb band will be visible, in case *racA* has been deleted with the *AopyrG* gene, a 4.3 kb band becomes apparent.

2.4. Discussion

The inactivation of the NHEJ pathway has been demonstrated to be a successful tool to perform targeted genetic manipulations in a very efficient manner and has paved the way for high-throughput functional genomics approaches in filamentous fungi. For example, the *A. niger kusA* deletion mutant MA70.15 has been proven to be a powerful recipient strain to generate gene deletions and to identify essential genes (Meyer *et al*., 2007). To broaden the choice of selection markers for gene-targeting approaches and to avoid uridine/uracil auxotrophic strains when using dominant selection markers (MA70.15 is *pyrG*$^{-}$), we deleted in this study the *kusA* gene in the prototrophic *A. niger* strain N402. The resulting strain MA78.6 has repeatedly been used in our lab for gene deletion approaches using the hygromycin cassette and showed similar HR frequencies as reported for strain MA70.15 (own unpublished results). In addition, both MA70.15 and MA78.6 have been cured for the *amdS* marker by FAA counter-selection, generating NC4.1 (Δ*kusA*, *pyrG*$^{-}$) and NC5.1 (Δ*kusA*), respectively. The now available strain collection of MA70.15, MA78.6, NC4.1 and NC5.1 allows the use of several well-established selection markers (*pyrG*, acetamidase, hygromycin, phleomycin), thus improving flexibility in making user-defined mutations. Furthermore, the generation of prototrophic strains in which at least four genes can be targeted without the need for curing any selection marker has now become feasible.

To further demonstrate the value of Δ*kusA* strains, we have exemplarily focused on deleting two UPR genes, namely *hacA* and *ireA*. The generation of Δ*hacA* and Δ*ireA*/*ireA* strains unambiguously illustrates the advantage of using a NHEJ-deficient strain as recipient for the isolation of mutants displaying a severe growth defect (Δ*hacA*) or for the deletion of

essential genes (*ireA*). Our findings that all of the randomly selected primary transformants of Δ*hacA* and Δ*ireA* were heterokaryons, support earlier observations that heterokaryon formation is improved in a Δ*kusA* background strain, probably as a result of less-favoured ectopic integration events (Meyer *et al*., 2007). The elegant heterokaryon rescue technique useful for studying gene functions in heterokaryons has been first described in *A. nidulans* (Osmani *et al*., 2006). This technique is especially valuable for the asexual fungus *A. niger*, as it is impossible to generate deletions in a diploid strain and to analyse its progeny after meiosis. A heterokaryon strain can be used instead, and functional analysis tests can be performed by transferring spores or pieces of heterokaryotic mycelium onto different growth plates ((Osmani *et al*., 2006; Todd *et al*., 2007) and this work).

Interestingly, the number of nuclei carrying the deleted gene and nuclei carrying the wild-type gene are unbalanced in the Δ*ireA*/*ireA* strains (Fig. 3B). Such a nucleus imbalance is not unusual in fungal heterokaryotic strains and has been reported, e.g. for *A. nidulans*, *A. niger* and *Neurospora crassa* (Punt *et al*., 1998; Pitchaimani and Maheshwari 2003; Ichinomiya *et al*., 2007; Todd *et al*., 2007). As shown for *N. crassa*, the proportion of nuclei depends on medium conditions, the number of sub-cultivations and the genetic locus affected (Pitchaimani and Maheshwari 2003). Unfortunately, such an imbalance limits the use of heterokaryons for haploinsufficiency screens and analyses, which are outstanding tools to study gene functions as shown for *S. cerevisiae* and *Candida albicans* (Giaever *et al*., 1999; Baetz *et al*., 2004; Martinez and Ljungdahl, 2004). It is conceivable, however, that a balance of both types of nuclei is re-adjustable in heterokaryons, e.g. by fine-tuning the selection pressure, an assumption that awaits experimental verification.

Another drawback of imperfect fungi such as *A. niger* is the circumstance that a defective NHEJ pathway cannot be restored by sexual crossing. However, the NHEJ pathway and its components are crucial for maintaining genome integrity in eukaryotes, especially in aging cells. DSBs can arise from intracellular reactive oxygen species, by replication folk collapse, during meiotic chromosome segregation or from exogenous attacks such as radiation and hazardous chemicals. Consequently, DSB repair mechanisms such as HR and NHEJ pathways are crucial to the survival of eukaryotes (Pardo *et al*., 2009). To avoid any detrimental and pleiotropic effects of a constantly inactive NHEJ pathway in *A. niger*, we also established strain MA169.4 harbouring a transiently disrupted *kusA* allele. Using two genes of our interest as an example (*srgA*, *racA*), we could show that MA169.4 is as efficient as MA70.15 with respect to introducing gene deletions. The advantage of MA169.4 over MA70.15 is, however, that the native *kusA* allele can easily be restored after the genetic engineering approach has been accomplished. The respective strains gained are then especially valuable when post-exponential or aging phenomena are in the focus of the research. In this context, it is worth mentioning that HR frequencies are not only dependent on the activity of HR and NHEJ, but are also strongly dependent on the gene locus. We have encountered that about 10% of the *A. niger* genes analysed so far in our group (>60 genes), are difficult to target, potentially because they are localised close to contig borders or within silenced heterochromatic DNA regions.

Complementation of gene deletion mutants with the wild-type gene copy is an essential control to prove the function of a gene of interest. There are different options to perform complementation analyses, such as ectopic or homologous integration of the gene

copy. Both strategies have their disadvantages, e.g. ectopic integration is difficult to perform in a NHEJ-deficient background, and homologous integration raises questions on the choice of the target locus as it has to ensure sufficient expression of the wildtype gene. In addition, we have encountered that many *A. niger* mutants are difficult to transform if genomic integration has been approached (e.g. Δ*hacA*, data not shown). The AMA1-based complementation strategy described here in this work appeared to be very straightforward and successful to complement gene deletions in *A. niger* (Fig. 4). Moreover, it has also some advantages over the other two options: (1) it is possible to transform AMA1-based plasmids at high frequencies; (2) transformation efficiencies are independent of the *kusA* background; (3) high transformation efficiencies can even be obtained for mutants which are difficult to transform and (4) the selection marker as well as the complementing gene can easily be removed, simply by growing the complemented strains for several generations on non-selective medium ((Gems *et al*., 1991; Gems and Clutterbuck 1993) and this work). Taken together, this study has expanded the *ku70* toolbox for *A. niger* by generating various recipient strains for flexible and improved functional gene analysis. In addition, the establishment of the AMA1-based plasmids pMA171 and pMA172 are not only new and valuable molecular tools for *A. niger*, they can moreover be generally implemented for usage in filamentous fungi as the individual gene cassettes present in both plasmids are functional in many Asco- and Basidiomycetes.

Chapter 3

Functional YFP-tagging of the essential GDP-mannose transporter reveals an important role for the secretion related small GTPase SrgC protein in maintenance of Golgi bodies in *Aspergillus niger*

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Abstract

The addition of mannose residues to glycoproteins and glycolipids in the Golgi is carried out by mannosyltransferases. Their activity depends on the presence of GDP-mannose in the lumen of the Golgi. The transport of GDP-mannose (mannosyl donor) into the Golgi requires a specific nucleotide sugar transport present in the Golgi membrane. Here, we report the identification and functional characterization of the putative GDP-mannose transporter in *A. niger*, encoded by the *gmtA* gene (An17g02140). The single GDP-mannose transporter was identified in the *A. niger* genome and deletion analysis showed that *gmtA* is an essential gene. The lethal phenotype of the *gmtA* could be fully complemented by expressing an YFP-GmtA fusion protein from the endogenous *gmtA* promoter. Fluorescence studies revealed that, as in other fungal species, GmtA localized as punctate dots throughout the hyphal cytoplasm, representing Golgi bodies or Golgi equivalents.

SrgC encodes a member of the Rab6/Ypt6 subfamily of secretion related GTPases and is predicted to be required for the Golgi to vacuole transport. Loss of function of the *srgC* gene in *A. niger* resulted in strongly reduced growth and the inability to form conidiospores at 37°C and higher. Furthermore, the *srgC* disruption in the *A. niger* strain expressing the functional YFP-GmtA fusion protein led to an apparent "disappearance" of the Golgi-like structures. The analysis suggests that SrgC has an important role in maintaining the integrity of Golgi-like structures in *A. niger*

3.1. Introduction

The secretory pathway of filamentous fungi, as in other eukaryotic cells, comprises a complex endomembrane network, consisting of a number of different interdependent organelles. Every organelle is specialized and involved in the different steps which induce protein folding, assembly, modification, processing and sorting that are required before secretory proteins are secreted (reviewed in Shoji *et al*., 2008). The interest in filamentous fungi for the production of (heterologous) proteins has grown due to their inherent high secretion capacity (Conesa *et al*., 2001; Punt *et al*., 2002); therefore a better understanding of the processes operating during the secretory pathway is indispensable. Translation of mRNA molecules by ribosomes starts in the cytosol and proteins targeted for secretion are directed to the Endoplasmic Reticulum (ER) via their signal sequence. Within the ER, proteins are folded and undergo modifications such as disulphide bridge formation, signal peptide processing and glycosylation at serine or threonine residues (O-linked glycosylation) or at asparagine residues (N-linked glycosylation) (Peberdy 1994; Gemmill and Trimble 1999). In order to ensure proper protein assembly, a quality control system operates in the ER and misfolded proteins are targeted to destruction by the proteasome via the Endoplasmic Reticulum Associated Degradation (ERAD) (Schrader *et al*., 2009; Hoseki *et al*., 2010) or vacuole (Veses *et al*., 2008). Correctly folded proteins are packed into Coat Protein complex II (COPII) vesicles and transported into the Golgi complex (Spang, 2009). Unlike the serial stack of flattened cisternae observed in higher eukaryotes, the Golgi in filamentous fungi is different, comprising unstacked and dispersed cisternae and therefore, usually referred to as Golgi-like structures or Golgi Equivalents (Howard, 1981; Breakspear *et al*., 2007; Hubbard and Kaminskyj, 2008; Pantazopoulou and Peñalva, 2009). Proteins are released into the *cis*-Golgi network and transported through the organelle to the *trans*-Golgi network (Orci *et al*., 2000) while undergoing further modifications, such as the attachment of additional mannose residues to O-linked sugar chains by different mannosyltransferases (Shaw and Momany, 2002; Goto, 2007; Kriangkripipat and Momany, 2009). Additionally to glycoproteins, glycolipids and glycophosphatidylinositol (GPI) anchors are also modified by the addition of mannose (Sipos *et al*., 1995). The GDP-mannose is the sugar donor for these reactions occurring in the Golgi and is synthesized in the cytosol (Berninsone and Hirschberg, 2000), which implies that GDP-mannose has to be transported into the Golgi lumen. In yeast, the transport of GDP-mannose is mediated via the GDP-mannose transporter Vrg4p (Dean *et al*., 1997). Nucleotide sugar transporters (NSTs) are predicted to contain 6-10 membrane spanning domains (Hirschberg *et al*., 1998), and in particular, yeast GDP-mannose transporter is predicted to contain 6-8 membrane domains (Gao and Dean, 2000; Nishikawa *et al*., 2002a). In *S. cerevisiae*, the N- and C- termini of the Golgi NST Vrg4p are located in the cytosol; the N-terminal is required for ER export and Golgi localization, whereas the C-terminal has been shown to be essential for its stability and oligomerization (Gao and Dean, 2000). Although localized in the Golgi, *S. cerevisiae* Vrg4p has been shown to recycle between this organelle and the ER (Abe *et al*., 2004). Secreted proteins have also been shown to be processed in the Golgi by resident proteases, a carboxypeptidase Kex1p, an endopeptidase (kex2p), and a dipeptidylaminopeptidase A (Cooper and Bussey, 1992; Nothwehr *et al*., 1993; Nakayama, 1997; Brenner and Fuller, 2002). Kex2p homologues have

also been studied in *Aspergillus niger* - KexB/PclA - (Jalving *et al*., 2000; Punt *et al*., 2003) and *Aspergillus nidulans* - KpcAp – (Kwon *et al*., 2001). Depending on targeting sequences, proteins leaving the Golgi are sorted to the plasma membrane, cell wall, vacuole or extracellular environment.

The transport steps within the secretory pathway are complex and involve the sorting of proteins into transport vesicles and delivery to the following compartment or organelle. Secretion related small Guanosine Triphosphate-binding Proteins (GTPases) belonging to the Rab/Ypt and Sar1/Arf families play an important role in the regulation of vesicular traffic within the secretory pathway (Segev, 2001). The Rab/Ypt and Sar1/Arf belong to the Ras superfamily of GTP-binding proteins which also include the following families: Ras, involved in cell growth and signalling; Rho, involved in cell growth and morphology; Ran, involved in nuclear transport (Wong *et al*., 1997; Takai *et al*., 2001; Wu *et al*., 2008); and Ral, only found in animal cells, that has been shown to be involved in exocytosis and actin-cytoskeleton dynamics (Jullien-Flores *et al*., 2000; Moskalenko *et al*., 2002). Different GTPases are involved in specific transport steps in the secretory system and have been described in *A.niger* (Punt *et al*., 2001). In this study, we have used the functional YFP-GmtA Golgi reporter to establish a role for the secretion-related GTPase *srgC* in the maintenance of Golgi bodies in *Aspergillus niger.*

3.2. Materials and methods

3.2.1. Strains and culture conditions

Aspergillus niger strains used in this study are listed in Table 1. Strains were cultivated in minimal medium (MM) (Bennett and Lasure 1991) or in complete medium (CM) as described (Carvalho *et al*., 2010) When required, 10 mM uridine or/and 100 μg/ml of hygromycin was added.

Table 1. *Aspergillus niger* strains used in this study.

Name	Genotype	Reference
N402	*csp, amdS*$^-$	Bos *et al*., 1988
MA70.15	*ΔkusA, pyrG*$^-$*, amdS*$^+$	Meyer *et al*., 2007
MA210.1	*ΔkusA, pyrG*$^-$ *,* Δ*gmtA*/heterokaryon	This study
REN2.1	Δ*gmtA*/heterokaryon complemented	This study
REN1.10	*ΔkusA, pyrG*$^+$*,* pREN1 *(*P*gmtA*-YFP::*gmtA*-T*gmtA*-pyrG*)	This study
MA160.1	REN1.10, Δ*srgC*	This study
MA161.6	REN1.10, Δ*gmtA*	This study
XWA16.1	N402, Δ*srgC*	This study
MA141.1	*ΔkusA, pyrG*$^+$, pMA141(P*gpdA*-GlaA2::sGFP-HDEL-T*trpC*-pyrG*)	This study

3.2.2. Molecular biological techniques

All basic molecular techniques were performed according to standard procedures (Sambrook and Russel 2001). *Escherichia coli* DH5α was used for transformations. DNA isolations (GeneJet Plasmid Miniprep Kit), molecular enzymes were obtained from Fermentas and used according to the supplied protocols. Phusion™ High-Fidelity PCR Kit (Finnzymes) was used according to manufacturer's instructions for PCR amplifications. Transformation of *A. niger*, genomic DNA extraction, screening procedures, diagnostic PCR and Southern analysis were conducted as recently described in detail (Meyer *et al*., 2010).

3.2.3. Deletion of the *Aspergillus niger gmtA* gene and complementation analysis

The *A. niger gmtA* gene (An17g02140) was deleted by replacing the coding region with the hygromycin selection marker. The deletion cassette was produced by amplification of the fragments corresponding to 5' (951 bp) and 3' *gmtA* (482 bp) flanking regions using primers indicated in Supplementary Table 1. The gene coding for hygromycin resistance (*hph*) was obtained by digesting pAN7.1 (accession number: Z32698, Punt *et al*., 1987) with *Xho*I and *Xba*I. A fusion PCR was performed to obtain the disruption cassette using 5' and 3' *gmtA* flanking regions and *hph* as template DNAs and gmtA-P1F/gmtA-P6R as outer primers (Supplementary Table 1), according to Szewczyk (2006). The *gmtA* deletion construct was transformed into MA70.15 and putative transformants were selected by incubating protoplasts on MM agar plates containing hygromycin. As no viable *gmtA* deletion mutants could be obtained after purification of the primary transformants, propagation and maintenance of *gmtA* heterokaryotic strains were done by transfer of mycelia from the primary transformants onto MM. Putative Δ*gmtA*::hyg/*gmtA* mutants were further analyzed by Southern blot. Due to the heterokaryotic nature of the putative Δ*gmtA* primary transformants, the isolation of genomic DNA was done by inoculating pieces of mycelium in MM containing hygromycin. For gene complementation analysis, the *A. niger gmtA* gene was amplified using primers GmtP1/P6 (Supplementary Table 1) and cloned into the autonomously replicating plasmid pMA172 (Carvalho *et al*., 2010) containing *pyrG* as a selection marker to give pRen2. pRen2 was transformed into a Δ*gmtA* heterokaryon strain and the resulting transformants were purified, examined by Southern blot analysis and transformant REN2.1 was selected for further studies.

3.2.4. Construction of YFP-GmtA fusion protein for Golgi visualization

The YFP-GmtA fusion constructed by fusion PCR as described (Szewczyk *et al*., 2006). Fragments containing the *gmtA* promoter region (951 bp), the YFP gene (747 bp) and coding region including terminator (1.8 kb) were amplified using primers listed in Supplementary Table 1 using N402 or pEYFP (Clontech®) as template DNA. The fusion PCR was performed using similar DNA amounts (≈50ng) of *gmtA* promoter region, YFP and *gmtA* + 3' flanking region and primers GmtAP11F and GmtAP12R and the PCR product was cloned into pJet1.2. An *Xba*I-*Xba*I *pyrG** fragment was obtained from pAF3 (Damveld *et al*., 2005) and cloned into this plasmid for targeted integration of the YFP-GmtA construct at the *pyrG* locus to give pRen1. The pRen1 was transformed into MA70.15 and putative

transformants were purified and subjected to Southern blot analysis. Transformant REN1.10, expressing a single copy YFP-GmtA at the *pyrG* locus was used as a recipient strain to disrupt the *gmtA* gene using the disruption cassette as described above. Primary transformants were randomly selected for further purification steps and subjected to Southern analysis. Transformant MA161.6 bearing a *gmtA* deletion and a functional YFP-GmtA fusion protein was selected for following studies.

3.2.5. Cloning and disruption of the *A. niger srgC* gene

A genomic library of *A. niger* N402 in λBlueSTARclone (Novagen) was screened with the *srgC* fragment (Genbank accession no. AJ278660 (cDNA clone F688#5)) (Punt *et al*., 2001). The *srgC* gene was located on a 3.8 kb *Not*I-*Bam*HI fragment and subcloned into the corresponding restriction sites of pBluescript-II SK to give pSrgC. From this subclone the complete *srgC* open reading frame (Genbank accession no. DQ213058), including 0.5 kb promoter and 0.8 kb terminator regions were determined. The *A. niger srgC* was disrupted by insertion of the hygromycin B resistance gene into the unique *Nhe*I (+75) restriction site. pSrgC was digested with *Nhe*I and a 3154 bp *Nhe*I-*Xba*I fragment from pAN7-1 (Genbank accession no. Z32698), containing the hygromycin B selection marker under control of the P*gpdA* promoter and T*trpC* terminator, was inserted. From the resulting plasmid pΔSrgC, the 5.0 kb *Not*I/*Kpn*I fragment was transformed into N402 and Ren1.10 strains. Hygromycin resistant transformants were purified and disruption of the *srgC* gene was confirmed by Southern blot analysis. Therefore, fungal genomic DNA was isolated as described by Meyer *et al*. (2010) and digested with *Bam*HI. Hybridisation was done with a 0.8 kb *Eco*RI-*Bam*HI fragment of the 3' end of *srgC* as a probe. Strains XWA16.1 (*ΔsrgC* in the N402 background) and MA160.1 (*ΔsrgC* in the Ren1.10 background) were chosen for further studies.

3.2.6. Construction of a reporter strain for ER visualization

To visualize the ER by GFP fluorescence, we used plasmid pXW2 that expresses the GLA::GFP-HDEL fusion protein (Gordon *et al*., 2000a) from the *gpdA* promoter. A 1.6 kb *BamH*I-*Xba*I fragment containing part of the GlaAG2 gene fused to the sGFP(S65T)::HDEL sequence including the *trpC* terminator sequence was excised from pAN56-2sGFP(S65T)::HDEL (Gordon *et al*., 2000a) and cloned in the corresponding restriction sites of vector pAN56-1 (EMBL Z32700) to give pXW2. For targeted integration at the *A. niger pyrG* locus, *pyrG** was used as a selection marker and cloned into pXW2 (as described above for pRen1). The resulting plasmid pMA141 (P*gpdA*-GlaAG2::sGFP-HDEL-T*trpC*-*pyrG**) was transformed into MA70.15 strain and transformant and MA141.1 which contains a single copy integration of pXW2 at the *pyrG* locus (confirmed by Southern blot) was chosen for further studies.

3.2.7. Microscopy

Conidiospores of N402 and XWA16.1 (*ΔsrgC*) ($1x10^4$ spores ml^{-1}) were inoculated in growth medium and poured in Petri dishes with sterile cover slips (Harris *et al*., 1994). Strains were grown at different temperatures (25ºC, 30ºC, 37ºC and 42ºC) for 24 hours and analyzed with a Zeiss Axioplan2 upright microscope. Pictures were taken using an AxioCam MRc5 digital camera coupled to the Axioplan microscope with standard DIC settings. Images were collected with AxioVision imaging software. For fluorescence microscopy, conidiospores of Ren1.10 and MA160.1 strains were inoculated in solid MM medium, incubated O/N (≈16h) at 25°C and analyzed with fluorescence Zeiss Axioscope using standard Fluorescein Isothiocynate (FITC) filters (ext. 450-495 em. 505-550) and infinity corrected plan-neofluar 60x/1.3 lenses.

