Microbiology (2012), **158**, 46–57 **DOI** 10.1099/mic.0.053132-0

Review

The cargo and the transport system: secreted proteins and protein secretion in *Trichoderma reesei* (*Hypocrea jecorina*)

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Trichoderma reesei (*Hypocrea jecorina*) is an efficient cell factory for protein production that is exploited by the enzyme industry. Yields of over 100 g secreted protein l^{-1} from industrial fermentations have been reported. In this review we discuss the spectrum of proteins secreted by *T. reesei* and the studies carried out on its protein secretion system. The major enzymes secreted by *T. reesei* under production conditions are those degrading plant polysaccharides, the most dominant ones being the major cellulases, as demonstrated by the 2D gel analysis of the secretome. According to genome analysis, *T. reesei* has fewer genes encoding enzymes involved in plant biomass degradation compared with other fungi with sequenced genomes. We also discuss other *T. reesei* secreted enzymes and proteins that have been studied, such as proteases, laccase, tyrosinase and hydrophobins. Investigation of the *T. reesei* secretion pathway has included molecular characterization of the pathway components functioning at different stages of the secretion process as well as analysis of the stress responses caused by impaired folding or trafficking in the pathway or by expression of heterologous proteins. Studies on the transcriptional regulation of the secretory pathway have revealed similarities, but also interesting differences, with other organisms, such as a different induction mechanism of the unfolded protein response and the repression of genes encoding secreted proteins under secretion stress conditions.

Introduction

The filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) is used widely in the enzyme industry as a production organism (for reviews see Nevalainen & Penttilä, 2004; Nevalainen *et al.*, 2004; Penttilä *et al.*, 2004; Kubicek *et al.*, 2009). It is primarily used for production of its native cellulolytic and hemicellulolytic enzymes, but also for production of heterologous proteins. In addition, *T. reesei* has served as an important model organism of fungal lignocellulose degradation. For example, most of the cellulase and hemicellulase enzymes were identified and characterized for the first time at genetic level in *T. reesei* (e.g. Shoemaker *et al.*, 1983; Penttilä *et al.*, 1986; Teeri *et al.*, 1987), the molecular structures of these enzymes have been solved (e.g. Rouvinen *et al.*, 1990; Divne *et al.*, 1998) and the gene regulation mechanisms controlling the expression of these enzymes have been elucidated (Kubicek *et al.*, 2009; Aro *et al.*, 2005) from *T. reesei*. All the *T. reesei* strains used for research or protein production are derived from a single natural isolate. Extensive mutagenesis and screening programs have created a whole pedigree of strains with improved enzyme production properties. The genome of *T. reesei* was sequenced by the Joint Genome Institute (Martinez *et al.*, 2008); the sequence is about 34 Mbp and it comprises about 9100 predicted gene models. Both the genome size and the gene number are smaller than that observed for other filamentous fungi. This is mostly explained by the fact that *T. reesei* has very little redundancy in its genome.

During the last two decades, *T. reesei* has been used as a host to express numerous recombinant proteins (reviewed by Nevalainen *et al.*, 2005). Early examples of this work include two mammalian proteins: bovine chymosin (Harkki *et al.*, 1989) and antibody Fab fragments (Nyyssönen *et al.*, 1993). Chymosin was produced at about 40 mg l^{-1} and Fab fragments at the most at about 150 mg l^{-1}. The Fab production was greatly improved when the heavy chain gene was fused with the native cellobiohydrolase CBHI serving as a secretion carrier to the Fab. Different enzymes from fungi and bacteria have also been produced in *T. reesei*, and their production levels have often been in the g l^{-1} range. Examples of these include the *Hormoconis resinae* glucoamylase (Joutsjoki *et al.*, 1993), the *Trichoderma harzianum* chitinase (Margolles-Clark *et al.*, 1996), the xylanase from the bacterium *Nonomuraea flexuaosa* (Paloheimo *et al.*, 2003) and the cutinase from *Coprinopsis cinerea* (Kontkanen *et al.*, 2009). The production levels of recombinant enzymes do not match the amounts of native enzymes produced. It has been reported that yields of over 100 g native enzymes l^{-1} can be obtained in industrial production (Cherry & Fidantsef,

2003). To our knowledge, this level is not surpassed by any other protein production system.