3.3. Results

3.3.1. *In silico* analysis of *Aspergillus niger gmtA*

The An17g02140 gene (*gmtA*) was identified in *A. niger* as a putative GDP-mannose transporter (Pel *et al*., 2007). We have compared different nucleotide sugar transporters (NSTs) from three *Aspergilli* with those of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Fig. 1). This analysis revealed that some clusters contain representative transporters from all five fungi in study such as those belonging to the subfamilies UGA (UDP-Galactose:UMP Antiporter, TPT (Triose-Phosphate Transporter), UAA (UDP-N-acetylglucosamine:UMP Antiporter), TPPT (Thiamine Pyrophosphate Transporter) and GMA (GDP-Mannose:GMP Antiporter). This analysis indicates that these NSTs are probably involved in conserved processes that take place in all five fungi analyzed (galactosylation, mannosylation, N-acetylglucoaminylation, etc.). The phylogenetic analysis (Fig. 1) shows that GmtA clusters together with the well characterized GDP-mannose transporters of *S. cerevisiae*, Vrg4p (Dean *et al*., 1997), and the recently identified putative GDP-mannose transporters in *Aspergillus nidulans*, GmtA and GmtB (Jackson-Hayes *et al*., 2008). GDP-mannose transporters could be indentified in the different classes of ascomycete and basiodiomycete species (Table S2).

Phylogenetic analysis of GDP-mannose transporters revealed that despite function conservation there is a clear divergence among Ascomycota and Basidiomycota phylums (Supplementary Fig. S1).

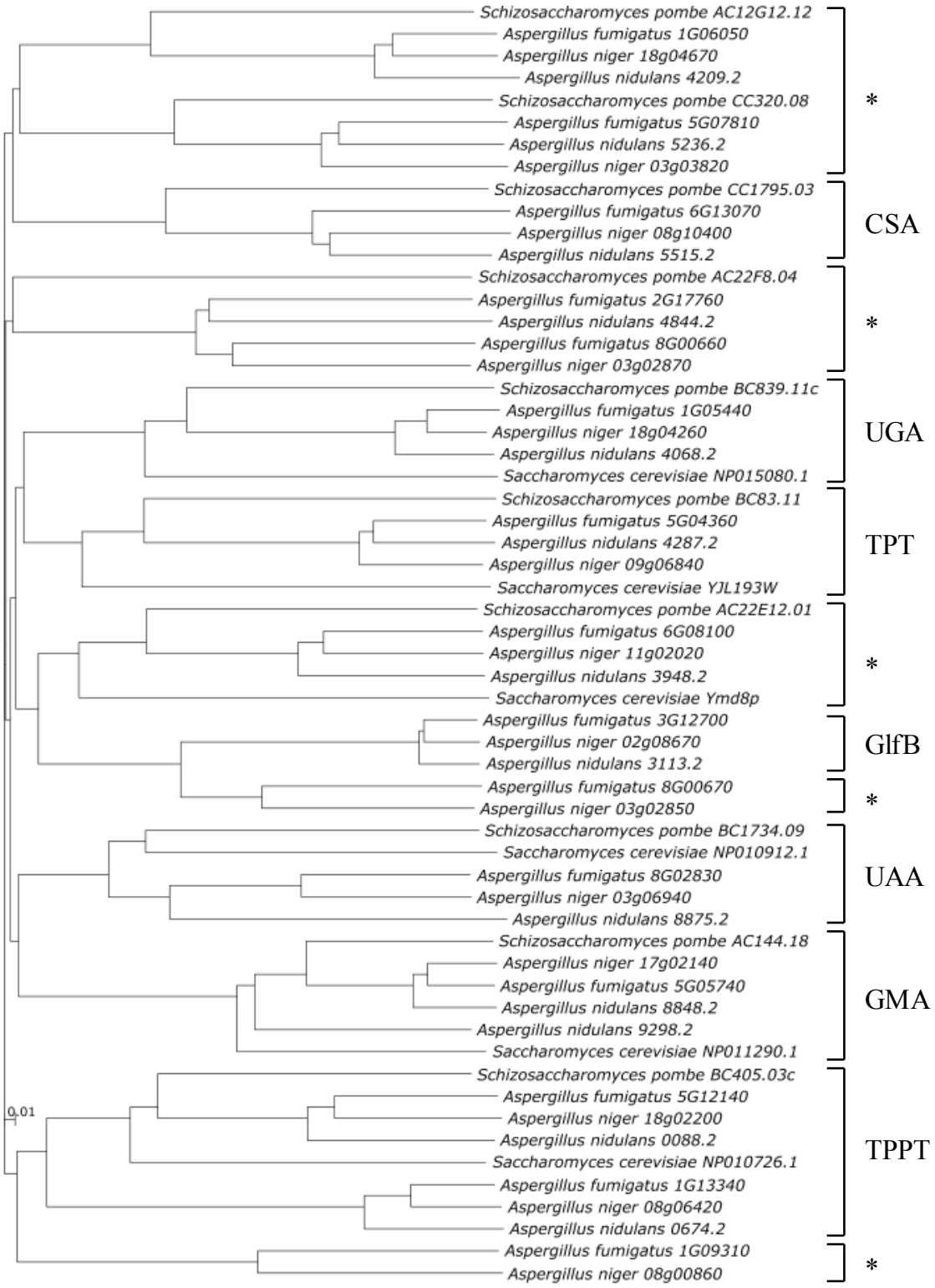

Figure 1. Phylogenetic tree of known and putative NSTs from *S. cerevisiae*, *S. pombe* and *Aspergillus species*. Alignment has been carried out using ClustalW and trees were designed using Archaeopteryx (http://www.phylosoft.org/archaeopteryx/). On the right of 1A is shown the family classification. TPT-Triose-phosphate Transporter; UAA-UDP-Nacetylglucosamine:UMP Antiporter; UGA-UDP-galactose:UMP Antiporter; CSA-CMP-Sialate:CMP Antiporter; GMA-GDP-mannose:GMP Antiporter; TPPT- Thiamine Pyrophosphate Transporter; GlfB- DUF250 domain membrane protein; * no subfamily attributed yet. The scale bar corresponds to a genetic distance of 0.01 substitution per position.

The protein sequence of the predicted GmtA orthologue in *A. niger* and its alignment with the two GDP-transporters described in *A. nidulans*, GmtA (AN8848.3) and GmtB (AN9298.3), and *S. cerevisiae* Vrg4p (YGL225W**)** sequence is shown in Fig. 2. The alignment reveals a high conserved region around the GALNK motif (marked with * in Fig. 2) that has been described to be unique to proteins closely related to Vrg4p and related GPD-mannose transporters (Gao *et al*., 2001; Nishikawa *et al*., 2002b; Jackson-Hayes *et al*., 2008). It has been proposed that the localization of NSTs in the Golgi depends on the lysine residues at the C-terminal of these proteins (Abe *et al*., 2004). In this way, the three lysine residues (KxKxxxK) found at the C-terminal of *A. niger* an *A. nidulans* GmtA proteins might be significant and have a role in its localization (marked with **:** in Fig. 2).

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An GmtA    MAEGKKTDDYTIQMDSIDQGNKSFEAPPPP-QPRSPPSGSLSNNPILPVLAYCGSSILMTVMNKYVLSG-TDFNLNFFLL  78
AN GmtA    MTDNRKPEDYTIEMDKLGQ-NKNYQAPPPPPQPRSSTASSISNNAALSVLAYCGSSILMTVMNKYVLS--SDFNLNFFLL  77
AN GmtB    ------------------------------------------------------------------------------  
Sc Vrg4    MSE-----------------LKTGHAGHNPW-------ASVANSGPISILSYCGSSILMTVTNKFVVN-LKDFNMNFVML  55

An GmtA    CIQSLVCIIAIQTCKSCGLITYRD-FSADEARKWFPITLLLIGMIYTGSKALQFLSIPVYTIFKNLTIILIAYGEVLWFG  157
AN GmtA    CVQSLVCIIAIQLCKACGLITYRD-FNLDEARKWFPITLLLIGMIYTGSKALQFLSIPVYTIFKNLTIILIAYGEVLWFG  156
AN GmtB    -----------MVCKKAGLIQNLGLFDLKKAQTWLPISLLLVGMIYTGNKALQFLSVPVYTIFKNLTIIVIAYGEVFMVG  69
Sc Vrg4    FVQSLVCTITLIILRILGYAKFRS-LNKTDAKNWFPISFLLVLMIYTSSKALQYLAVPIYTIFKNLTIILIAYGEVLFFG  134

An GmtA    GSVTGLTLFSFGLMVLSSIIAAWAD----IKHAVESNGDATAKVSTL---NAGYIWMLVNCLCTSSYVLGMRKRIKLTNF  230
AN GmtA    GSVTNLTLFSFGLMVFSSIIAAWAD----IKHAIESSGDATSKVSTL---NAGYIWMLINCLCTSSYVLGMRKRIKLTNF  229
AN GmtB    GSVKPLALLSFGLMVLSSVVAAWADIQ--IATAATAKASSDSAVATLSALNAGYAWMGTNVVFSASYALGMRRVIKKTNF  147
Sc Vrg4    GSVTSMELSSFLLMVLSSVVATWGDQQAVAAKAASLAEGAAGAVASF---NPGYFWMFTNCITSALFVLIMRKRIKLTNF  211

An GmtA    KDFDTMFYNNLLSIPVLIVLSAFLEDWSSTNVNRNFPPMDRNSIVFAMIISGLSSVFISYTSAWCVRVTSSTTYSMVGAL  310
AN GmtA    KDFDTMFYNNLLSIPVLIVGSGILEDWSPANVARNFPSADRNGIMFAMIISGLSTVFISYTSAWCVRVTSSTTYSMVGAL  309
AN GmtB    DNWDVMFYNNLLSVPILLLSSLLVEDWSSENLQRNFPAESRQSLVIGIFYSGVAAIFISYCTAWCVRATSSTTYAMVGAL  227
Sc Vrg4    KDFDTMFYNNVLALPILLLFSFCVEDWSSVNLTNNF----SNDSLTAMIISGVASVGISYCSGWCVRVTSSTTYSMVGAL  287
                                                                                  **** ****

An GmtA    NKLPIAISGLIFFDAPVTFPSVSAIVVGFVSGIVYAVAK-IKQNAKPRTGVLPTANPPVSASSQSMRDSLRS  381
AN GmtA    NKLPIALSGLIFFDAPVTFPSVSAIMVGFVSGIVYAVAK-IKQNAKPKVGILPTTN-PVSASSQSMRDSLRS  379
AN GmtB    NKLPIAVAGIVFFAAPVTFGSVSAIVLGFISGLVYTWAK-STGA----------------------------  270
Sc Vrg4    NKLPIALSGLIFFDAPRNFLSILSIFIGFLSGIIYAVAKQKKQQAQP----------------------LRK  337
           ****                                 :  :   :
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Figure 2. Alignment of the proteins of *Aspergillus niger* (An) GmtA, *Aspergillus nidulans* (AN) GmtA and GmtB and *Saccharomyces cerevisiae* (Sc) Vrg4. Identical residues on all four proteins are shaded and the GALNK motif is indicated by the (*). The three lysine residues (KxKxxxK) found at the C-terminal of *A. niger* and *A. nidulans* GmtA proteins are marked as (**:**). *A. nidulans* sequences were retrieved from CADRE (http://www.cadre-genomes.org.uk/Aspergillus_nidulans/).

Nucleotide sugar transporters are structurally conserved proteins predicted to contain 6-10 membrane spanning domains and both N- and C- termini localized at the cytoplasmic side (Hirschberg *et al*., 1998; Gerardy-Schahn *et al*., 2001), and the *S. cerevisiae* GDP-mannose transporter is predicted to contain 8 membrane domains (Gao and Dean 2000; Nishikawa *et al*., 2002a). Algorithm based predictions (http://www.cbs.dtu.dk/services/TMHMM/) for *A. nidulans* GmtA indicates the presence of 10 TMDs (Supplementary Fig. S2A) whereas for *A. niger* GmtA 9 TMDs are predicted (Supplementary Fig. S2B). The different number of TMDs implies a different topology of the GMT proteins. Assuming that the cytosolic localization of the C-terminus is conserved, it implies that the N-terminus of the *A. nidulans* protein is cytosolic, whereas the N-terminus of the *A. niger* GmtA is in the lumen of the Golgi. However, we propose that in *A. niger*, as it

has been determined for other NSTs (Eckhardt *et al*., 1999; Gerardy-Schahn *et al*., 2001), the localization of both termini should be in the cytosol side; therefore an uneven number of TMD seems unlikely. It also appears that the algorithm might have failed to predict a TMD between 3 and 4 (Supplementary Fig. S2B) and therefore, we suggest that *A. niger* GmtA, in agreement to what has been predicted for e.g. *A. nidulans* (Supplementary Fig. S2A) and *S. pombe* (data not shown), contains 10 TMDs.

3.3.2. The *gmtA* gene is essential for *A. niger*

In order to study the function of *gmtA* in *A. niger*, we deleted this gene using hygromycin resistance as a selection marker (for details see Materials and Methods) and used the heterokaryon rescue technique (Osmani *et al*., 2006) to show that *gmtA* is an essential gene. After transforming strain MA70.15 with the deletion cassette, no obvious phenotype was observed for the primary transformants obtained (data not shown) and 12 primary transformants were randomly selected for purification. Conidiospores from the primary transformants were transferred onto new selective medium (MM containing hygromycin (note that conidia of *A. niger* are uninucleate)) and the majority of the primary transformants (10/12) formed no colonies after transfer on selective medium. These results indicated that most of the primary Δ*gmtA* transformants were heterokaryons, containing nuclei with the genotype *gmtA*/hygB^{-} and nuclei with the genotype Δ*gmtA*/hygB^{+}. Propagation of the ten Δ*gmtA/gmtA* transformants was only possible when mycelium was transferred (data not shown), suggesting that Δ*gmtA* strains are only viable as heterokaryons and that *gmtA* is an essential gene. Conidia derived from the putative heterokaryons were also plated on MM supplemented with 1.2 M sorbitol; however this osmotic support was not successful in obtaining a *gmtA* deletion strain (data not shown). Southern analysis confirmed the heterokaryotic nature of one of the primary transformants, MA210.1 (Supplementary Fig. S3, lane 2), where signals corresponding to the presence of the *gmtA* allele and the presence of nuclei with a disrupted *gmtA* locus were observed. To prove that the lethal phenotype observed for the Δ*gmtA* strain is due to the absence of this gene, a complementation experiment was performed. To do so, pRen2, an AMA1-based vector (Carvalho *et al*., 2010) containing the *gmtA* ORF and conferring uridine prototrophy, was transformed into the Δ*gmtA* heterokaryotic strain as described in the Materials and Methods section. Putative transformants were selected and purified on MM without uridine but with hygromycin. Southern analysis was performed in one of the transformants (REN2.1) (Supplementary Fig. S3, lane 3), which confirmed the gene deletion and the presence of the complementing gene on the AMA-vector. The difference in band intensities between the complementing plasmid and *gmtA* deletion (Supplementary Fig. S3, lane 3) could suggest that more than one plasmid copy is present per nucleus as previously proposed (Verdoes *et al*., 1994b; Carvalho *et al*., 2010). Growth assays on plate revealed that the lethal *gmtA* deletion phenotype was largely rescued by pRen2 bearing the *gmtA* gene (Fig. 3).

It should be noted that full complementation using an autonomously replicating vector is not always found, due to plasmid instability. Alternatively, as Southern analysis indicated the presence of more than one *gmtA* copy, also overexpression of *gmtA* in the cells may have caused the somewhat aberrant growth phenotype observed.

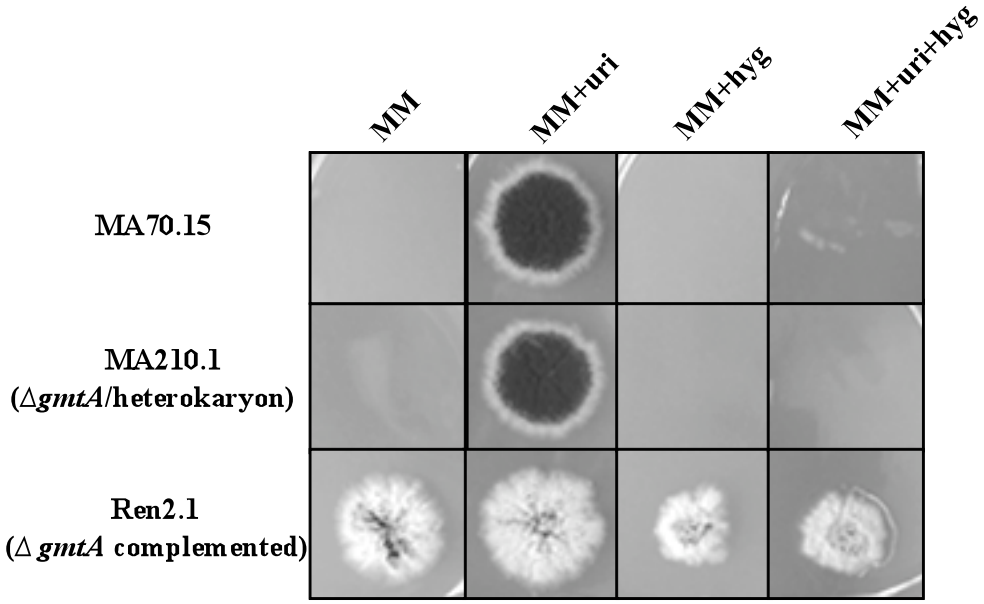

Figure 3. Phenotypic analysis of *ΔgmtA/gmtA* heterokaryons and respective complemented strain. About 10^4 spores were spotted on the different types of media indicated and incubated at 30°C for 3 days. Uri = uridine; hyg = hygromycin.

3.3.3. Disruption of the *srgC* gene in *A. niger* affects morphology and is required for conidiation at high temperatures

Small GTPases of the Ypt/Rab subfamily have been shown to be required at distinct steps during the secretory pathway. In *A. niger* ten members of the Ypt/Rab subfamily have been identified in the genome sequence (Pel *et al*., 2007), and only the function of SrgA (Sec4-orthologue) has been studied in some detail (Punt *et al*., 2001). SrgB (Ypt1-orthologue) is an essential gene and has not been studied further (A.F.J. Ram, unpubl.). In this study, the function of the *srgC* gene was examined in relation to Golgi related functions. SrgC is expected to be involved in the transport of proteins from the Golgi to the vacuole as Ypt6 and Rab6 are the closest homologues of SrgC in *S. cerevisiae* and mammalian cells, respectively (Supplementary Figure 4).

To examine the phenotypic consequences of a null allele of *srgC* in *A. niger*, a *srgC* disruption strain (*ΔsrgC)* was constructed in the wild-type (WT) strain N402. Two types of hygromycin transformants were observed. The majority of transformants grew as WT and a small number of colonies (less than 1%) were slow growing and displayed a compact colony phenotype. Several strains that either grew normally or compact were purified and analyzed by Southern blot, confirming the proper disruption of the *srgC* gene in the transformants with a compact growth phenotype (data not shown). From several *srgC* disruption strains, XW16.1 was selected for further phenotypic analysis. Growth of the XW16.1 strain at different temperatures was strongly reduced at 25ºC, 30ºC and 37ºC. At 42ºC the phenotype was even more pronounced (Fig. 4A). At 37°C and 42ºC the *ΔsrgC* mutant failed to conidiate (Fig. 4A). More detailed microscopical analysis of morphology of the *ΔsrgC* strain was carried out

by inoculating spores in liquid medium followed by growth for 24 hours at different temperatures. The results indicate that normal hyphal extension is severely disturbed with increasing temperatures which resulted in morphologically aberrant germlings (Fig. 4B). The hyphal compartments of the *ΔsrgC* strain, defined as a part of the hyphae that is bordered by two septa, were considerably shorter than in WT (7.7 ± 2.3 µm versus 31.9 ± 3.72 µm). Hyphal compartments of the *ΔsrgC* strain were also wider than those of the WT strain (5.15 ± 0.48 µm versus 3.4 ± 0.48 µm) (Quantified at 30°C). Septum formation itself seems unaffected in the *ΔsrgC* mutant as septa were clearly visible in the *ΔsrgC* strain.

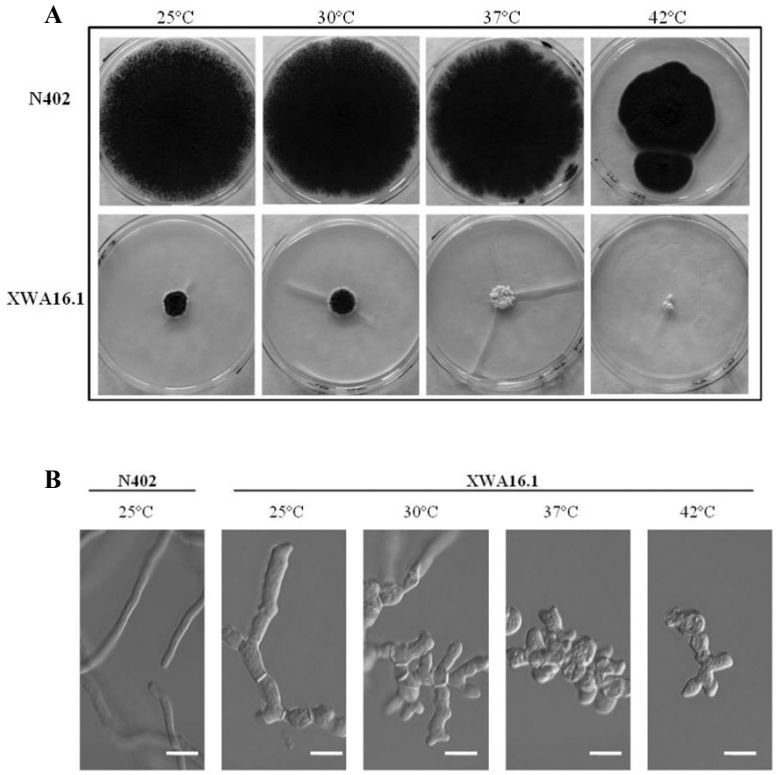

Figure 4. Phenotypic analysis of the *srgC* disruptant mutant (XWA16.1). Spores were spotted on MM agar plates with glucose as a carbon source and incubated 14 days at four different temperatures: 25°C, 30°C, 37°C and 42°C. (A) *A. niger* WT cells (N402) at the upper row and the *srgC* mutant at the lower row. (B) Microscopic analysis of N402 and XWA16.1 strains cultivated for 22h at the temperatures indicated. Scale bar represents 10 µm.

3.3.4. GmtA locates to Golgi equivalents

To determine the intracellular localization of the GmtA protein, a plasmid expressing an YFP labelled wild-type *gmtA* was made as described in Materials and Methods and transformed into MA70.15. Southern analysis confirmed the correct integration at the *A. niger pyrG* locus in the strain Ren1.10 (Supplementary Fig. S5A). To test the functionality of the YFP-GmtA fusion protein, we also deleted the WT *gmtA* gene in Ren1.10 strain and

selected strain MA161.6. Southern analysis confirmed, in this strain (MA161.6), the *gmtA* gene deletion at the WT locus (Supplementary Fig. S5B). This strain bearing the YFP-GmtA fusion protein had no effects on growth or morphology (data not shown) and proved to fully complement the *gmtA* phenotype.

From microscopic analysis we observed that both Ren1.10 (data not shown) and MA161.6 (Fig. 5A) displayed a punctate localization in the hyphae cytoplasm in apical cells. This has been described as Golgi equivalents in other fungi (Nishikawa *et al*., 2002b; Abe *et al*., 2004; Jackson-Hayes *et al*., 2008) using the respective *gmtA* homologues tagged with a fluorescent marker. Additionally, we also observed a gradient of higher intensity of putative Golgi structures towards the tip, a Golgi polarization characteristic that has been shown in other fungi (Rida *et al*., 2006; Hubbard and Kaminskyj, 2008; Pantazopoulou and Peñalva, 2009). In sub-apical cells, the YFP-GmtA fluorescent signal was observed in vacuoles (Fig. 5A). From these observations, we infer that in *A. niger* GmtA localization represents Golgi structures in *A. niger* in actively growing tips cells, and that YFP-GmtA relocates in non actively growing cells to the vacuoles.

3.3.5. Disruption of *A. niger srgC* affects Golgi equivalents formation

To elucidate the role of the Ras GTPase SrgC in GmtA localization and overall Golgi function we disrupted *srgC* in the *A. niger* strain expressing the YFP-GmtA fusion protein (Ren1.10 strain). As observed for the disruption of *srgC* in the N402 background, disruption of *srgC* in the Ren1.10 strain resulted in the formation of compact and slow growing colonies and Southern analysis was performed to verify proper disruption of the *srgC* gene in the YFP-GmtA expression strain (data not shown). Microscopy analysis revealed that the punctate structures representing Golgi-structures in the WT strain (Fig. 5A) were lost in the *srgC* mutant (Fig. 5B), which suggests that this integrity of the Golgi-like structures is affected by the *srgC* disruption (strain MA160.1). We have also tagged the ER of *A. niger* by making a strain (MA141.1) expressing the fusion protein GlaA::GFP containing the C-terminal tetrapeptide HDEL. This fusion construct directed GFP to a tubular network within the hyphae (Fig. 5C). In strain MA160.1 (*ΔsrgC*), instead of the punctate structures, the pattern of YPF-fluorescence resembles ER-like structures (Fig. 5B and C).

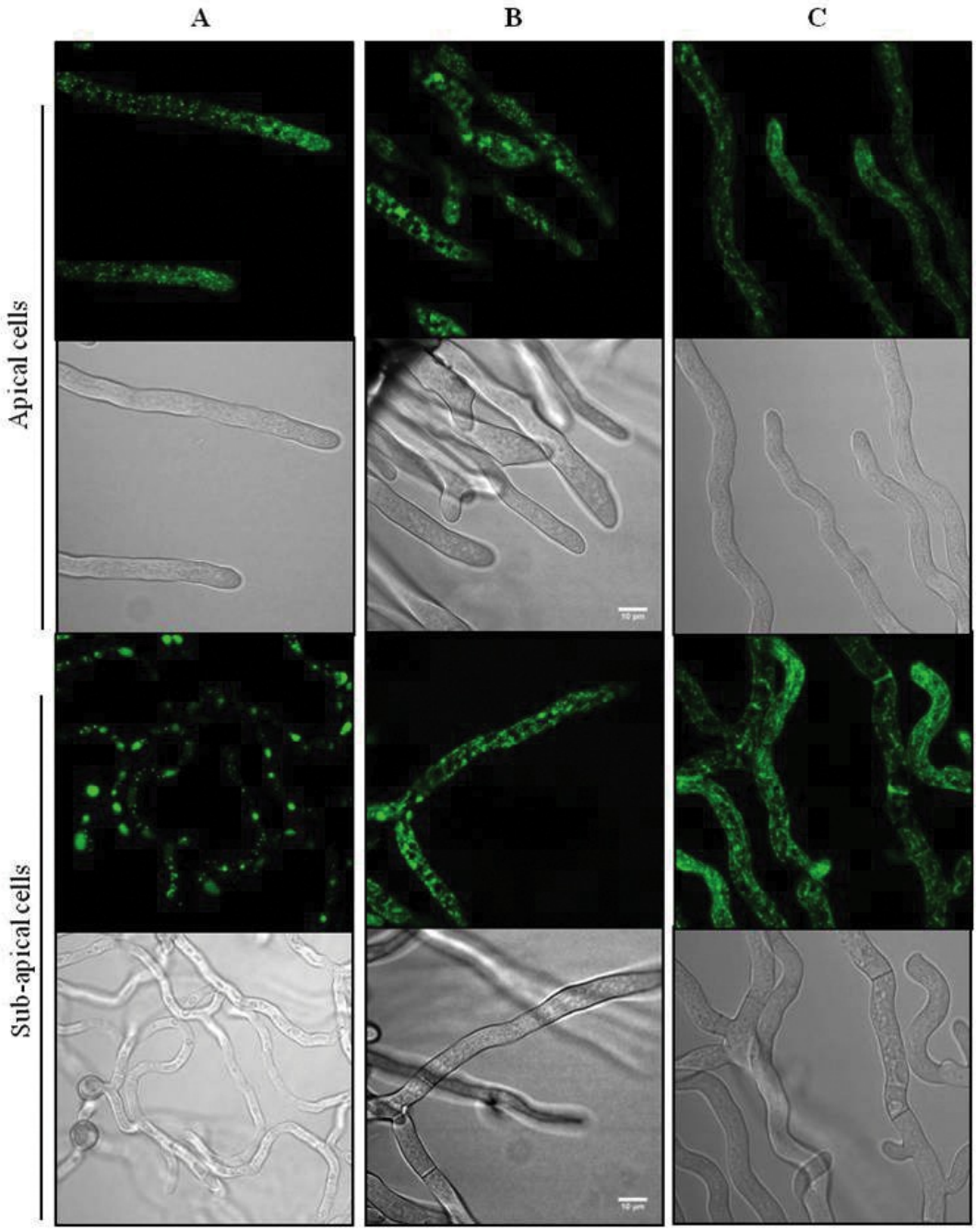

Figure 5. Subcellular localization of the GmtA in *Aspergillus niger*. (A) Strain MA161.6 bearing the YFP-GmtA reporter construct showing a punctate distribution typical of Golgi equivalents; (B) strain MA160.1 bearing the YFP-GmtA reporter construct and the *srgC* disruption showing a disappearance of the punctate Golgi pattern and localization of YFP-GmtA at structures resembling the ER as observed in strain MA141.1 carrying GlaA2::sGFP-HDEL as an ER marker (C).

3.4. Discussion

This report describes the identification and characterization of a putative GDP-mannose transporter, GmtA, in *A. niger*. Within the *A. niger* genome, 14 putative NSTs were identified (Fig. 1). Among them, we find a single gene copy that codes a putative GDP-mannose transporter. Interestingly, some clusters seem to lack representative genes in *S. cerevisiae* (some transporters with no subfamily attributed yet and CMP-Sialate:CMP Antiporter (CSA)) and might represent specific processes that occur in filamentous fungi and *S. pombe*, but are absent in *S. cerevisiae*. Moreover, our analysis (Fig. 1) also shows that some NSTs are unique to filamentous fungi, like the recently identified GlfB protein which has been recognized as an UDP-galactofuranose specific transporter (Gal*f*) (Engel *et al*., 2009). Galactofuranosylation, a protein and lipid modification that is absent in Ascomycete yeasts, is important as these modifications have been shown to affect growth, virulence and pathogenicity of fungi (Varki, 1993; Schmalhorst *et al*., 2008). Phylogenetic analysis of putative GDP-mannose transporters from different fungi revealed that despite function conservation of *gmtA* orthologue genes, there is a divergence between the Ascomycota and Basidiomycota phylums (Supplementary Fig. S1). Furthermore, among the Ascomycota there is also divergence that results in the clustering of species belonging to the classes Euromycetes (*Aspergilli*), Sordariomycetes (e.g. *Neurospora crassa* and *Magnaporthe grisea*) and Saccharomycetes (e.g. *S. cerevisiae* and *Candida sp.*). Worthy to note is the presence of a GmtA orthologue in the homobasidiomycete *Schizophyllum commune* despite the fact that Golgi mannosyltransferases, as well as other enzymes involved in glycosylation reactions in this organelle, are absent in this and other homobasidiomycetes (Berends *et al*., 2009). Unlike *Aspergillus nidulans* (Jackson-Hayes *et al*., 2008), in all other *Aspergillus* species, including *A. niger*, only one *gmtA* copy in the genome is present. Interesting to notice is the separation of the two Gmt proteins of *Aspergillus nidulans* (Supplementary Fig. S1), as GmtB clusters with the Sordariomycetes. Jackson-Hayes and co-works (2010) have shown that GmtA and GmtB co-localize at Golgi equivalents, although they perform distinct functions at different steps of development. All predicted GDP-mannose transporters, including the *A. niger* GmtA protein (Fig. 2), contain the GALNK motif which has been shown to be required for binding GDP-mannose (Gao *et al*., 2001).

Our results show that *gmtA* is an essential gene, similar to what has been shown in *S. cerevisiae* and *Candida albicans* (Poster and Dean, 1996; Nishikawa *et al*., 2002a). This result implies that, as described for other species and contrary to *Aspergillus nidulans* (Jackson-Hayes *et al*., 2008) and *Cryptococcus neoformans* (Cottrell *et al*., 2007), which contain two *gmt* genes able to complement each other, in *A. niger* there is no other functionally redundant gene besides *gmtA* that encodes a GDP-mannose transporter. The complementation of the lethal Δ*gmtA* phenotype with an autonomously replicating plasmid containing the WT *gmtA* gene was successful (Fig. 3). Although Hashimoto *et al*. (2002) have shown that in *S. cerevisiae* overexpression of *VRG4* did not have a significant effect on cell growth or GDP-transport activity, in *A. niger* overexpression of *gmtA* (suggested by the presence of multicopies of the plasmid (Supplementary Fig. S3, lane 3)) seems to affect fungal growth. However, at this point one cannot rule out that the expression of GmtA from the AMA-based plasmid on its own might result in a growth phenotype.

The ability of GFP fusions to monitor organelles and protein trafficking has been demonstrated in numerous systems (March *et al*., 2003). Several studies in fungal species have demonstrated that the cellular localization of the GDP-mannose transporter is the Golgi or at least its functional equivalent (Nishikawa *et al*., 2002b; Abe *et al*., 2004; Jackson-Hayes *et al*., 2008). Deletion of *gmtA* in a the *A. niger* strain expressing the YFP::GmtA had no effects on growth or morphology, demonstrating that the fluorescent tagged GmtA was fully functional as it was able to fully complement the lethal phenotype of the *gmtA* deletion strain. In this strain, GmtA localization was monitored by fluorescent microscopy revealing the YFP::GmtA protein localized in punctate spots (Fig. 5A). Our results are consistent with what has already been illustrated for yeast and filamentous fungi as a typical GDP-mannose transporter, and other Golgi markers, punctate cellular distribution (Dean *et al*., 1997; Gao and Dean, 2000; Nishikawa *et al*., 2002b; Abe *et al*., 2004; Arakawa *et al*., 2006; Breakspear *et al*., 2007; Jackson-Hayes *et al*., 2008; Pantazopoulou and Peñalva, 2009). The identification of a putative lysine cluster in the C-terminal region (Fig. 2, indicated with **:**) which has been implicated as responsible for location in the Golgi (Abe *et al*., 2004) led us to create a YFP tagged GmtA construct in which the fluorescent protein located in the N-terminal, in order not to disturb this potential Golgi retention site. However, unlike what has been described for GmtA-GFP fusions (C-terminal tagging) in *A. nidulans* (Jackson-Hayes *et al*., 2008), we did not observe any ring-shaped structures and the different localization of the GFP tag might be responsible for the observed difference.

In this study we analyzed the function of one of the secretion related small GTPases (SrgC) in the secretory pathway related to the Golgi compartment, by disruption of the *srgC* gene in the YFP-GmtA reporter strain (Ren1.10). *A. niger* SrgC belongs to the Ypt6p/Rab6p subfamily and the yeast/mammalian orthologues (Supplementary Figure 4), have been shown to act in endosome-to-Golgi and intra-Golgi retrograde transport steps and mutations in the *YPT6/RAB6* genes result in defect in vacuolar biogenesis and aberrant vacuolar morphogenesis (Tsukada and Gallwitz, 1996; Li and Warner, 1996; Mayer *et al*., 1996; Luo and Gallwitz, 2003). Loss of function of the *srgC* gene in *A. niger* resulted in strongly reduced growth (Fig. 4A) and morphologically, this strain was characterized by the presence of short, thickened hyphal compartments and irregular branching patterns (Fig. 4B) and the lack of mature vacuoles (not shown). In *S. cerevisiae*, deletion of *YPT6* has also an effect on vacuolar morphology; in the *ypt6* mutant, several small vacuoles instead of a single organelle found in WT cells were found (Tsukada and Gallwitz 1996). Golgins are proteins with a role in a variety of membrane-membrane and membrane-cytoskeleton tethering events at the Golgi apparatus that contribute to its organization, architecture and function. These events are all regulated by small GTPases of the Rab and Arl families (reviewed in Ramirez and Lowe, 2009). With the disruption of *srgC* in our Golgi reporter strain (MA160.1), the punctate distribution typical for the fungal Golgi-equivalent in WT cells (Fig. 5A) was no longer observed. Instead, GmtA seems to localize in a more tubular network-like structure within the cell (Fig. 5B), which resembles the network that has been described for the ER has been previously described (Khalaj *et al*., 2001; Fig. 5C). However, further studies are needed to determine GmtA localization under *ΔsrgC* conditions. Fridmann-Sirkis and co-workers (2004) have shown that the mammalian golgin TMF (Sgm1 in *S. cerevisiae*) binds to Rab6 and contributes to the organization of the Golgi. In that study the authors showed that the

reduction of the TMF levels with RNAi treatment leads to the mislocalization of the Golgi throughout the cells as well as morphological changes in its structure (Fridmann-Sirkis *et al*., 2004). As Rab6 is responsible to recruit TMF and the absence of this protein influences the morphology of the Golgi, it is tempting to speculate that the opposite scenario would have the same effect: the absence of *srgC* (the homologue of Rab6) would fail to recruit an TMF homologue in *A. niger* and have similar effects as those reported by Fridmann-Sirkis *et al*. (2004) and also observed in our study. Besides TMF, Rab6 has been shown to recruit another golgin, bicauldal-D, that has been suggested to mediate the movement of Golgi membranes along microtubules by binding to dynactin (Short *et al*., 2002). We propose that in *A. niger* SrgC is also a protein with multiple functions playing key roles in the secretory pathway. Thus, the putative interference with golgins recruitment, together with the disruption of endosome recycling and proper vesicle traffic affects the maintenance and steady-state distribution/organization of the Golgi equivalents in *A. niger*, explaining the distinctive fluorescent patterns observed between the WT MA161.6 (Fig. 5A) and the *ΔsrgC* MA160.1 (Fig. 5B) strains.

Chapter 4

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

Neuza Carvalho, Mark Arentshorst, Rolf Kooistra, Hein Stam, Cees Sagt, Cees van den Hondel & Arthur Ram

Appl Microbiol Biotechnol. (2011) **89**:357-73

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.

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	Δ*kusA*::*amdS*$^{+}$ in AB4.1 *pyrG*$^{-}$	-	Meyer *et al*., 2007
MA78.6	Δ*kusA*::*amdS*$^{+}$ in N402	-	Carvalho *et al*., 2010
NC5	FAA-resistant derivative from MA78.6Δ*kusA*, *amdS*$^{-}$)	-	Carvalho *et al*., 2010
MA97.2	Δ*derA::amdS* in NC5	-	this study
MA98.1	Δ*doaA::amdS* in NC5	-	this study
MA94.3	Δ*hrdC::amdS* in NC5	-	this study
MA95.9	Δ*mifA::amdS* in NC5	-	this study
MA96.6	Δ*mnsA::amdS* in NC5	-	this study
MV3.2	pBB19-3*pyrG** in MA70.15 (Δ*kusA*::*amdS*$^{+}$*)*	pGpdA-Gla514-Gus-*pyrG**	this study
MA99.3	FAA-resistant derivative from MV3.2	pGpdA-Gla514-Gus-*pyrG**	this study
MA110.1	Δ*derA::amdS* in MA99.3	pGpdA-Gla514-Gus-*pyrG**	this study
MA111.3	Δ*doaA::amdS* in MA99.3	pGpdA-Gla514-Gus-*pyrG**	this study
MA112.10	Δ*hrdC::amdS* in MA99.3	pGpdA-Gla514-Gus-*pyrG**	this study
MA113.2	Δ*mifA::amdS* in MA99.3	pGpdA-Gla514-Gus-*pyrG**	this study
MA114.7	Δ*mnsA::amdS* in MA99.3	pGpdA-Gla514-Gus-*pyrG**	this study
MA115.1	Δ*derA* FAA-resistant derivative from MA97.2	-	this study
MA116.2	Δ*hrdC* FAA-resistant derivative from MA94.3	-	this study
MA117.1	Δ*mifA* FAA-resistant derivative from MA95.9	-	this study
MA118.2	Δ*mnsA* FAA-resistant derivative from MA96.6	-	this study
MA119.1	Δ*hrdC,* Δ*derA::amdS* (Δ*derA::amdS* in MA116.2	-	this study
MA120.1	Δ*hrdC,* Δ*doaA::amdS* (Δ*doaA::amdS* in MA116.2	-	this study
MA122.4	Δ*hrdC,* Δ*mnsA::amdS* (Δ*mnsA::amdS* in MA116.2	-	this study
MA123.7	Δ*mifA,* Δ*derA::amdS* (Δ*derA::amdS* in MA117.1	-	this study
MA124.2	Δ*mifA,* Δ*doaA::amdS* (Δ*doaA::amdS* in MA117.1	-	this study
MA125.1	Δ*mifA,* Δ*hrdC::amdS* (Δ*hrdC::amdS* in MA117.1	-	this study
MA127.3	Δ*mnsA,* Δ*derA::amdS* (Δ*derA::amdS* in MA118.2	-	this study
MA128.1	Δ*mnsA,* Δ*doaA::amdS* (Δ*derA::amdS* in MA118.2	-	this study
MA130.3	Δ*mnsA,* Δ*mifA::amdS* (Δ*mifA::amdS* in MA118.2	-	this study
MA131.1	Δ*derA,* Δ*doaA::amdS* (Δ*doaA::amdS* in MA115.1	-	this study
AB4-1dglaA36#3	[pBB19-3]#3 multicopy transformant	Multicopy pGpdA-Gla_{514}-Gus	Punt *et al*,. 1994, 1998
MA134.64	Δ*kusA*::*amdS*$^{+}$ in AB4-1dglaA36#3 (Δ*ku70*, *amdS*$^{-}$)	Multicopy pGpdA-Gla_{514}-Gus	this study
MA135.3	FAA-resistant derivative from MA134.64	Multicopy pGpdA-Gla_{514}-Gus	this study
MA136.18	Δ*derA::amdS* in MA135.3	Multicopy pGpdA-Gla_{514}-Gus	this study
MA137.2	Δ*doaA::amdS* in MA135.3	Multicopy pGpdA-Gla_{514}-Gus	this study
MA139.6	Δ*mifA::amdS* in MA135.3	Multicopy pGpdA-Gla_{514}-Gus	this study
MA140.8	Δ*mnsA::amdS* in MA135.3	Multicopy pGpdA-Gla_{514}-Gus	this study
AB1.13#72	[phIL6-3A]#72pAN7-1	IL6 (P*gpd*A)	Broekhuijsen *et al.* 1993
AB1.13#54	[pAN56-3hIL6]#54pAN7-1	GLA::IL6 (P*gpd*A)	Punt *et al*., 1998
AB1.13#38	[pAN56-4hIL6]#38pAN7-1	GLA::kex::IL6 (P*gpd*A)	Punt *et al*., 1998
D15	[pGpdA-GlaA::tPA]#25	GLA::kex::tPA (P*gpd*A)	Wiebe *et al*., 2001
MGG029#25	[pGlaA-MNP1.i]#25	MnP1 from *Phanerochaete chrysosporium*	Conesa *et al*., 2000
MGG029#13	[pGlaA-GlaA::MNP1]#13	Mnp1 from *P. chrysosporium* expressed as GlaA fusion protein	Conesa *et al*., 2000
B36	[pAB6-10]#36	Contain over 80 copies of the Glucoamylase gene	Verdoes *et al*., 1993
AR1.1	[pPgla-Gla_{514}::GFP]	Glucoamylase-GFP	Gordon *et al*., 2000a
XW2.2.1	[pPgla-Gla_{514}::GFP-HDEL]	Glucoamylase-GFP fusion with ER targeting sequence	Gordon *et al*., 2000a
MA23.1.1	[pPgpd-CPY_{31}::GFP]	CpyA- GFP fusion expressed from *gpdA* promoter	Weenink and Ram, unpublished
NW5.1	[pPgpd-CwpA::GFP]	CwpA-GFP from *A. niger*	Damveld and Ram, unpublished
XW5.2	[pPgla-Gla_{514}::POX2]	Laccase from *Pleurotus ostreatus*	Weenink *et al*., 2006
XW6.1	[pPgla-Gla_{514}]	-	Weenink *et al*., 2006

IL6-interleukine 6; tPA-tissue plasminogen activator; MnP1-manganese peroxide; 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 KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO_3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4, 174 nM 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 $NaNO_3$ 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 Russel 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 $1x10^4$ 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 1×10^5 until 1×10^1) 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 *Asc*I 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 pJet*PyrG*Asc*I. Finally, the *pyrG** fragment was isolated with *Asc*I and cloned into the unique *Asc*I site in pBB19-3 to give pBB19-3*pyrG**. 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 2×10^7 spores on MM plates containing 1% (w/v) of glucose as a carbon source, without $NaNO_3$ 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.