The cellulolytic and hemicellulolytic enzymes find applications in many industrial sectors, including textile, food, feed, pulp, and paper and detergent industries. The recent trend of replacing the use of fossil raw materials in fuel and chemical production by lignocellulosics has attracted a lot of new interest on the lignocellulose degradation enzymes and their production systems. In this review we discuss the selection of extracellular proteins produced by *T. reesei* and the secretion system delivering these proteins to the outside of the cells.

Proteins secreted by *T. reesei*

The spectrum of enzymes secreted by *T. reesei* depends on the composition of its culture medium. When the fungus is grown in a medium with complex plant-derived substances such as cellulose or spent grain, it produces a complex enzyme mixture composed mostly of cellulases and hemicellulases. The main categories of cellulases are the cellobiohydrolases releasing cellobiose from the ends of the cellulose chain, endoglucanases cleaving the chain from the middle, and β-glucosidases converting the cellobiose and small oligosaccharides to glucose. The main categories of hemicellulases are the endoxylanases and endomannanases cleaving the main chains of xylan and mannan, respectively, and the side chain and substitution cleaving activities including arabinofuranosidases, α-glucuronidases, α-galactosidases and acetyl xylan esterases. These enzymes have been classified in carbohydrate active enzyme (CAzyme) categories (data publicly available at http://www.cazy.org/).

The genome content of the carbohydrate active enzyme categories of *T. reesei* has been analysed (Martinez *et al.*, 2008). It was observed that on average it has lower numbers of glycosyl hydrolases, carbohydrate esterases, polysaccharide lyases and carbohydrate-binding module-containing enzymes than the *Ascomycete* species with sequenced genomes. *T. reesei* has 200 genes encoding glycosyl hydrolases, and the glycosyl hydrolase families involved in cellulose or hemicellulose degradation clearly have less members in *T. reesei* than in the other filamentous fungi with sequenced genomes. Thus it was concluded by Martinez *et al.* (2008) that the reason for the good lignocellulose degradation capabilities of *T. reesei* is not the versatility of its enzyme system, but it may be the efficiency of its enzyme production machinery. It is also possible that *T. reesei* is specialized for degradation of only certain types of plant biomasses in nature, and does not need a very versatile enzyme system. As the inventory of hydrolytic enzymes in the genome has been extensively discussed by Martinez *et al.* (2008), we do not go into more detail in this review.

Apart from enzymes involved in lignocellulose degradation, a few other secreted proteins have been analysed from *T. reesei* at the molecular level. These include two proteases, the major aspartic protease named trichodermapepsin (homologue of *Aspergillus niger pepA*; Mäntylä *et al.*, 1998) and the major serine protease named trypsin-like serine protease 1 (TSP1; Dienes *et al.*, 2007). The *T. reesei* genome has six gene models encoding putative multicopper oxidases. One of these was expressed under the strong *cbh1* promoter and characterized (Levasseur *et al.*, 2010). It showed activity on the typical laccase substrates ABTS and syringaldazine. Laccases of *Bacidiomycetes* are involved in lignin degradation. However, the fact that *T. reesei* has a laccase gene does not necessarily mean that it could degrade or modify lignin; the enzyme might have a different function. In a similar way to the laccase, a putative tyrosinase gene found in the genome sequence was cloned and expressed under the *cbh1* promoter (Selinheimo *et al.*, 2006). This tyrosinase has been found to have applications as a cross-linking agent in food processing (Selinheimo *et al.*, 2007; Mattinen *et al.*, 2008). Neither the laccase nor the tyrosinase enzyme can be detected in the culture supernatant under standard culture conditions; they could only be characterized through overexpression experiments.