Table 2. Primers used throughout this study. Restriction enzymes added are underlined.

Primer name	Sequence (5' to 3')	Amplification of	Restriction Enzyme
pDER9Eco	gcgaattctgcaccccactggggcatttactgc	derA 3' flank	*Eco*RI
pDER10Hin	gcaagctttaatcccgcacaagaagatacc	derA 3' flank	*Hind*III
pFDERMB	gcaacgcgttgcaaaggatcctccgcgtaatcgctc	derA 3'' flank	*Bam*HI
pRDERKpn	tcggtacctcggatgaggtcagagcatgctttaatc	derA 3'' flank	*Kpn*I
pDER3Not	tggcggccgcggtacgcacgctgaacgtcg	derA 5' flank	*Not*I
pDER4Bam	ggggatccttgatgggtagtagagttgcga	derA 5' flank	*Bam*HI
pdoaHinb	ctgatcgctaagcttttgcaagagctgaaccaacacgtc	doaA 3' flank	*Hind*III
pdoaAsc	gcaaggcgcgcctacgtagagaatgaaggtcaaagtg	doaA 3' flank	*Asc*I
pdoaEco	cgtagaagattgttgaattcctgaacaatggc	doaA 5' flank	*Eco*RI
pdoaHina	agaagcttagatcttgaactcaggcatacatagaccag	doaA 5' flank	*Hind*III
pdoaNot	cgatagtagcggccgcaatgtgaagtgacgataaaggtg	doa 5'' flank	*Not*I
pdoaMlu	catacgcgtggccctccaaagaagcggagatcttgaactc	doaA 5'' flank	*Mlu*I
pHRD7Not	ttgcggccgcagcctgcaggtcgatccccttc	hrdC 5' flank	*Not*I
pHRD8Mlu	tacgcgtcggaaggcttcttgggcgtaatg	hrdC 5' flank	*Mlu*I
phrdhin	caagctttgctgcggaatgcagcgctggctcttatc	hrdC 3' flank	*Hind*III
phrdasc	gggcgcgccccttgatatgcaatgggaatggattgtg	hrdC 3' flank	*Asc*I
phrdMlu	gacgcgttttgctgcggaatgcagcgctggctcttatc	hrdC 3'' flank	*Mlu*I
phrdKpn	catggtacccgtcttcgcggtggtcgtcaaggcg	hrdC 3'' flank	*Kpn*I
pmifNot	cacgcggccgctgatcacggaatcggatcaaccgaggaagc	mifA 5' flank	*Not*I
pmifXmaa	ggcccggggttacctgaagctccccgcggcattggagcag	mifA 5' flank	*Xma*I
pmifEco	ccgaattccgcaccagggcaggctcctctgtaccttctc	mifA 3' flank	*Eco*RI
pmifAsc	aaggcgcgccgcagtagatatatgttgcgctaatagactaag	mifA 3' flank	*Asc*I
pmifXmab	cacccgggcaggctcctctgtaccttctctcgtcaccaaac	mifA 3'' flank	*Xma*I
pmifKpn	aaggtaccttgcccagttgactgcgtgccaggtggtgc	mifA 3'' flank	*Kpn*I
pmnsNot	tagcggccgcccacccccaatctacttatgctcatataatg	mnsA 5' flank	*Not*I
pmnsXmaa	gcggtccccggggaggggattgttcagggagttggag	mnsA 5' flank	*Xma*I
pmnsEco	caccgaattcaatgtcgacgaccctcgcgtcatggaaacagac	mnsA 3' flank	*EcoR*I
pmnsAsc	gcggcgcgccacgacgtgtatatataacgaggaaacg	mnsA 3' flank	*Asc*I
pmnsXmab	cacccgggatcacctacttcaatgtcgacgaccctcgcg	mnsA 3'' flank	*Xma*I
pmnsKpn	gcggtaccgcctccgtattgaatacatggtcttcg	mnsA 3'' flank	*Kpn*I
pNC43	ggcgcgcctcggtcgctcactgttcct	*pyrG**	*Asc*I
pNC44	ggcgcgccgacggagtagccgagagcaa	*pyrG**	*Asc*I

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 P*gpdA* 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 *Not*I and *Asc*I 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 chemiluminescence kit (Bio-Rad), according to manufacturer's instructions. The Gus-antibody was kindly provided by Prof. P. Punt (TNO,

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).

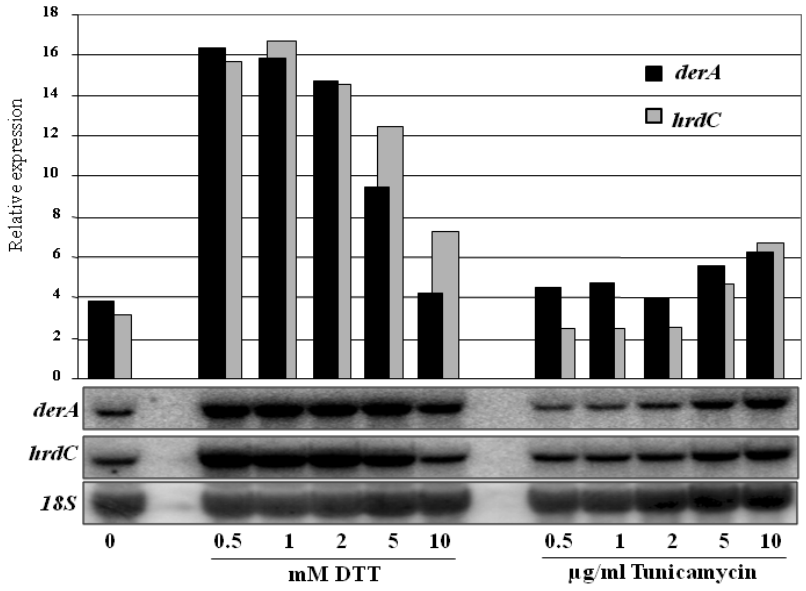

Figure 1. Induction of two genes involved in the ERAD pathway (*derA* and *hrdC*) by the presence of increasing concentrations of DTT or tunicamycin stress agents. Samples for Northern analysis were collect after 16h growth on liquid CM (1% glucose) at 30°C. On the Y-axis is the relative expression of *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.

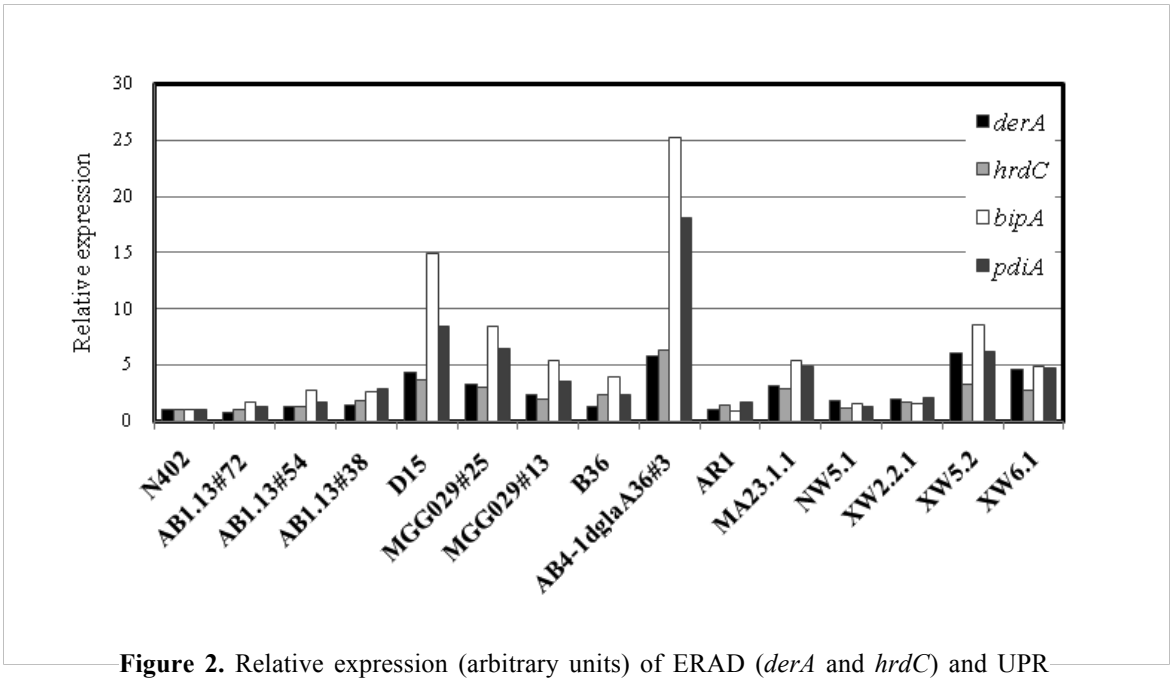

Figure 2. Relative expression (arbitrary units) of ERAD (*derA* and *hrdC*) and UPR (*bipA* and *pdiA*) reporter genes in strains expressing different heterologous proteins (see Table 1 for details). Samples for Northern blot analysis were collected from these strains grown for 16h at 30°C in liquid 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 (pBB19-3*pyrG*)* 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).

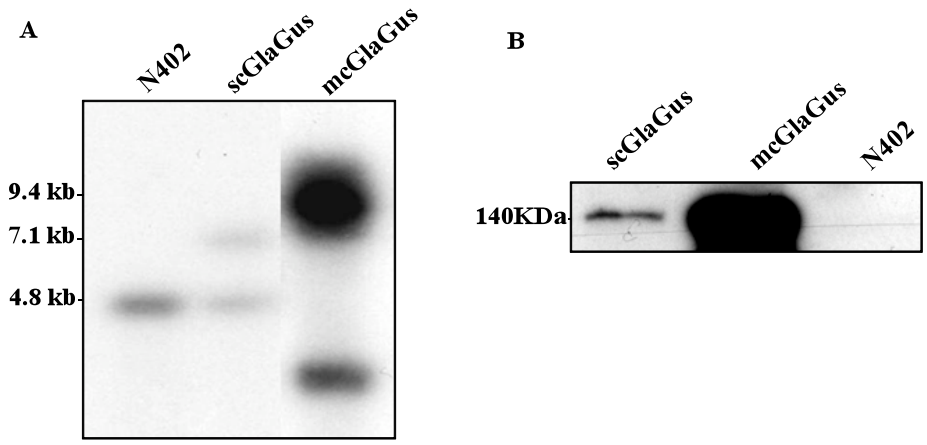

Figure 3. (A) Southern blot analysis of the GlaGus copy number in mcGlaGus strain. Genomic DNA was digested with *Nco*I and probed with a probe annealing within the glucoamylase ORF. Expected band size for endogenous glucoamylase is 4.8 kb; for the scGlaGus strain 7.1 kb and 4.8 kb bands are expected. 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.

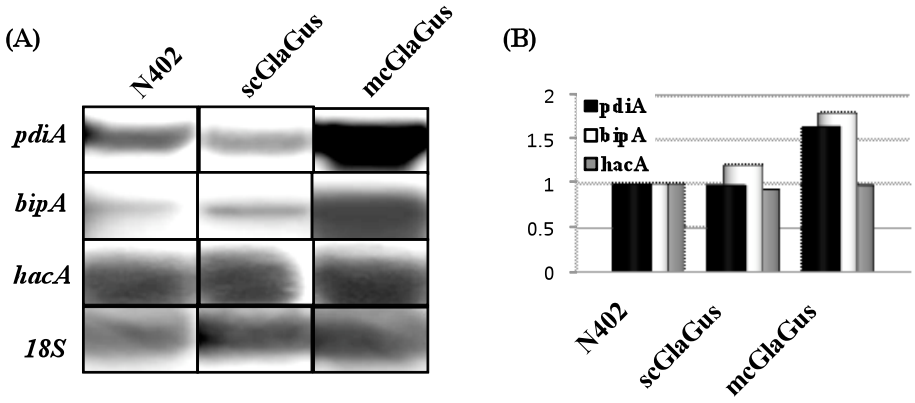

Figure 4. (A) Northern blot analysis of mRNA levels of UPR target genes on strains containing either a single copy or multi copy GlaGus genes in comparison to N402. Total RNA was extracted from mycelia grown for 24h at 30°C in CM. (B) The UPR target genes expression levels were normalized using N402 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; Δ*kusA*, *amdS*⁻), the scGlaGus strain (MA99.3; Δ*kusA, amdS*⁻*, scpBB19-3pyrG*)* and the mcGlaGus strain (MA135.3 (Δ*kusA, 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).

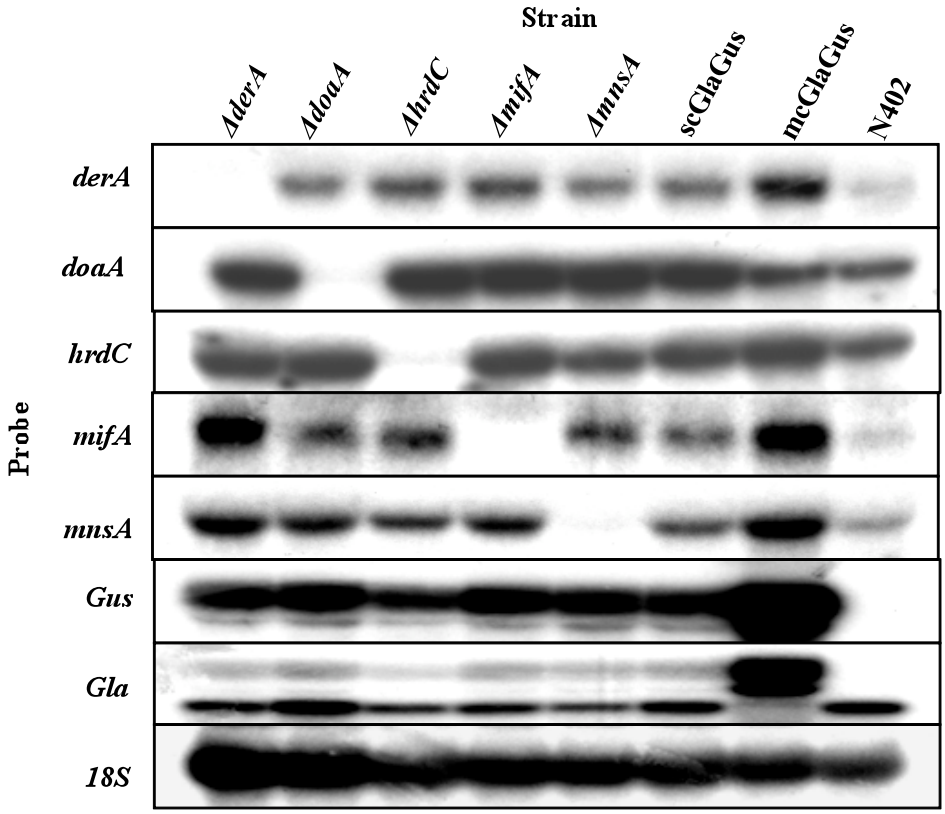

Figure 5. Expression analysis of different genes in the five ERAD deletion strains on the scGlaGus background, scGlaGus parental strain, mcGlaGus and N402. Total RNA was extracted from mycelia grown for 24h at 30°C in CM. RNA (5µg) was separated by agarose gel electrophoresis, blotted and hybridized with ^{32}P-labeled probes specific for the genes indicated. 18S rRNA was used as loading control.

The morphological and growth effects of the disruption of these ERAD genes in the 14 *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*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*derA* shows a more apparent growth defect than the other deletions.

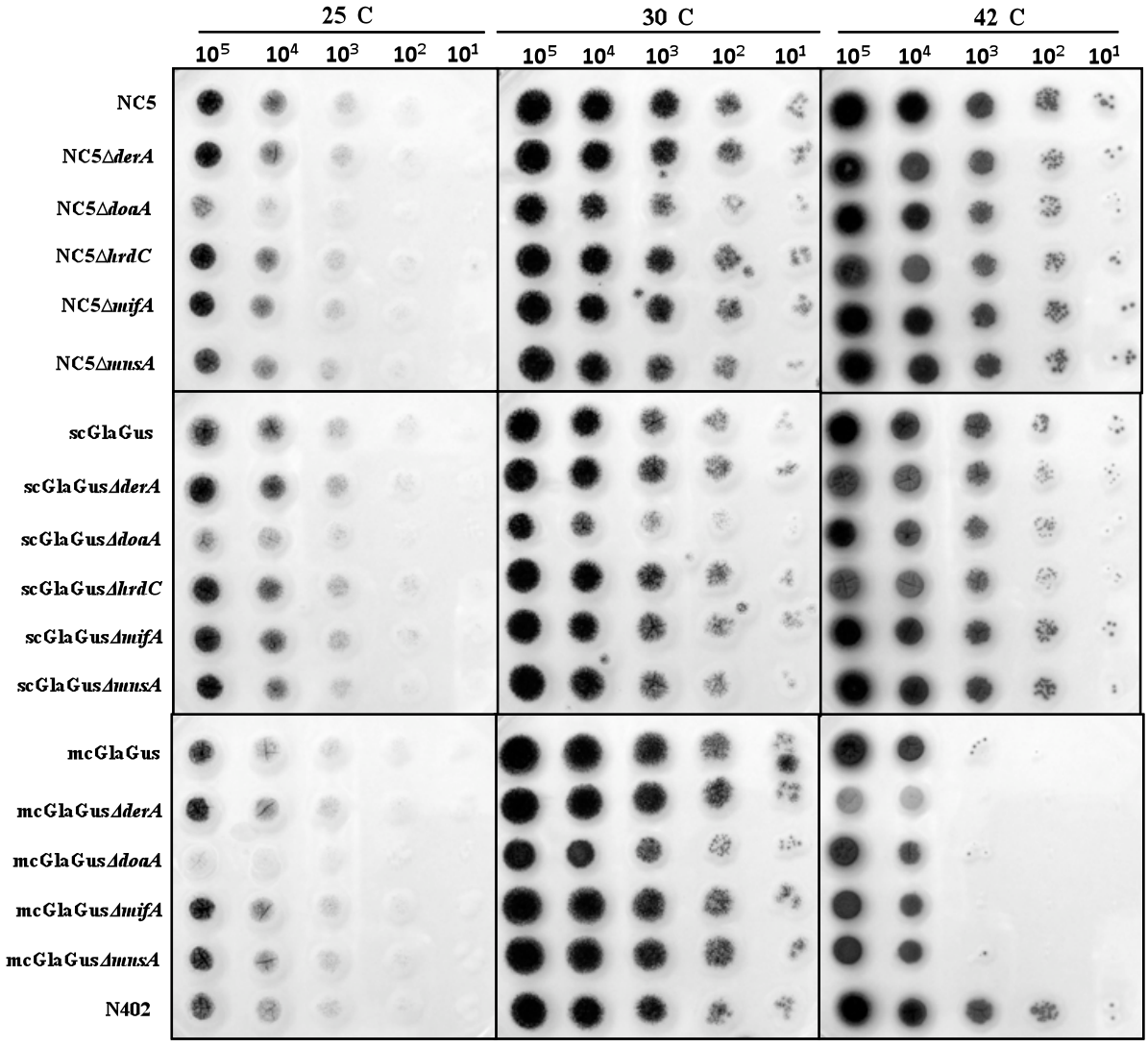

Figure 6. Growth assay at different temperatures of parental strains NC5, scGlaGus and mcGlaGus and respective ERAD deletions. Spore serial dilutions were spotted onto solid MM and incubated under the given conditions. Growth was monitored for 3 days.

Subsequently, the sensitivity of the ΔERAD 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 Δ*doa*A mutant was affected in the absence of DTT, but the Δ*doa*A 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 Δstrains becomes more resistant towards DTT. As also observed in Fig. 6, the mcGlaGusΔ*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.

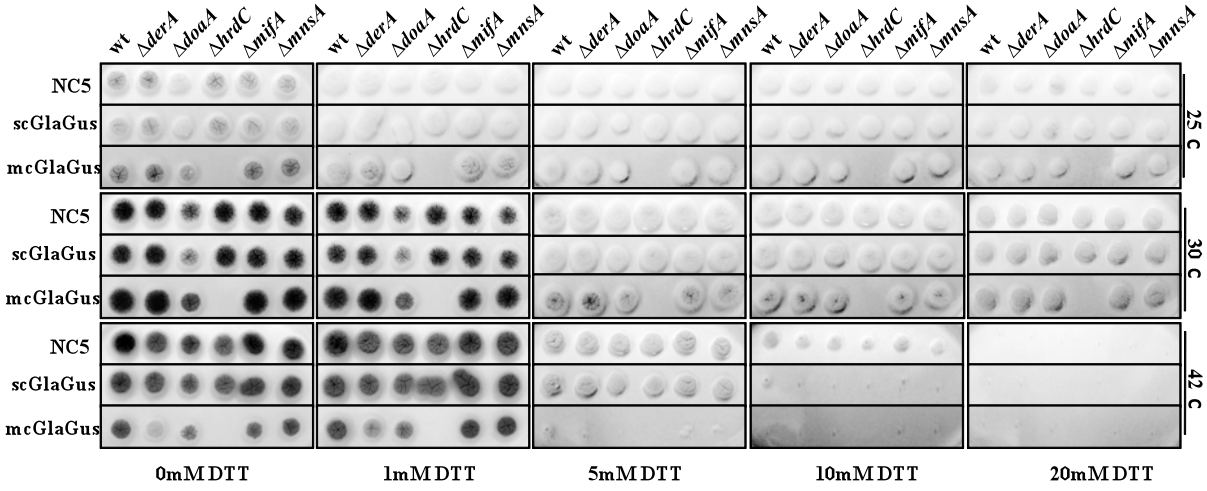

Figure 7. Comparison of colony morphology of parental strains and respective ERAD deletion mutants incubated at different temperatures (indicated on the right) and in the presence of increasing concentrations of the stress agent DTT (indicated at the bottom). 10^4 spores per 10 µl of each strain were 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 Δ*doaA*) 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 *amdS* marker used to disrupt the first ERAD gene. Then, the ERAD deletion constructs were transformed into these new 5 ERAD deletions (*amdS*$^-$) 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 Δ*doaA*, Δ*mifA* and Δ*mnsA* 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.

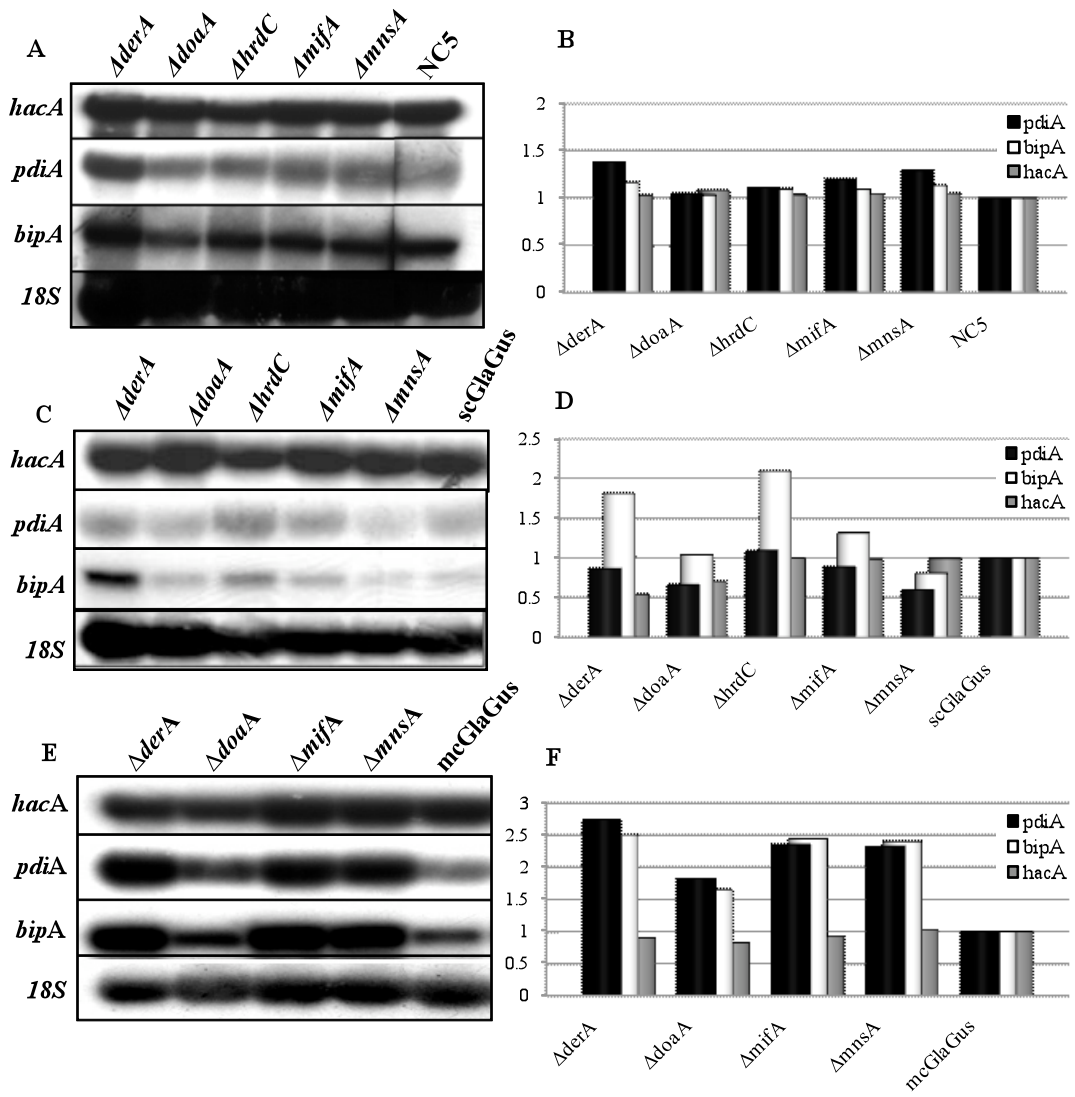

Figure 8. Northern blot analysis of ERAD deletion strains in NC5 (A), scGlaGus (C) and mcGlaGus (E). Total RNA was extracted from mycelia grown for 24h at 30°C in CM. RNA (5µg) was separated, blotted and hybridized with ^{32}P-labeled probes specific for the genes indicated. (B, D, F) Quantification of the mRNA expression levels of *hacA*, *pdiA* and *bipA*. Signals were corrected for loading differences using 18S. The UPR target genes expression levels were then normalized using the respective parental strain values as reference.

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.

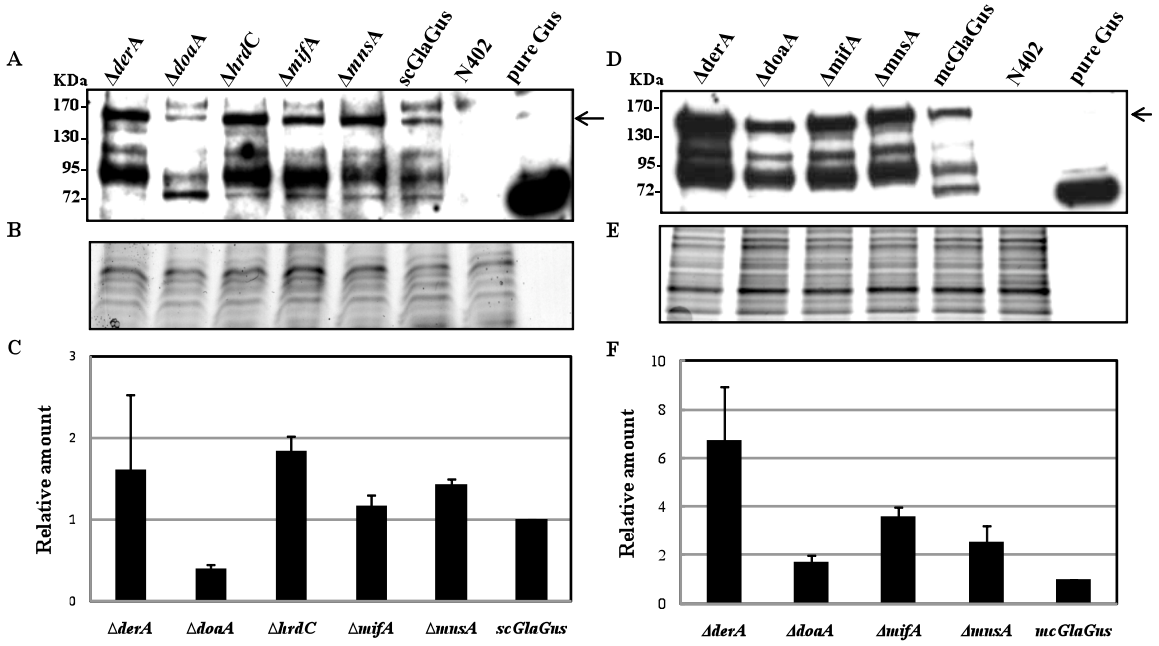

Figure 9. Effect of deletion of ERAD components on the amount of GlaGus fusion protein in total protein extracts. Western analysis of GlaGus amounts in total protein of mycelium samples of scGlaGus (A) and mcGlaGus (D) ERAD deletion strains. Samples were grown in CM for 24h at 30°C. 10µg of total protein 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; Broekhuijsen *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 42ºC suggesting that the temperature stress (42ºC 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 42ºC, 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

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 *ΔdoaA* 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.

Chapter 5

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

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(Manuscript in preparation)

Abstract

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

5.1. Introduction

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

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

or mammalian homologues, in addition to the intron splicing, the *hacA* mRNA of *A. niger*, *Aspergillus nidulans* and *Trichoderma reesei* is truncated at the 5'-end during UPR induction (Saloheimo *et al*., 2004; Mulder *et al*., 2006). However, Mulder and Nikolaev (2009) showed that in *A. niger* truncation of *hacA* is not a requirement for induction of the pathway. Once translated, HacAp migrates into the nucleus where it binds to palindromic UPR elements at the promoter regions of UPR targets (Mulder *et al*., 2006).

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

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

In this study, we present a genome-wide overview of the HacA responsive genes by comparing the transcriptomic profiles of *A. niger* strain expressing the wild-type *hacA* gene with a genetically engineered *A. niger* strain that expresses only the activated form of the HacA transcription factor. The comparison revealed that HacA is a master regulator, coordinating several processes within the secretory pathway such as the induction of protein folding, protein glycosylation and intracellular transport. Additionally, we discovered that constitutive activation of HacA results in the down regulation of the AmyR transcription factor and the AmyR regulon, which includes the most abundantly produced extracellular

glycoproteins, thereby reducing import of new proteins into the ER. The downregulation of the AmyR regulon revealed by the genome wide expression analysis was phenotypically confirmed as the $HacA^{CA}$ mutant displayed a strongly reduced growth phenotype on starch plates.

5.2. Material and Methods

5.2.1. Strains and culture conditions

Aspergillus niger strains used throughout study (Table 1) were cultivated in minimal medium (MM) (Bennett and Lasure, 1991) containing 1% (w/v) of glucose (or other as indicated) as a carbon source, 7 mM KCl, 11 mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO_3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4, 174 nM EDTA; or in complete medium (CM) containing, in addition to MM, 0.1% (w/v) casamino acids and 0.5% (w/v) yeast extract. When required, 10 mM uridine was added. The glucose minimal medium used for bioreactor cultivations has been described previously (Jorgensen *et al*., 2010).

Table 1. *Aspergillus niger* strains used in this study.

Strain	Genotype	Reference
N402	*cspA1 derivative of ATCC9029*	Bos *et al., 1988*
MA70.15	*ΔkusA::amdS*$^{+}$ *in AB4.1 pyrG*$^{-}$	Meyer *et al., 2007*
NC1.1	Wild type *hacA* in MA70.15, *pyrG*$^{+}$	This study
NC2.1	Constitutive active *hacA* in MA70.15, *pyrG*$^{+}$	This study
YvdM1.1	Δ*amy*R *in AB4.1 pyrG*$^{+}$	Yuan *et al*., 2008a
XY3.1	Δ*inu*R *in AB4.1 pyrG*$^{+}$	Yuan *et al*., 2008b

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

To replace to endogenous *hacA* gene on the *hacA* locus with a constitutive activated allele of the *hacA* gene, a replacement cassette was constructed. As a control, a similar replacement cassette was made with the wild-type *hacA* gene. To construct the *hacA* reference strain, three PCR fragments consisting of the *hacA* gene including promoter and terminator regions, the *Aspergillus oryzae pyrG* selection marker and a *hacA* terminator region were cloned into pBluescript-SK. Subsequently, this plasmid was used as template to introduce the mutations that led to a constitutive active *hacA* allele by site directed mutagenesis (according to Quick Change II site directed mutagenesis protocol, Stratagene). To construct the wild-type *hacA* replacement construct the *A. niger hacA* gene (accession number: AY303684), including about 0.6 kb promoter and 0.6 kb of terminator regions, was

amplified by PCR using N402 genomic DNA as template and primers NC8 and NC11 (Table 2) to which *NotI* and *XhoI* restriction sites were added, respectively. The amplified gene was cloned into pTZ57R/T (Fermentas) and sequenced. The *hacA* terminator region (≈1 kb) was amplified by PCR using N402 genomic DNA as template and primers NC1 and NC2, to which *SalI* and *KpnI* restriction enzymes were added, respectively. The fragment was cloned into pGEM-T easy (Promega) and sequenced. For PCR amplification, Phusion™ High-Fidelity PCR Kit (Finnzymes) was used according to manufacturer's instructions. The *AopyrG* gene (≈2kb) was PCR amplified using pAO4-13 (de Ruiter-Jacobs, 1989) as template DNA and primers NC7 and pAOpyrG-GA5rev, to which *XhoI* and *SalI* restriction sites were added, respectively. The fragment was cloned into pGEM-T easy (Promega) and sequenced. The fragments corresponding to the *hacA* terminal region and *pyrG* were digested from the plasmids using the respective restriction enzymes mentioned above and cloned in a 3-way ligation step into pBlue-SK, previously digested with *XhoI-KpnI* to give pBS-pyrG-3'hac. To obtain the final construct, the *hacA* gene was digested from pTZ57R/T using *NotI-XhoI* and cloned into pBS-pyrG-3'hac, previously digested with the same enzymes. The final construct, named pHAC, was linearized with *NotI* and transformed into the *A. niger* MA70.15 strain. Transformants with a targeted integration of the construct at the *hacA* locus were screened by Southern blot analysis.

To obtain a strain only expressing the constitutively active *hacA* gene, a construct was made lacking the 20 nucleotide intron (see introduction for details) using the site-directed mutagenesis technique. Mutagenic oligonucleotide primers NC31 and NC32 (Table 2) were designed, surrounding each side of the intron region. PCR was performed using *PfuUltra* HF DNA polymerase (Stratagene), the pHAC (≈10 ng) as template and conditions as follows: initial denaturation of 1 min at 95°C, 18 cycles of 30 sec denaturation at 95°C, annealing at 55°C for 30 sec and elongation for 8 min and 30 sec at 68°C. Afterwards, PCR products were digested with *Dpn*I for one hour at 37°C, for destruction of parental methylated and hemimethylated plasmid DNA. The mixture was directly used for *E. coli* transformation. Plasmid pConstHac was analyzed by restriction enzymes and sequencing, confirming the absence of the 20 nt intron. This construct was linearized with *Not*I and then transformed into *A. niger* MA70.15. Southern analysis of putative transformants carrying the wild-type *hacA* and the constitutively active *hacA* was performed by digesting the genomic DNA with *Nhe*I and probing with a 0.6 kb probe corresponding to the *hacA* 3'-flanking region. Transformants NC1.1 containing expressing the wild-type *hacA* and NC2.1 expressing the activated *hacA* form at the endogeneous *hacA* locus were chosen for further studies and we will refer to these strains as the $HacA^{WT}$ (wild-type) and $HacA^{CA}$ (Constitutive Active) strains, respectively. The absence of the intron in the NC2.1 strain was further confirmed by PCR analysis using genomic DNA as template, together with primers phac1 and phac2 (Table 2) using Taq polymerase (Fermentas).

Table 2. Primers used throughout this study.

Name	Sequence
pNC1	ACGCGTCGACGCTGTTGAGGTTCCGGCTGTA
pNC2	GGGGTACCAATCTTCAGAGCGCGCCAG
pNC7	CCGCTCGAGGGATCTCAGAACAATATACCAG
pAOpyrG-GA5rev	ACGCGTCGACCCGCTGTCGGATCAGGATTA
pNC8	ATAAGAATGCGGCCGCCTCCATACCACTTTGTGCTAG
pNC11	CCGCTCGAGGGCGCATGAGAGAGTTAGG
pNC31	CGTGACACAACATCCTCCAGCGGTGTTGTGCGACCTCCAGTGTCCGTCGCTGG
pNC32	CCAGCGACGGACACTGCAGGTCGCACAACACCGCTCCAGGATGTTGTGTCACG
phac1	CTTCTCCTACCCTAACTCCT
phac2	TCAAAGAGAGAGAGGGCA

5.2.3. Bioreactor cultivation conditions

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

5.2.4. Biomass concentration and substrate determination

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

5.2.5. RNA isolation and quality control

Mycelium intended for gene-expression analyses was separated from culture medium and frozen in liquid nitrogen within 15-20 s from sampling RNA was extracted from mycelium and snapfrozen in liquid nitrogen using TRIzol reagent (Invitrogen). Frozen ground mycelium (≈200 mg) was directly suspended in 800 µl Trizol reagent and vortexed vigorously for 1 min. After centrifugation for 5 min at 10000 × g, 450 µl of the supernatant was transferred to a new tube. Chloroform (150 µl) was added and after 3min incubation at room temperature, samples were centrifuged and the upper aqueous phase was transferred to a new tube to which 400 µl of isopropanol was added, followed by 10 min incubation at room temperature and centrifugation for 10 min at 10000 × g. The pellet was washed with 75% (v/v) ethanol and finally dissolved in 100 µl H_2O. RNA samples for micro-array analysis were additionally purified on NucleoSpin RNA II columns (Machery-Nagel) according to the manufacturer's instructions. RNA quantity and quality was determined on Nanodrop spectrophotometer.

5.2.6. Microarray analysis

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

5.2.7. Transcriptomic data analysis

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

5.2.8. Enrichment analysis of Gene Ontology (GO) terms

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

5.3. Results

5.3.1. Construction and analysis of a strain expressing a constitutively activated form of *hacA*

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

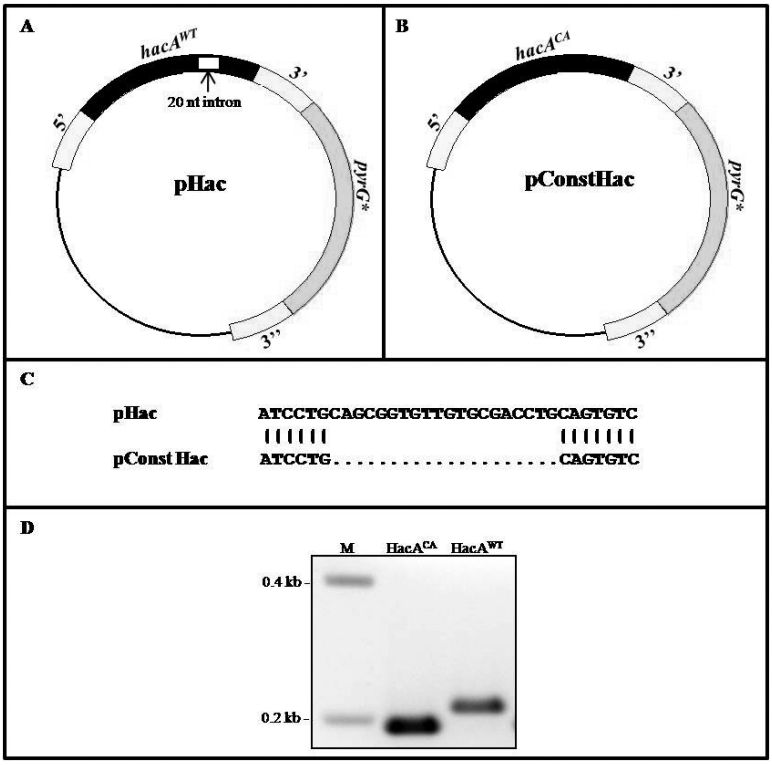

Figure 1. Schematic representation of the plasmids pHAC (A) and pConstHac (B) (Note: fragment sizes are not on scale). (C) Sequence alignment of pHAC and pConstHAC showing the absence of the 20 nt intron on pConstHac. (D) PCR amplification of gDNA of HacA^{WT} (NC1.1) and HacA^{CA} (NC2.1) transformants. Primers were designed about 100 bp upstream and 100bp downstream of the hacA intron region, giving rise to a band of 200 bp for HacA^{CA} and 220 bp for HacA^{WT}. Sizes of the DNA Marker (M) are indicated.

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

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

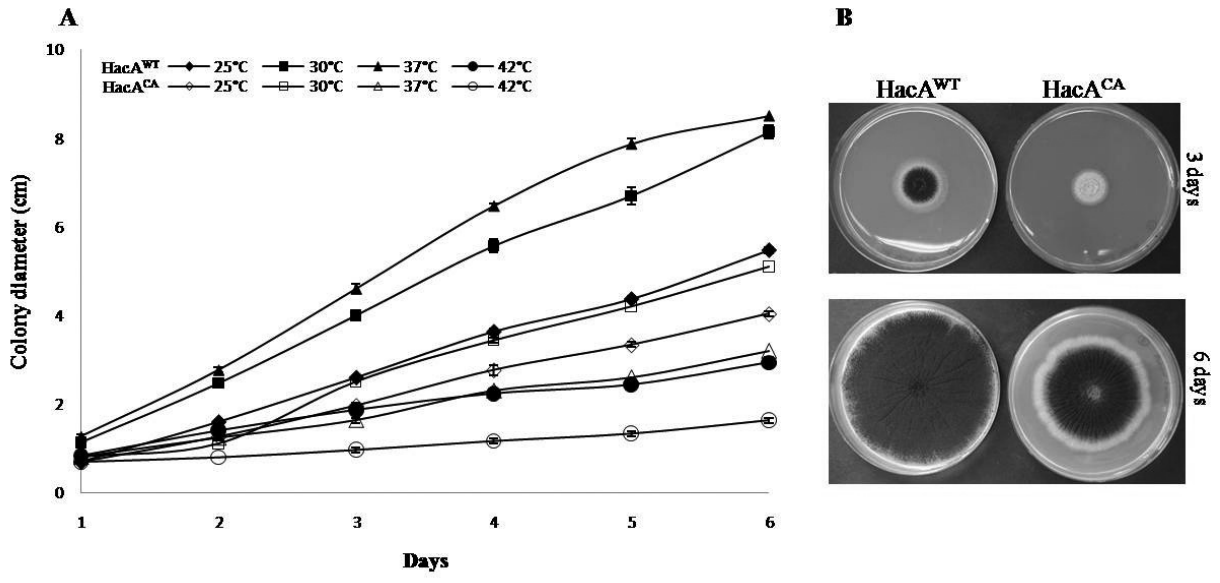

Figure 2. (A) Differences on colony size (diameter) of HacA^{WT} and HacA^{CA} strains growing at different temperatures. 10^4 spores were spotted on solid CM plates and growth was monitored for 6 days. (B) Strains phenotype on CM after 3 and 6 days of growth at 30°C. HacA^{CA} phenotype is characterized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

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

Growth of triplicate batch cultures of HacA^{WT} and HacA^{CA} was characterized as filamentous and highly reproducible. The growth kinetics of a representative culture of each strain is shown in Fig. 3 and results from all cultures are given in the supplemental material (Suppl. Fig. 1).

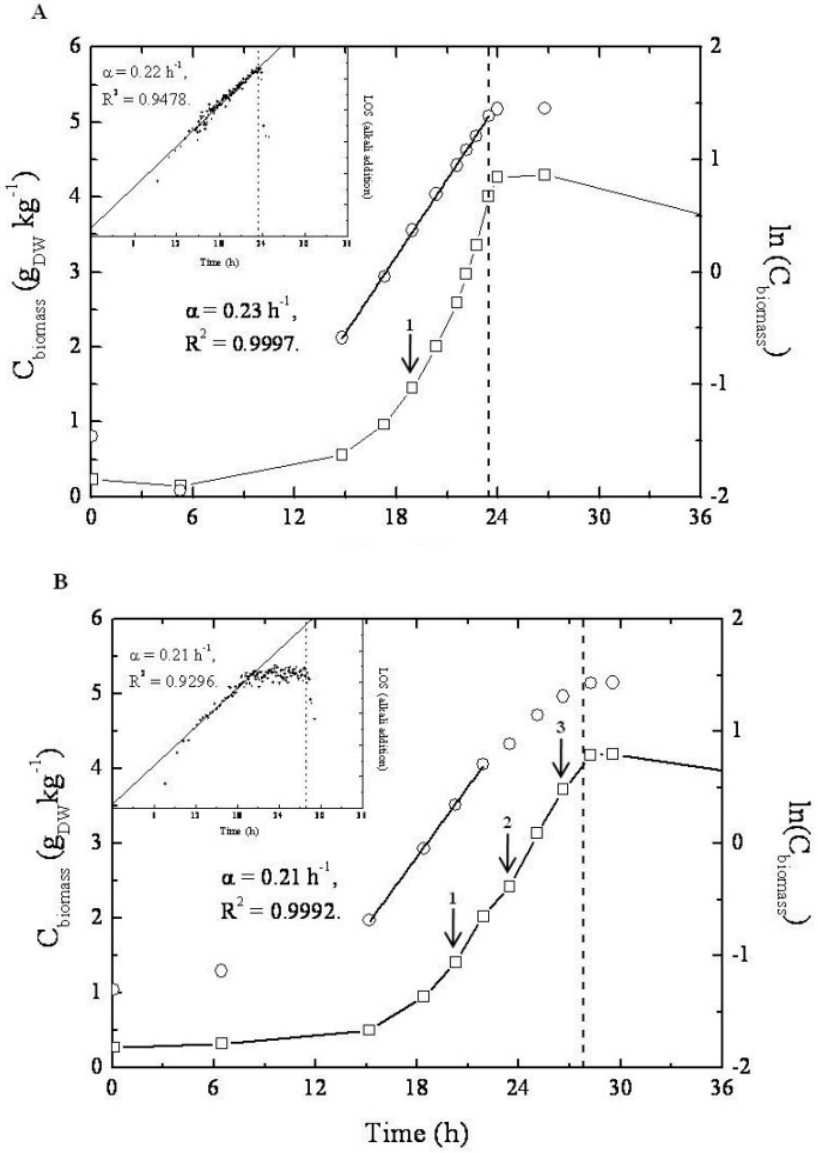

Figure 3. Growth profiles of one of the triplicate *A. niger* HacA^{WT} (A) and HacA^{CA} (B) batch cultures. Dry weight biomass concentration ($g_{DW}kg^{-1}$) as a function of time (h) illustrates the growth of the cultures. The maximum specific growth rate for each culture was determined from the 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). Dash-line represents the end of the exponential growth phase (depletion of glucose). Arrows indicate time-points where mycelium was harvested for transcriptomic analysis.

Cultures of the HacA^{WT} strain exhibited exponential growth with a specific growth rate (μ) of 0.22 ±0.01 h^{-1} (n=4) from exit of lag phase to depletion of glucose (Fig. 3A). Initial growth of HacA^{CA} was similar to that of the HacA^{WT}; it was exponential with a μ of 0.21 ±0.01 h^{-1} (n=3). However, after 21-22 h of batch cultivation, when half of the glucose was consumed, the growth kinetics shifted from exponential to apparently linear (Fig. 3B). It was not clear from the relatively few determinations of biomass concentration whether growth was truly linear in the second phase but this was strongly supported by analysis of the growth-dependent alkali addition (inset Fig. 3A, B). We established a concordance between growth and alkali added to maintain constant pH in the cultures (not shown), and used this as an indirect measure of growth as described previously by Iversen *et al.* (1994). Linearity was then confirmed by log-transformation of alkali addition rates using the computer recorded titrant addition data and the LOS program (Poulsen *et al.*, 2003). During exponential growth, growth yield on substrate (Y_{xs}) was comparable in both strains: 0.53±0.02 for HacA^{WT} and 0.52±0.04 for HacA^{CA}.

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

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

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

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

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

↑ up-regulated ; ↓ down-regulated

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

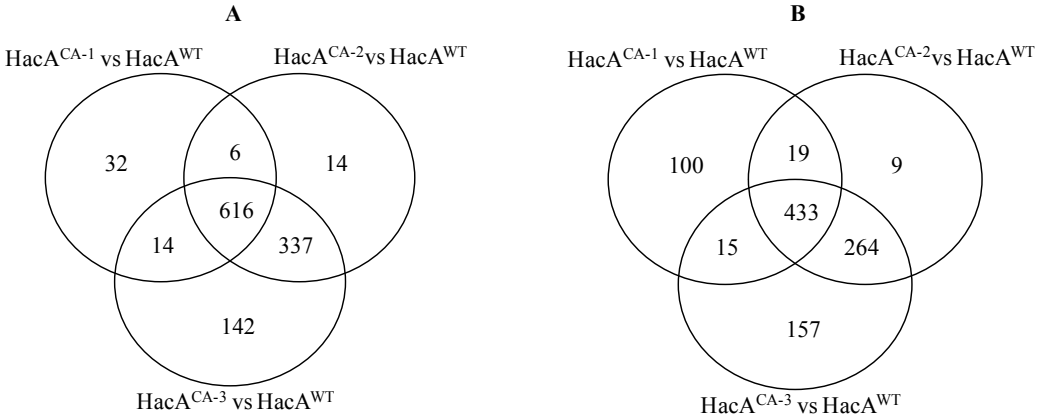

Figure 4. Venn diagrams of the number of overlapping and non-overlapping induced (A) or repressed (B) genes on *A. niger* HacA^{CA} mutant strain at different time points in comparison to HacA^{WT} strain.

From the 616 up-regulated genes (Supplementary Table S1B) we were able to retrieve 598 upstream regions. These upstream regions were analysed for the presence of UPRE sequences (5'-CAN(G/A)NTGT/GCCT-3', Mulder *et al*., 2006). From the up-regulated genes in the HacA^{CA} strain, we found 47 genes that contained at least one UPRE sequence within the 400 bp region up-stream their start codon (Supplementary Table S1C). Compared to the frequency of UPRE in the 400 bp upstream region of the remaining non up-regulated genes (457 out of 13156) a statistical significant enrichment ($p \leq 5.4\times10^{-7}$) was assessed with the Fisher's exact test (one-sided). Although this analysis indicates a statistical enrichment for genes containing a HacA binding site in the promoter region of HacA induced

genes, it shows that only about 10% of the $HacA^{CA}$ induced genes contain an UPRE. It suggests that either the currently used HacA binding consensus site is too stringent and that additional sequences allow HacA to bind, or that additional transcription factors are involved in the induction in response to the constitutive activation of HacA. The data set of HacA induced genes with a putative UPRE site include genes related to protein folding (as previously described by Mulder *et al*., 2006), lipid metabolism, transport within the cell, glycosylation, ER quality control as well as a large set of genes that code for hypothetical and unknown function proteins (Supplementary Table S1C).

5.3.4. Identification of biological processes enriched in the transcriptomic profiles of $HacA^{CA}$ strain

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

In the $HacA^{CA}$ strain we found enriched GO terms linked to ER processes, such as those related to entry in the ER: signal particle recognition, cleavage of signal sequence, and translocation (e. g. Sec61 and related subunits). In parallel to the processes that mediate the recognition, targeting and entering the ER, enrichment of GO terms that include a large number of genes involved in the subsequent events of protein folding and quality control are also observed. The category of protein folding includes the well known HacA targets such as *bipA*, *pdiA*, *tigA* and *prpA* (Mulder *et al*. 2006). After being synthesized and folded properly in the ER, proteins are packed in vesicles and transported to the Golgi and from there on, further transported to reach their final intra- or extra-cellular destination. Our analysis identified a number of genes that encode proteins that take part in the vesicle/trafficking machinery such as those involved in ER-to-Golgi (COPII associated components), Golgi-to-ER (COPI transport vesicles, Sec components) and Golgi to endosome transport. Additionally, genes involved in exocytosis were also induced (Fig. 5). GO-terms related to processes involving protein glycosylation, were up-regulated in the $HacA^{CA}$ strain. The processes include genes involved in sugar nucleotide synthesis, oligosaccharyl synthesis (ALG-genes) and transfer (OST-complex) of the preassembled oligosaccharide to certain asparagine residues (*N*-glycosylation). In addition, genes related to the addition of *O*-glycans

(genes homologous to the *S. cerevisiae* Pmt-family and Kre2-family of mannosyltransferases) were also up-regulated. Finally, several genes related to the synthesis and transfer of Glycosylphosphatidylinositol (GPI) anchors to proteins were found to be up-regulated. Supplementary Table 6 lists the differentially expressed genes with a proposed function in relation to protein glycosylation or GPI-anchor attachment. In addition, the constitutive activation of HacA has a pronounced effect on the transcription of genes involved in phospholipid metabolism and includes proteins that are homologous to proteins involved in ergosterol biosynthesis as well as proteins involved in fatty acids and inositol metabolisms (Table S7). Categories containing less GO-terms included terms related to intracellular pH regulation and terms related to glutathione catabolic processes (Table S8).

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

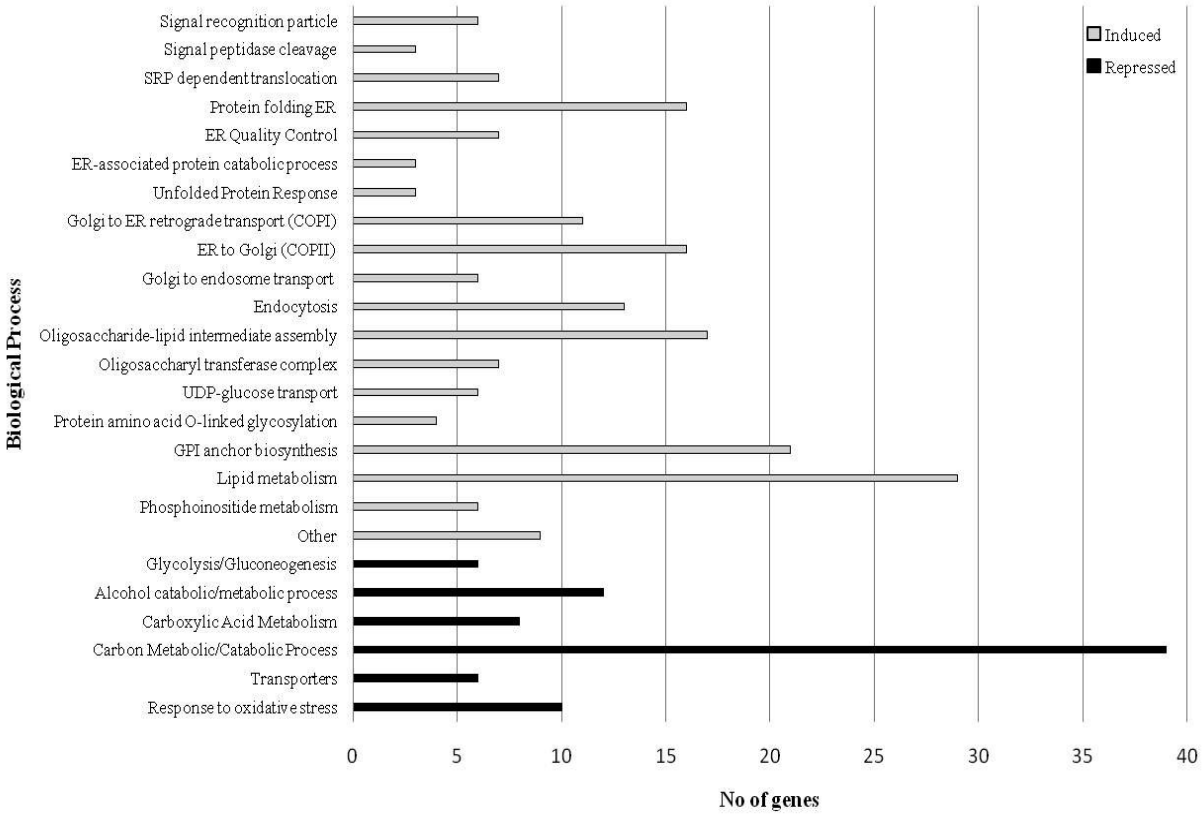

Figure 5. Main significant induced and repressed biological processes in the $HacA^{CA}$ mutant strain in comparison to $HacA^{WT}$ strain.

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

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

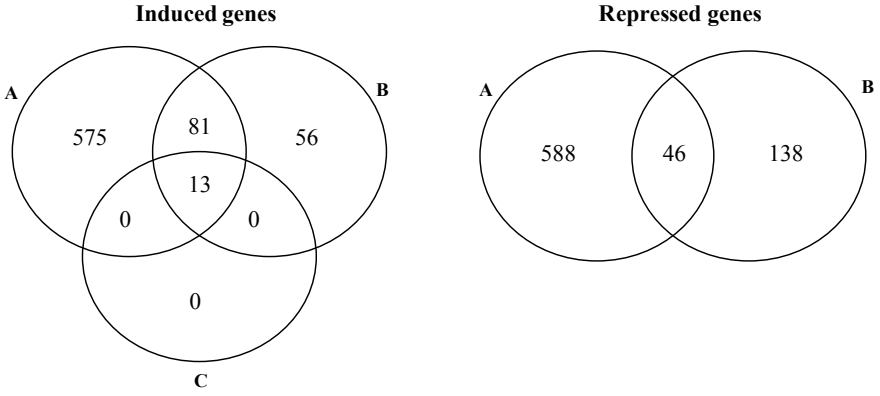

Figure 6. Venn diagrams of the number of overlapping and non-overlapping induced or repressed genes of *A. niger* $HacA^{CA-1}$ /$HacA^{WT}$(A) and genes from Guillemette *et al*. (2007) induced (or repressed) at least in two conditions (B) or induced in all conditions (C).

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

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

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

Table 4. Expression values of *A. niger* ERAD genes.

Gene ID	Gene name	Description	Fold change		
			$HacA^{CA-1}$/ $HacA^{WT}$	$HacA^{CA-2}$/ $HacA^{WT}$	$HacA^{CA-3}$/ $HacA^{WT}$
An15g00640	*derA*	strong similarity to hypothetical protein GABA-A receptor epsilon subunit – *C. elegans*	**4.0**	**6.0**	**6.4**
An01g12720	*hrdC*	similarity to tumour suppressor TSA305 protein of patent WO9928457-A1 – *H. sapiens*	**3.3**	**3.9**	**4.0**
An01g14100	*mifA*	weak similarity to stress protein Herp – *M. musculus*	**3.1**	**4.3**	**4.6**
An18g06220	*mnsA*	strong similarity to alpha-mannosidase MNS1 – *S. cerevisiae*	**4.2**	**4.7**	**5.0**
An08g09000		strong similarity to ubiquitin-like protein DSK2 – *S. cerevisiae*	**1.8**	**1.7**	**1.9**
An16g07970		similarity to autocrine motility factor receptor Amfr – *M. musculus*	**2.9**	**2.9**	**3.1**
An03g04340		strong similarity to ER membrane translocation facilitator Sec61 – *Y. lipolytica*	**2.6**	**2.6**	**2.6**
An04g01720		similarity to DnaJ protein SIS1 – *C. curvatus*	**1.8**	**2.3**	**2.2**
An12g00340		similarity to alpha 1,2-mannosidase IB – *H. sapiens*	**3.2**	**2.9**	**3.1**
An04g00360		strong similarity to transport vesicle formation protein Sec13p – *S. cerevisiae*	**2.1**	**2.1**	**2.1**
An09g06110		strong similarity to ubiquitin conjugating enzyme ubcp3p – *S. pombe*	1.4*	**1.6**	**1.7**

* Not significantly differentially expressed

For instance, the *der1* homologue (*derA*, An01g00560), involved in transport of unfolded proteins out of the ER (Ye *et al*., 2001), is 4.0-fold induced; *hrd3* (*hrdC*, An03g04600), involved in recognition and presentation of the substrate for degradation (Plemper *et al*., 1999), is 3.3-fold induced. The *mifA* (An01g14100) gene, a homologue of mammalian *herp1/mif1* protein and suggested as the link between the UPR and ERAD pathways (van Laar *et al*., 2001), 3.1-fold induced. Furthermore, *mns1* (*mnsA*, An18g06220), a mannosidase that by removal of 1,2 α-mannose units targets the substrate to degradation (Tremblay and Herscovics, 1999), is 4.2-fold induced. In comparison to Travers *et al.* (2000), our study allowed us to unravel the regulation of other ERAD related genes in relation to UPR, such as *mns1*, *mif1*,a *DSK2* homologue (An08g09000, putatively encoding a ubiquitin-like protein) (1.8-fold induction) and another putative α-mannosidase (An12g00340, 3.2-fold induced).

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

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

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

Gene ID	Gene name	Description	Fold change		
			$HacA^{CA-1}$/ $HacA^{WT}$	$HacA^{CA-2}$/ $HacA^{WT}$	$HacA^{CA-3}$/ $HacA^{WT}$
Starch regulation					
An04g06910	*amyR*	transcription regulator of maltose utilization AmyR – *A. niger*	**-3.3**	**-3.3**	**-3.3**
An01g06900		weak similarity to transcription activator AmyR – *A. oryzae*	-1.7*	1.4*	**2.1**
An09g03100	*amyA*	strong similarity to alpha-amylase precursor AMY – *A. shirousamii*	**-5**	**-5**	**-5**
Starch degradation					
An11g03340	*aamA*	acid alpha-amylase – *A. niger*	**-370**	**-50**	**-50**
An04g06920	*agdA*	extracellular alpha-glucosidase – *A. niger*	**-5**	**-10**	**-10**
An01g10930	*agdB*	extracellular alpha-glucosidase – *A. niger*	**-10**	**-10**	**-10**
An03g06550	*glaA*	glucan 1,4-alpha-glucosidase – *A. niger*	**-10**	**-25**	**-25**
An04g06930	*amyC*	extracellular alpha-amylase – *A. niger*	**-10**	**-25**	**-25**
Sugar uptake					
An02g03540	*mstC*	strong similarity to hexose transport protein HXT3 – *S. cerevisiae*	**-2**	**-2**	**-2**
An15g03940		strong similarity to monosaccharide transporter Mst-1 – *A. muscaria*	**-2.5**	**-2**	**-1.7**
An09g04810		strong similarity to high affinity glucose transporter HGT1 - *K. lactis*	**-5**	**-10**	**-10**
An11g01100		strong similarity to high-affinity glucose transporter HGT1 - *K. lactis*	**-5**	**-5**	**-5**
An12g07450	*mstA*	Sugar/H+ symporter	**-5**	**-10**	**-10**

*Not significantly differentially expressed.

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

As predicted from the transcriptomic and similar to the Δ*amyR* strain, $HacA^{CA}$ was unable to grow on the plate containing starch as sole carbon source. With the aim of testing if this reduced growth was specific for growth on starch or if it would apply to other complex

carbohydrates, we performed a similar test on other polymers, inulin, xylan and pectin and respective monomeric substrates, fructose, xylose and galacturonic acid (Fig 8). These results show that the HacA^{CA} strain is growth impaired when challenged to assimilate nutrients from complex substrates, although this was not so evident when grown on inulin, but growth of the HacA^{CA} strain was clearly further reduced on xylan and pectin, suggesting that the down-regulation of extracellular enzyme expression is not limited to the amylolytic genes, but also for xylanolytic and pectinolytic genes.

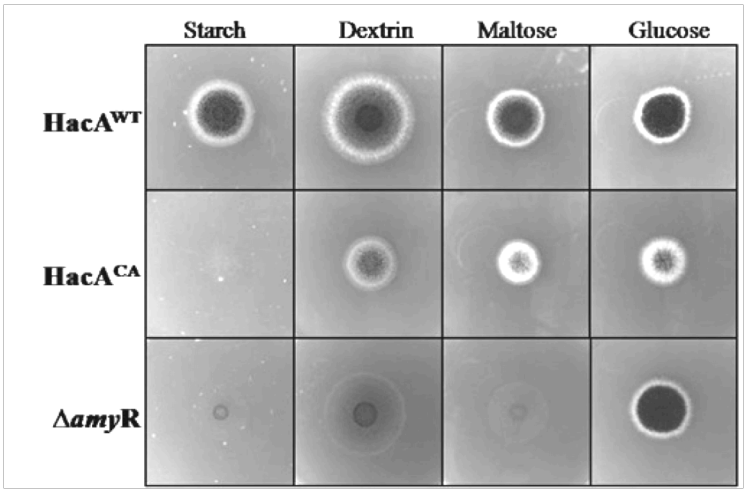

Figure 7. Effects of the constitutive activation of the UPR on the utilization of starch and starach related carbon sopurces. The wild-type strain (HacA^{WT}), the strain containing a constitutive active form of *hacA* (HacA^{CA}) and the AmyR disruptant (Δ*amyR*) strain were grown on MM containing 1%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

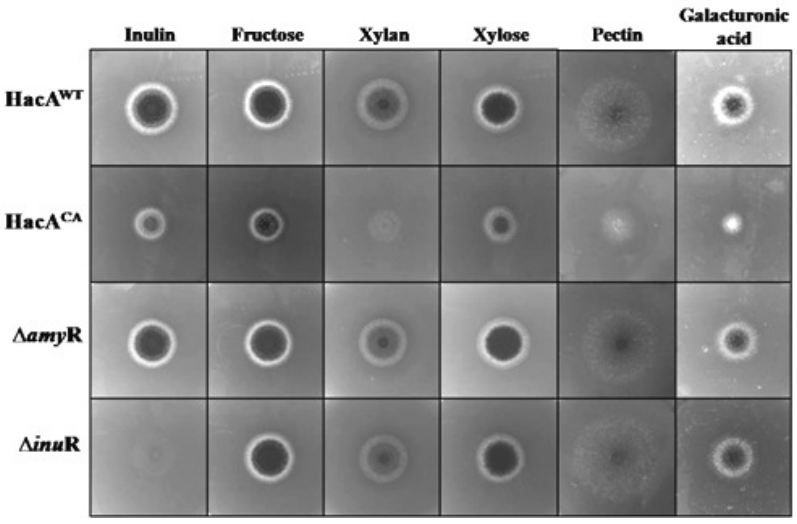

Figure 8. Effects of the constitutive activation of the UPR on the utilization of different polimeric and monomeric carbon sources. The wild-type strain (HacA^{WT}), the strain containing a constitutive active form of *hacA* (HacA^{CA}) the *amyR* disruptant (Δ*amyR*) and *inuR* disruptant (Δ*inuR*)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

5.4. Discussion

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

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

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

GO enrichment analysis on the induced set of genes showed that all the well-known UPR target genes related to folding are represented in the $HacA^{CA}$ dataset, and include genes encoding the chaperone BipA, and homologues of LhS1p (An01g13220), P58PK (An11g11250) and Scj1p (An05g00880), as well as the protein disulfide isomerases PdiA, PrpA and TigA . Glycosylation also appeared as one of the enriched categories. Several

aspects of protein glycosylation including the categories of oligosaccharide-lipid assembly, oligosaccharyl transferase complex, UDP-glucose transport, O-linked glycosylation and GPI anchor biosynthesis (Fig. 5), were up-regulated indicating that the cell responds to ER stress by increasing the capacity to glycosylate proteins. The induction of genes associated with lipid metabolism (Supplementary Table S7) suggests a proliferation of the ER to bear the increase of proteins that reside in this organelle, as also indicated in UPR studies of *S. cerevisiae* (Travers *et al*., 2000).

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

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

5.4.2. New leads on the RESS mechanism

The accumulation of misfolded protein in the ER leads to a selective down-regulation of genes encoding secreted proteins in fungi and plants (Pakula *et al*., 2003; Martínez and Chrispeels, 2003; Al-Sheikh *et al*., 2004; Wang *et al*., 2010). This phenomenon is termed REpression under Secretion Stress (RESS). In these studies, associated with the UPR activation by chemical induction is the down-regulation of transcription encoding extracellular enzymes that include cellulases and xylanases in *T. reesei* (Pakula *et al*., 2003) and glucoamylase in *A. niger* (Al-Sheikh *et al*., 2004) amongst other genes encoding secreted proteins (Guillemette *et al*., 2007). The mechanism by which the down-regulation is mediated is unknown, but *glaA* promoter studies in *A. niger* indicated that a promoter region between 1 and 2 kb upstream of translational start is important and a direct mediation of RESS through the UPR was questioned (Al-Sheikh *et al*., 2004). RESS has been recognized as an effort from the cells to prevent the entry and overload of newly synthesized proteins into the already "full" ER (Pakula *et al*., 2003; Al-Sheikh *et al*., 2004; Wang *et al*., 2010). In our study, the activation of UPR target genes by introducing the constitutive active form of the

HacA transcription factor lead to the down-regulation of not only glucoamylase (*glaA*), but also other genes coding for starch-degrading enzymes that include acid α-amylase (*aamA*), α-glucosidases A and B (*agdA* and *agdB*) and α-amylase C (*amyC*), suggesting a down-regulation of the AmyR regulon and sugar transporters (Table 5). A phenotypic demonstration of this down-regulation was provided by the inability of the $HacA^{CA}$ strain to growth on starch (Fig. 7). Growth assays on other polymeric substrates (Fig. 8) suggested that the down-regulation might not to be specific for starch but is relevant to other sugar polymers including xylan (Fig. 8). We speculate that HacA has a role in controlling the transcription of genes that encode the transcriptional activator AmyR (starch), and possibly XlnR (xylan). Such mechanisms include the possibility that HacA binds directly to the *amyR* promoter region (binding motif to be determined), and serves as a repressor. It will be of interest for future studies to determine the molecular mechanism that results in the downregulation of AmyR and AmyR targets genes in response to HacA activation.

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

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

To prevent the influx of proteins into the ER in mammalian cells, a mechanism of translation attenuation is activated that is mediated by PERK. This transcription factor mediates the phosphorylation of eIF2 (eukarytotic translation initiation factor) which in turn leads to the arrest of protein translation. The eIF2 is also required for the translation of selective mRNAs such as the Activating Transcription Factor-4 (ATF4) (Vattem and Wek, 2004). ATF4 is involved in the regulation of UPR genes involved in ERAD, metabolism and apoptosis (Fels and Koumenis, 2006). Gcn4/CpcA are the ATF4 homologues of *S. cerevisiae* and filamentous fungi, respectively. Both *S. cerevisiae* and *A. niger* lack an obvious PERK homologue. Gcn2p phosphorylates eIF2 leading to a global reduction on protein synthesis and stimulation of Gcn4 translation, that has been shown to control amino acid biosynthesis (Hinnebusch, 1993). Although this resembles the PERK function, Gcn2p–eIF2 phosphorylation is only attributed to amino acid starvation and not to ER stress (Harding *et al*., 2000). In *S. cerevisiae*, the involvement of Gcn2 and Gcn4 in UPR has been shown (Patil *et al*., 2004). In our transcripromic profiles, Gcn2 homologue (An17g00860) is not differentially expressed, whereas *cpcA* (An01g07900) shows ≈2 fold higher expression in comparison with the wild-type strain. According to our results, the activation of *cpcA* is likely to occur in a Gcn2-independent way and it is tempting to speculate that in filamentous fungi a similar PERK-eIF2-ATF4 pathway may exist. ATF4 is involved in glutathione biosynthesis (Harding *et al*., 2003) and glutathione-s-transferases have been shown to be up-regulated under ER stress conditions (Gilmore and Kirby, 2004). According to our data, the homologue to human glutathione-s-transferases 3 (An12g03580) is 2-fold induced in $HacA^{CA-1}$ and 2.6 fold induce in the later time points. What we also observe is that as in the case of ATF4-regulated genes, not all the genes involved in glutathione metabolism are affected under

secretion stress situation (Harding *et al*., 2003), as for example asparagine synthase (An01g07910) or glutathione reductase (An03g03660) that are not differentially expressed. Similar results have been observed in *Trichoderma reesei* (Arvas *et al*., 2006). Another interesting observation is the 4-fold induction of the human homologue RNA-activated protein kinase inhibitor P58 (An11g11250). In mammals, P58 is induced via ATF6, a transcription factor also involved in the regulation of UPR chaperones and apoptosis (no homologue in fungi), and it is an important component on the regulation of PERK-eIF2-ATF4 pathway, attenuating the UPR (van Huizen *et al*., 2003). The up-regulation of P58 has been shown in studies characterizing the UPR under different conditions (Guillemette *et al*., 2007; Jørgensen *et al*., 2009); however, the role and (putative) involvement of a fungi P58 homologue in this pathway remains to be elucidated. ATF6, that induces XBP1 (HacA homologue), also possesses the ability to impel lipid biosynthesis and expansion of the ER (Bommiasamy *et al*., 2009). The identification of these potential regulatory genes involved in mediating the HacA response in this study, has given multiple new leads for further research to better understand the mechanism of how *A. niger* reacts to secretion stress.

Chapter 6

General Discussion

6.1. General discussion

Aspergillus niger is a widely used host for the industrial production of enzymes as a result of its natural and high secretion capacity of proteins to the extracellular environment. As a result of many years of investigation and strain improvement, the production of many *A. niger* native enzymes have successfully improved resulting in high production levels, whereas researchers still face several problems in reaching satisfactory production levels of heterologous proteins. Many studies have been focusing on the understanding of the metabolism and physiology alongside with genetic engineering of many steps of the secretory pathway (reviewed in Fleissner and Dersch, 2010). A main bottleneck for heterologous protein production is attributed to difficulties of the host in the folding and maturation of foreign proteins in the ER. An increase bulk and flux of proteins in the ER often results in protein misfolding and/or misassemble, triggering an increase in the ER folding machinery and quality control mechanisms, a condition known as ER stress or protein secretion stress (Guillemette *et al*., 2007). This thesis investigates the effects of the modulation of different components of the *A. niger* secretory pathway, with special emphasis on the Unfolded Protein Response (UPR) and Endoplasmic Reticulum Associated Degradation (ERAD) pathways and its relation to protein secretion efficiency. The main conclusions and arisen questions from our research will be addressed and discussed in more detail in the following sections.

6.1.1. The double-edged sword of HacA activation

The UPR is a signal transduction network activated by ER stress or perturbations in the ER homeostasis. Removal of a 20 nt intron from the *hacA* mRNA by IreA activates the UPR pathway (Mulder *et al*., 2004), which affects a large number of genes (Guillemette *et al*., 2007). In Chapter 5 we have explored the physiological and transcriptomic consequences of an *A. niger* strain bearing a constitutive active *hacA* form, therefore expecting a full activation of genes under HacA control and other components of the UPR pathway. In Fig. 1 is depicted a schematic representation of pleiotropic effects of HacA in *A. niger* and its presumed involvement in the down- regulation of genes encoding secretory enzymes.

Like in the *S. cerevisiae* UPR, HacA binding results in a direct activation and transcriptional up-regulation of folding enzymes (Travers *et al*., 2000; Gasser *et al*., 2007). Mulder and co-workers (2006) have define a set of chaperones and foldases that contain putative UPR elements or UPR binding sites in their promoter region, which include *bipA*, *cypB*, *pdiA*, *prpA*, *tigA* and the transcription factor *hacA* itself. Studies in *S.cerevisiae*, *A. niger* and in mammalian cells where Hac ortologs (Hac1/HacA/XBP1) and the UPR have been studied have shown that this pathway is not solely a linear response engaging transcription factor activation followed by folding machinery up-regulation, but involves the up-regulation of many other genes involved in different processes throughout the secretory pathway, such as glycosylation, intracellular vesicular transport between organelles, membrane proliferation, ERAD, among others (Travers *et al*., 2000; Shaffer *et al*., 2004; Kimata *et al*., 2006; Arvas *et al*., 2006; Gasser *et al*., 2007; Guillemette *et al*., 2007; Bommiasamy *et al*., 2009). Analysis of our data set (Chapter 5) revealed that only about 10% of the $HacA^{CA}$ up-regulated genes seem to contain an UPRE sequence in the proximity of the

start codon (≤-400bp), making it unlikely that the large set of HacA^{CA} induced genes will be under direct HacA regulation. From this point of view, it seems more plausible that more regulators will have a role in activating different secretory machinery through a cascade signal activated by HacA or unidentified HacA target(s). Following research should focus on the identification of regulatory elements that coordinate the expression of the genes involved in protein synthesis and secretion.

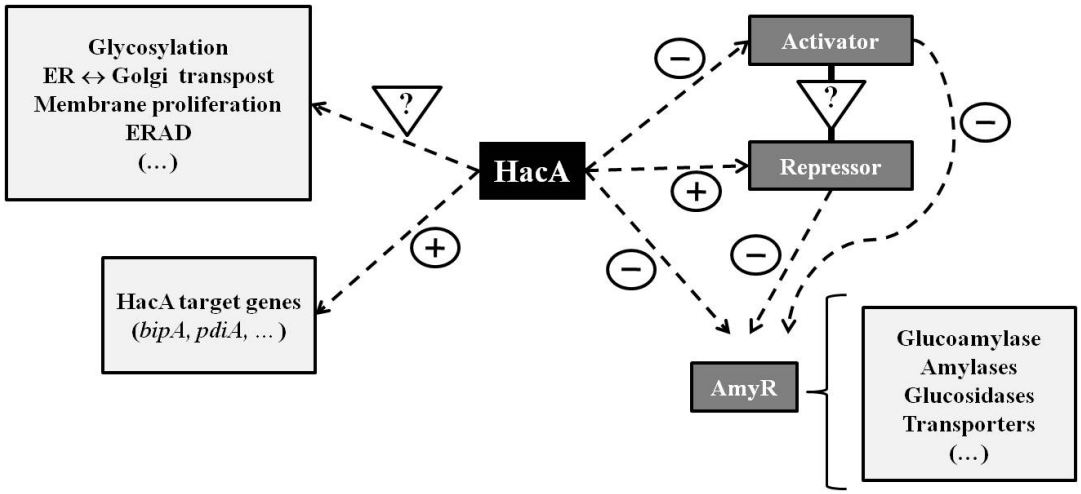

Figure 1. Schematic overview of the consequences of a constitutive activation of the UPR transcriptional regulator HacA and putative models for its role on RESS.

The repression of extracellular enzymes transcription has also been related to the activation of the UPR pathway and describes the phenomena known as response to secretion stress (RESS). The down-regulation of secreted enzymes such as glucoamylase (Al-Sheikh *et al*., 2004), xylanases and cellulases (Pakula *et al*., 2003) reflects an effort from the fungi to prevent the entry and overload of newly synthesized proteins into the ER. In our study (Chapter 5) we have shown that down-regulation is not confined to single carbohydrate degrading enzymes, like glucoamylase, but extends to the transcription factor and its regulon, in our case the AmyR regulon. These results obtained by transcriptomic analysis could be experimentally shown by the inability of HacA^{CA} to grow on starch (Chapter 5, Fig. 7). The mechanism(s) that regulates the repression of AmyR and its target genes under constitutive HacA activation is not known but some hypothesis can be considered. Al-Sheikh and co-workers (2004) have shown that repression involves a 1.0-2.0 kb promoter region of the *glaA* gene. As no UPRE were described in this region, a direct binding and repression by HacA to these secreted enzymes, transporters or *amyR* itself seems unlikely. We speculate that AmyR repression might come from the activation of an unidentified repressor, directly or indirectly involving HacA. Alternatively, the down regulation of an unidentified AmyR activator also seems plausible (Fig. 1). These putative repressor or activator proteins may be present in the

large set of genes of unknown function found to be differentially expressed in the HacA^{CA} strain (Chapter 5, Supplementary Table S1). The RESS mechanism resembles the mammalian UPR branch regulated by PERK. Upon UPR activation, this transcription factor mediates a mechanism which prevents the influx of new proteins into the ER by mRNA translation attenuation (Fels and Koumenis, 2006).

Although we did not obtain evidence for ER membrane proliferation in the HacA^{CA} strain (unpublished data), up-regulation of lipid-biosynthesis related genes in the HacA^{CA} strain suggests an expansion of ER to harbour and increase the protein folding capacity in this organelle. The involvement of XBP1 (the HacA orthologue in mammalian cells) in events beyond ER stress response has been well described in professional secretory mammalian systems, suggesting a broader role for XBP1 (Shaffer *et al*., 2004; Bommiasamy *et al*., 2009). The idea of a "physiological UPR" activated in secretory cells different from a "stress UPR" activated by a disturbed ER homeostasis, as described for secretory mammalian systems (Gass *et al*., 2002), might represent a new UPR paradigm in filamentous fungi and has not been associated with the *S. cerevisiae* UPR. Recent evidence for a "physiological UPR" in *A. niger* comes from the study of Jørgensen and co-workers (2009). The high secretion capacity of extracellular enzymes by *A. niger* comes from its saprophyte lifestyle. The transcriptomic response when growing on maltose (Jørgensen *et al*., 2009) much resembles the "physiological" UPR in mammalian systems (Gass *et al*., 2002; Shaffer *et al*., 2004) and comprises a large overlap of the gene set observed under constitutive *hacA* activation (Chapter 5). Hence, the transcriptional regulation of secretory pathway genes reflects a general mechanism for modulation of secretion capacity in response to the conditional need for extracellular enzymes. Hence, although *A. niger* shares many common features with the *S. cerevisiae* UPR, new evidences point to a closer relation to the UPR system as it has been described secretory mammalian cells. The impaired growth of Δ*hacA* (Chapter 2) and reduced growth of HacA^{CA} (Chapter 5) suggests that the activity of HacA should be fine tuned and controlled to sustain optimal growth.

6.1.2. Can heterologous protein production benefit from constitutive *hacA* activation?

Aspergillus niger is frequently used as a microbial cell factory for the production of heterologous proteins, not only for being able to take care of post-translational modifications essential for protein activity, but mostly because of its natural high secretion capacity. However, producing a foreign proteins alongside with the complexity of folding, trafficking and secretion processes often results in undesirable lower yields and/or accumulation of unfolded proteins in the ER, which can be toxic for the cells. Increasing knowledge on the UPR pathway and its association with the increase of folding related enzymes, ER proliferation and overall secretory pathway expansion (Travers *et al*., 2000; Arvas *et al*., 2006; Guillemette *et al*., 2007; this thesis), led to the study and modulation of individual UPR components to increase heterologous protein production. Some studies in *S. cerevisiae* have shown, for example, that overproducing BiP increases the secretion yields of several heterologous proteins (Shusta *et al*., 1998; Harmsen *et al*., 1996; Kim *et al*., 2003). Similar results were found in other fungi, not only when overexpressing BiP (Lombraña *et al*., 2004),

but also other folding enzymes like Pdi (Robinson et *al*., 1994; Moralejo *et al*., 2001; Smith *et al*., 2004) and calnexin (Conesa *et al*., 2002; Klabunde *et al*., 2007). On the other hand, some studies revealed that is not always the case that increasing the folding machinery has benefits on protein production (Harmsen *et al*., 1996; Robinson *et al*., 1996; van Gemeren *et al*., 1998; Ngiam *et al*., 2000; Smith *et al*., 2004) and can even be detrimental (van der Heide *et al*., 2002). These studies show that the yields of heterologous protein obtained is variable and much depends on the properties of the protein expressed. Other approaches have focused on enhancing the secretory pathway by overexpressing the UPR transcriptional regulator HAC1/HacA. In *S. cerevisiae*, overexpressing *T. reesei* HAC1 improved the secretion yields of *Bacillus* α-amylase, but had no effects on *T. reesei* endoglucanase I secretion; whereas disruption of *HAC1* in *S. cerevisiae* decreased the secretion of these two heterologous proteins (Valkonen *et al*., 2003a). In *P. pastoris*, expressing *S. cerevisiae HAC1* increased the secretion rate of a Fab antibody fragment (Gasser *et al*., 2006). More recently, Guerfal and co-workers (2010) have shown that overexpressing Hac1p in *P. pastoris* could slightly improve the surface expression levels of mouse interferon-γ, human thrombomodulin and human erythropoietin but not the levels of human interferon-β. Overproduction of *hacA* in *A. awamori* increased *T. versicolor* laccase and *preprochymosin* production (Valkonen *et al*., 2003b).

We previously showed that the expression of a Glucoamylase-Glucuronidase (GlaGus) fusion construct in *A. niger* did not lead to a successful secretion of this protein into the growth medium (Chapter 4). To examine whether constitutive activation of HacA had a positive effect on the secretion of the GlaGus protein the GlaGus fusion construct was transformed to the $HacA^{CA}$ and $HacA^{WT}$ strains (Chapter 5). For both strains a transformant was selected that contained a single copy of the GlaGus construct (pBB19-3pyrG*, Chapter 4). Expression is driven by the *gpdA* promoter. Western blot analysis (Fig. 2) was performed on medium samples and total protein extract samples. No GlaGus protein could be detected in the culture medium in both strains using the Gus antibody (data not shown).

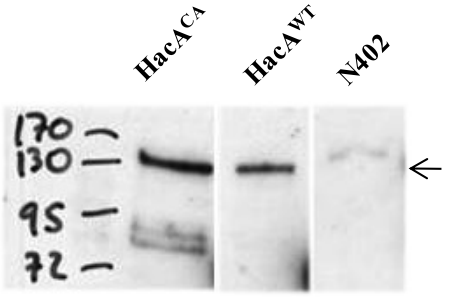

Figure 2. Effects of a constitutive activation of HacA on the amounts of GlaGus fusion protein in total intracellular protein extracts. Western analysis was performed on samples from $HacA^{CA}$, $HacA^{WT}$ and N402 (negative control) grown in CM for 24h at 30°C. Ten micrograms of total protein were separated by gel electrophoresis and immunodetected with an anti-Gus antibody. Detection was carried out through a chemiluminescence reaction for 30 minutes. Arrow indicates the expected GlaGus band size.

Fig. 2 illustrates that when using an antibody against Gus, relatively low amounts of intracellular GlaGus could be detected in both $HacA^{CA}$ and $HacA^{WT}$ strains. No activity could be measured in protein extracts or culture media of either strains expressing GlaGus (data not shown). This suggests that production of this heterologous fusion protein is difficult (low amounts) in the wild-type strain and doesn't seem to be much improved by a constitutive activation of HacA. Furthermore, the Gus antibody recognized smaller proteins in the protein extract of the $HacA^{CA}$ strain which might suggest that the proteolytic fragments are more stable in the $HacA^{CA}$ strain. A possible explanation might be the increased abundance ER-chaperones in the $HacA^{CA}$ strain that prevent rapid degradation. We cannot rule out the possibility that glycosylation of Gus might interfere with its enzymatic activity.

Can heterologous protein production benefit from artificial activation of HacA? Results from literature are controversial but most seem to point out a beneficial effect of HacA activation on production levels. The results presented in Fig. 2 are preliminary but suggest that β-glucuronidase could be one of the heterologous where the production and secretion cannot be improved by manipulating *hacA* activation. On the other hand, even though the $HacA^{CA}$ shows a limited growth phenotype and most of the central metabolism is repressed (Chapter 5); it is still capable to support similar levels of heterologous protein (GlaGus) as observed in the $HacA^{WT}$ strain (Fig. 2), and therefore this topic deserves further attention. It is clear from previous studies and this preliminary study that constitutive activation of *hacA* does not per definition results in higher production levels. It is likely that each heterologous protein to be produced is unique and will challenge the host in a different way.

6.1.3. ERAD: the only way to destruction?

The UPR and the ERAD are two pathways which have been shown to be linked and cooperatively work when the ER is overloaded with misfolded proteins (Travers *et al*., 2000; Casagrande *et al*., 2000; Ng *et al*., 2000; Friedlander *et al*., 2000). The ERAD pathway is part of a complex ER quality control (ERQC) system that monitors protein folding and assembly as well as detects and targets terminally misfolded proteins for destruction (reviewed in Sayeed and Ng, 2005). Studies in *S. cerevisiae* and mammalian systems have identified several ERAD components and collectively infer that ERAD substrates are recognized, targeted, retrotranslocated, polyubiquitylated and finally degraded by the 26S proteasome (reviewed in Vembar and Brodsky, 2008). In *A. niger* the ERAD pathway is still poorly understood. In Chapter 4 we have given the first steps on the identification and characterization of ERAD components that act at different levels within this pathway. Surprisingly, none of the ERAD genes studied – *derA*, *mnsA*, *mifA*, *doaA* and *hrdC* – was found to be essential in *A. niger*. Moreover, phenotypic differences from the deletions and the wild-type strains were limited to the Δ*doaA* strain. Deletion of *doaA* in *A. niger* resulted in an irregular morphology and reduced sporulation phenotype. In *S. cerevisiae*, Doa1p plays a role in the ubiquitin-dependent protein degradation by a direct interaction with Cdc48p (Ogiso *et al*., 2004; Mullally *et al*., 2006). It has also been shown in *S. cerevisiae* that Doa1 has a role

in cell morphology (Kunze *et al*., 2007) and in the response to DNA damage (Hanway *et al*., 2002); therefore, the Δ*doaA* phenotype observed may not entirely relate to ERAD. These (phenotypic) results did not vary when the deletion strains were challenged to express or over-express a heterologous protein, although degradation seemed to be compromised in some strains (Chapter 4). The lack of a clear phenotype when deleting individual ERAD genes seems coherent with the idea that under normal growth and no ER stress conditions this pathway is not activated (only basal levels). However, the production of heterologous proteins is usually associated with ER stress; and the often low protein production yields obtained are attributed, to some extent, to degradation mechanisms by the host (Gouka *et al*., 1997).

According to *S. cerevisiae* model, Hrd3p is responsible for the activity and stability of Hrd1p (E3 ubiquitin ligase) (Plemper *et al*., 1999b; Gardner *et al*., 2000). Der1p is part of the Hrd1 complex and it has been attributed a role in the retrotranslocation of targeted proteins to be delivered to the proteasome (Lilley and Ploegh 2004; Goder *et al*., 2008). The Sec61p is the retrotranslocation channel associated with ERAD substrates during degradation (Gillece *et al*., 2000). In the absence of DerA, Sec61p might act as an alternative retrotranslocon for Der/Hrd substrates. Additionally, in *S. cerevisiae*, some substrates of this complex have been shown to be targeted to destruction by a HRD/DER independent pathway (Haynes *et al*., 2002). If such pathway exists in *A. niger* remains to be investigated but could account for an alternative degradation pathway if HrdC fails to activate the HdrA complex.

In mammalian cells, *Mif1* is an UPR target and contains an ER stress-responsive element (van Laar *et al*., 2000). Its function seems to be to mediate the translocation of the 26S proteasome from the cytoplasm to the ER membrane upon ER stress (van Laar *et al*., 2001). Moreover, studies have shown MIF1 forms a complex with HDR1 (Schulze *et al*., 2005) and that the knockdown of this gene leads to the stabilization of ERAD substrates (Hori *et al*., 2004). *Mns1* codes for a α1,2-mannosidase which is responsible for the cleavage of the α1,2-mannose from misfolded glycoproteins, an event that will lead the protein to be eliminated by the ERAD machinery (Cabral *et al*., 2001; Termine *et al*., 2009). *Mns1* disruption or inhibition has shown to stabilize glycoproteins (Jakob *et al*., 1998; Liu *et al*., 1999). If both *mifA* and *mnsA* deletions also stabilize ERAD substrates in *A. niger*, it is possible that these substrates are no longer recognized as misfolded and continue their journey to the following organelle. On the other hand, under Δ*mifA* and Δ*mnsA* conditions, ERAD substrates might accumulate in ER sub-compartments, as the ERACs (ER-associated compartments) proposed for *S. cerevisiae* (Huyer *et al*., 2004). These ERACs are suggested to be retaining sites where some misfolded proteins are targeted to in order not to interfere with normal cellular functions (Huyer *et al*., 2004). Similar structures (Quality Control compartments) have been described in some mammalian cells but are often associated with the development of diseases (Kopito, 2000; Markossian and Kurganov, 2004; Rodriguez-Gonzalez *et al*., 2008). In *S. cerevisiae* , the ERACs have no negative effects on protein traffic through the ER nor lead to UPR induction; and the retained substrates will eventually be degraded via the proteasome (Huyer *et al*., 2004) or autophagy (Fu and Sztul, 2009).

Alongside with the ERAD-proteasome, vacuoles represent, among other functions, the second degradation system (Klionsky *et al*., 1990). Vacuoles contain many proteases (e.g. vacuolar proteases proteinase A (Pep4p), carboxypeptidase Y (CPY), proteinase B (Prb1p),

carboxypeptidase S (CPS)) that are relatively nonspecific and directed toward degradation of a large variety of substrates (reviewed in van den Hazel., 1996). Substrates are directed to the vacuole for degradation by different routes, e.g., plasma membrane proteins are targeted to the vacuole in both a constitutive manner, as a mean of continually refreshing the amino acids population; or a signal dependent manner, in order to down-regulate signalling or transport functions (reviewed in Li and Kane, 2009). Vacuoles are also involved in constitutively removing cytosolic and organellar proteins, and under starvation conditions basal levels of autophagy possibly account for the degradation of these proteins (Mizushima and Klionsky, 2007). To understand the possible role of vacuole in the degradation of heterologous proteins, it will of interest to identify mutants in which the biosynthesis or transport step to the vacuole is hampered. As the vacuole is an essential organelle such approaches require the generation of conditional mutants in which the vacuolar function can be blocked temporarily.

Impairing the ERAD pathway results in ER-retention of degradation-substrates (Chapter 4) and this could become toxic for the cells. The fact that *A. niger* is able to cope with this may suggest the existence of alternative mechanisms or by-pass pathways to relief the cells from the accumulation of misfolded proteins. The strategy of modulating the ERAD pathway as an approach of strain improvement for heterologous protein production requires a deeper knowledge on "if" and "how" these alternative mechanisms in filamentous fungi co-operate to deliver substrates for destruction.

Summary
&
Samenvatting
&
Supplementary material

Summary

In its natural habitat, *Aspergillus niger* usually grows on complex plant biopolymers. To be able to consume these nutrients, the fungus secretes a wide range of enzymes like amylases, xylanases, pectinases, cellulases, proteases, to break down the polymers into smaller molecules (monosaccharides or amino acids), that can be then taken up by the cell. The high secretion capacity of *A. niger* has drawn a lot of research towards the production of homologous and heterologous proteins to be used in different branches of the food, medical and textile industries. While the production yields of homologous protein are often high, the production of heterologous proteins has revealed more problematic. Several studies suggest that these problems usually arise post-translationally, within different steps of the secretory pathway. A major bottleneck is the protein synthesis and subsequent folding of secretory proteins that takes place in the ER. If the flow of new proteins into this organelle is higher than the rate at which the proteins are folded and delivered to the next organelle in the pathway, proteins begin to accumulate in the ER lumen, posing a risk to the cell. To counteract this stress, the UPR pathway is triggered, which results in activating the HacA transcription factor responsible for the induced expression of foldases and chaperones that support protein folding. At the ER, proteins also encounter quality control checkpoints that validate their aptness to continue in the secretory pathway. Misfolded proteins are recognized and targeted to the ERAD pathway to become degraded by the proteasome.

In this thesis we have focused our studies towards a better understanding of different processes involved in the protein secretion pathway that might act as bottlenecks for homologous and heterologous protein production. We have given particular attention to the molecular mechanisms of folding and quality control that take place in the ER, in order to be able to improve and develop new strategies for heterologous protein production by *A. niger*.

In **Chapter 1** a general outline is given of the secretory pathway of eukaryotes, with special attention to the UPR and ERAD pathways. Additionally, an overview of the commonly pointed bottlenecks that have been encountered on protein production, together with the current tools used by the modern science to broaden the knowledge on filamentous fungi. To generate gene knock-out mutants in filamentous fungi through homologous recombination is very time consuming which hampers the functional analysis of predicted genes from genome sequences. In **Chapter 2** we present several methods that will boast functional genomics in *A. niger*. We describe the construction of a set of strains where the *kusA* gene has been deleted, resulting in recipient strains in which deletions of essential and non-essential genes can be easily achieved, as shown in the case of the *ireA* and *hacA* genes, respectively. To overcome the disadvantages of having a compromised NHEJ pathway, we also describe the construction of a transiently disrupted *kusA* strain. For complementation of Δ*kusA* mutants we present an approach based on autonomously replicating plasmids, in which the mutant phenotype can be maintained or lost by regulating (on/off) the selective pressure. **Chapter 3** describes the cloning and functional characterization of the *A. niger gmtA* gene. Phylogenetic analysis showed that *gmtA* clusters together with well characterized GDP-mannose transporters from other fungal species and YFP tagging of GmtA proved its localization at Golgi equivalents. The isolation and characterization of a secretion related

small GTPase, *srgC*, is also described. The Δ*srgC* mutant phenotype showed reduced growth and the inability to form conidiospores at high temperatures and in addition, our microscopy results point to a role of SrgC in the organization, morphology and Golgi functions. **Chapter 4** describes the effect of the deletion of five genes involved in ERAD under i) normal growth conditions, ii) conditions of chemically induced ER stress and iii) by expressing an UPR-inducing heterologous protein (Glucoamylase-Gus). The results indicate that a functional ERAD pathway is not required for normal growth, but a defective ERAD pathway increases intracellular levels of the heterologous protein in study, indicating that this quality control pathway might impose a bottleneck on heterologous protein production by being (partially) responsible for their degradation. In **Chapter 5,** we have focused our study on i) the physiology of a genetically engineered *A. niger* strain that expresses only the activated form of the transcription factor HacA and ii) its transcriptome profile obtained using Affymetrix GeneChip analysis. GO enrichment analysis reflected a broader role of HacA in the cells, leading on one hand to the enrichment of secretory pathway processes (e.g.: folding and secretion) and an expansion of the endomembrane system ((phospho)lipid and inositol metabolism); on the other hand, a putative involvement of HacA on the negative regulation of extracellular enzymes expression (RESS mechanism) to counteract secretion stress. In the last Chapter (**Chapter 6**) we summarize and discuss the main conclusions of this thesis and look ahead on how our studies on UPR and ERAD might help us to further improve heterologous protein production in *A. niger*

Samenvatting

Aspergillus niger groeit in zijn natuurlijke omgeving op complexe plantaardige biopolymeren. Om deze voedingsstoffen te kunnen consumeren, scheidt de schimmel een verscheidenheid aan enzymen uit, zoals amylases, xylanases, pectinases, cellulases en proteases, om de polymeren af te breken in kleinere moleculen (monosacchariden of aminozuren) die door de cel kunnen worden opgenomen. De hoge secretie capaciteit van *A. niger* heeft er voor gezorgd dat er veel onderzoek wordt gedaan naar de productie van homologe en heterologe eiwitten die kunnen worden gebruikt in verschillende takken van de medische-, textiel- en voedingsindustrie. De hoeveelheid geproduceerde homologe eiwitten is vaak groot, terwijl de opbrengst van heterologe eiwitten vaak problematisch laag is. Verscheidene studies wijzen erop dat deze problemen post-translationeel zijn en plaatsvinden tijdens verscheidene stappen in de secretie route. Een van de grootste problemen is de eiwitsynthese en de hierop volgende vouwing van secretie eiwitten in het endoplasmatisch reticulum (ER). Als de stroom van nieuwe eiwitten groter is dan het tempo waarin deze eiwitten kunnen worden gevouwen en afgeleverd bij het volgende organel in de route, vindt er eiwit accumulatie plaats, wat stress veroorzaakt in de cel. Om deze stress tegen te gaan wordt de Unfolded Protein Response (UPR) route geactiveerd, resulterend in activatie van de transcriptie factor HacA, welke verantwoordelijk is voor de geïnduceerde expressie van foldases en chaperones betrokken bij eiwit vouwing. In het ER worden eiwitten ook gecontroleerd of ze geschikt zijn om de secretieroute in te gaan. Niet goed gevouwen eiwitten worden herkend en via de ER Associated Degradation (ERAD) route en afgebroken in het proteasoom.

In dit proefschrift hebben we onze studie gericht op het beter begrijpen van de verschillende processen die betrokken zijn bij eiwit secretie en mogelijk de oorzaak zijn van lage eiwitproductie. We hebben vooral onze aandacht gericht op het moleculaire mechanisme van eiwit vouwing en kwaliteitscontrole in het ER, om zo in staat te zijn nieuwe strategieën voor heterologe eiwitproductie in *A. niger* te ontwikkelen en te verbeteren.

In **Hoofdstuk 1** wordt een algemeen overzicht gegeven van de secretie route in eukaryoten, met speciale aandacht voor de UPR en ERAD routes. Bovendien worden de knelpunten besproken die zich voordoen bij eiwitproductie, samen met de huidige technieken die in de moderne wetenschap worden gebruikt om de kennis van filamenteuze schimmels te vergroten. Het verkrijgen van gen deletie mutanten door middel van homologe recombinatie is tijdrovend en hierdoor limiterend voor de functionele analyse van voorspelde genen in genoom sequenties. In **Hoofdstuk 2** presenteren we verschillende methoden die de functionele genomics in *A. niger* kunnen verbeteren. We beschrijven de constructie van een aantal stammen waarin het kusA gen is gedeleteerd, resulterend in stammen waar deleties van essentiële en non-essentiële genen gemakkelijk kan worden verwezenlijkt, zoals met de *ireA* en *hacA* genen aangetoond is. Om de nadelen van een gecomprimeerde NHEJ route te overbruggen beschrijven we ook de constructie van een omkeerbare *kusA* deletie stam. Voor complementatie van Δ*kusA* mutanten stellen we een benadering voor die gebruik maakt van autoreplicerende plasmiden waarbij het mutante fenotype behouden of verwijderd kan worden door regulatie van selectiedruk. **Hoofdstuk 3** beschrijft het kloneren en de

functionele analyse van het *A. niger gmtA* gen. Fylogenetische analyse laat zien dat *gmtA* clustert bij de uitgebreid gekarakteriseerde GDP-mannose transporters van andere schimmelsoorten en YFP-labeling van GmtA bewijst dat de locatie ervan bij Golgi equivalenten ligt. De isolatie en karakterisatie van een secretie gerelateerde kleine GTPase, *srgC*, is ook beschreven. Het fenotype van de Δ*srgC* mutant heeft verminderde groei en het onvermogen conidiosporen te vormen bij hoge temperaturen en daarbij laten onze microscopie resultaten zien dat SrgC een rol speelt in de organisatie, morfologie en Golgi functies. **Hoofdstuk 4** beschrijft het effect van de deletie van vijf genen die betrokken zijn in ERAD onder i) normale groei condities ii) condities van chemisch geïnduceerd ER stress en iii) door expressie van een UPR-geïnduceerd heteroloog eiwit (Glucoamylase-Gus). Het resultaat wijst erop dat een functionele ERAD route niet noodzakelijk is voor normale groei maar een defecte ERAD route verhoogt het intracellulair niveau van het onderzochte heterologe eiwit. Dit wijst erop dat deze kwaliteitscontrolerende route een knelpunt veroorzaakt voor heterologe eiwit productie door (gedeeltelijk) verantwoordelijk te zijn voor hun degradatie. In **Hoofdstuk 5** focussen we onze studie op i) de fysiologie van een genetisch gemanipuleerde *A. niger* stam dat alleen de geactiveerde vorm van transcriptiefactor HacA tot expressie brengt en ii) het transcriptoom profiel verkregen door het gebruik van Affymetrix GeneChip analyse. GO verrijkingsanalyse reflecteert een bredere rol van HacA in de cellen, leidend aan de ene kant tot verrijking van de secretie routes (bv. vouwen en secretie) en een expansie en van endomembraan systeem ((fosfo)lipide en inositol metabolisme); aan de andere kant een vermeende rol van HacA op de negatieve regulatie van extracellulaire enzym expressie (RESS mechanisme) om secretie stress tegen te gaan. In het laatste hoofdstuk (**Hoofdstuk 6)** geven we een samenvatting en discussie over de hoofdconclusies van dit proefschrift en kijken we verder naar hoe onze studies over UPR en ERAD ons kunnen helpen het heterologe eiwit productie in *A. niger* verder te verbeteren.

Supplementary material

The supplementary material of this thesis will be available via https://openaccess.leidenuniv.nl/handle/1887/1 and comprises the following:

Chapter 3:

Table S1. Primers used in this study.

Table S2. List of selected GDP-mannose transporters from a diversity of fungi. Protein sequences of these were used for the construction of a phylogenetic tree (Supplementary Fig. S1).

Figure S1. Phylogenetic tree of fungal GDP-mannose transporters from a selection of taxonomically diverse fungi.

Figure S2. Predicted transmembrane topology of the respective GDP-mannose transporters (GmtA) of *Aspergillus nidulans* and *Aspergillus niger*.

Figure S3. Southern analysis of *gmtA* deletion and respective complemented strain.

Figure S4. Phylogenetic tree of secretion related small GTPases from *Saccharomyces cerevisiae* (ScYPT/Sec4) and *Homo sapiens* (HsRab) and *Aspergillus niger*.

Figure S5. Southern analysis of YFP-GmtA integration at the *pyrG* locus and respective *gmtA* deletion.

Chapter 5:

Table S1A. All differentially expressed genes in $HacA^{CA}$. (Available only upon request: A.F.J.Ram@biology.leidenuniv.nl)

Table S1B. Overview of the 616 $HacA^{CA}$ up-regulated genes in the 3 time points.

Table S1C. $HacA^{CA}$ up-regulated genes that contain at least one UPRE sequence.

Table S2. GO analysis of biological processes enriched in the up-regulated set of genes in $HacA^{CA}$.

Table S3. GO analysis of biological processes enriched in the down-regulated set of genes in $HacA^{CA}$.

Table S4. Expression values of selected genes related to enriched GO terms of ER associated processes.

Table S5. Expression values of selected genes related to enriched GO terms associated with vesicle transport within the cell.

Table S6. Expression values of selected genes related to enriched GO terms associated with glycosylation processes.

Table S7. Expression values of selected genes related to enriched GO terms associated with lipid metabolic processes.

Table S8. Expression values of selected genes related to the GO terms “hydrolase activity”, “glutathione catabolic processes” and “vacuolar acidification”.

Table S9. Expression values of selected down-regulated genes related to enriched GO terms.

Table S10. Commonly induced and repressed genes in the HacA^{CA} strain and *A. niger* strains treated with DTT and Tunicamycin and expressing tPA (Guillemette *et al*., 2007).

Fig. S1. Growth profiles of *A. niger* HacA^{WT} and HacA^{CA} triplicate batch cultures.

Fig. S2: Network maps of related up-regulated GO-terms.

Fig. S3: Network maps of related down-regulated GO-terms.

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Publications & Curriculum Vitae

Publications

- **Carvalho ND**, Arentshorst M, Weenink XO, Punt PJ, van den Hondel CA, Ram AF (2011). Functional YFP-tagging of the essential GDP-mannose transporter reveals an important role for the secretion related small GTPase SrgC protein in maintenance of Golgi bodies in *Aspergillus niger*. Fungal Biol. 115:253-64.

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Curriculum Vitae

Neuza Daniela Silva Pinheiro Carvalho was born in April 9th, 1983 in Fafe, Portugal. She attended primary school at Escola Primária de Serafão, and high school at Escola EB 2,3 Carlos Teixeira and Escola Secundária de Fafe in the city of Fafe. From 2001 to 2005 she studied in the Universidade do Minho, Braga, Portugal where she completed a "Licenciatura" (Degree) in Applied Biology. While completing her degree, in March-July 2005 she did an Erasmus training period involved in the project "Inulin Modifying Network in *Aspergillus niger*: functional analysis of SucB and localization of InuR" under the supervision of Xiao-Lian Yuan (PhD student) and Dr. Arthur Ram in the Institute of Biology Leiden (IBL) at Leiden University in the Netherlands. In November 2005 she obtained her Degree in Applied Biology with the final classification of 16 (sixteen). From December 2005 until April 2010 she worked as a PhD student in the department of Molecular Microbiology and Biotechnology under the supervision of Dr. Arthur Ram and Prof. Dr. Cees van den Hondel in the Institute above mentioned, and the results are presented in this book.

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Neuza