Hydrophobins are amphipathic proteins specific for filamentous fungi (reviewed by Linder, 2009). They are surface active proteins that are partially attached to the cell wall and partially secreted to the medium. There are six hydrophobin genes found in the *T. reesei* genome (Kubicek *et al.*, 2008), and three hydrophobins have been characterized at molecular level: HFBI (Nakari-Setälä *et al.*, 1996), HFBII (Nakari-Setälä *et al.*, 1997) and HFBIII (Kisko *et al.*, 2007). HFBI and HFBII appear to have diverse functions; HFBI is involved in hyphal development and HFBII in sporulation (Askolin *et al.*, 2005). Hydrophobins have been utilized successfully as purification tags of fusion proteins in aqueous two-phase separation (Linder *et al.*, 2004).

Proteome analysis of secreted proteins

Proteome analysis methods have been applied to elucidate the variety of extracellular proteins produced by *T. reesei.* Commercial enzyme mixtures produced by *T. reesei* have been analysed by using both 2D gel electrophoresis-based methods (Vinzant *et al.*, 2001; Fryksdale *et al.*, 2002) and mass spectrometry methods (Chundawat *et al.*, 2011) in order to obtain information about the protein components contributing to the measured enzyme activities in the preparations.

2D gel-based proteomics has also been used to analyse and compare proteins produced in the cultures of different strains of *T. reesei* (Herpoël-Gimbert *et al.*, 2008; T. M. Pakula and others, unpublished data). The majority of the secreted cellulases and hemicellulases predicted by the genome sequence (Martinez *et al.*, 2008; http://genome.jgi-psf.org/Trire2/Trire2.home.html) or previously characterized (Foreman *et al.*, 2003, and references therein) have been identified in the proteome analyses of the cultures of *T. reesei* (Table 1, Fig. 1). Cellulases and hemicellulases form the major part of the proteins produced, the major

cellulases (CBHI/Cel7A, CBHII/Cel6A, EGI/Cel7B, and EGII/Cel5A) being the most prominent ones in the secretome. Only a few other proteins have been described by the studies, including, e.g. a cellulose-binding and an expansin-type protein and a few examples of proteases.

T. reesei has gone through extensive strain development via mutagenesis, in order to improve protein production by the fungus. Comparison of the hypercellulolytic strains Rut-C30 and CL847, originating from different lineages of mutagenesis of the wild-type strain QM6a, revealed differences in the enzyme profiles produced by the strains, in both the quantity of the proteins and the number of proteins detected (Herpoël-Gimbert *et al.*, 2008). In general, the protein pattern produced by CL847 was more diverse, especially regarding the xylan-degrading activities and minor components of the protein mixture, whereas Rut-C30 was reported to produce more of the major cellobiohydrolase CBHI. The result was in accordance with the enzyme activities measured from the cultures, Rut-C30 was shown to produce more activity against the cellulosic substrates, filter paper and carboxy methyl cellulose, and CL847 was shown to produce more xylanase and β-glucosidase activities under the conditions studied.

We have carried out secretome analysis of a strain from which the genes encoding the major cellulases have been deleted and compared the produced protein pattern to the one produced by the parental strain (T. M. Pakula and others, unpublished data). Additional proteins normally masked by the cellulases were detected in the 2D gel analysis. 2D maps of the secretome of the strain with the *cbh1*, *cbh2*, *egl1* and *egl2* gene deletions and the parental strain with wild-type cellulase production are shown in Fig. 1. Comparison of the strains indicated that deletion of the abundantly expressed major cellulases favours production of other proteins in the culture medium. More abundant production of many other secreted proteins was detected in the deletion strain than in the parental strain. In particular, the production of the xylanase XYNIV, the β-glucosidase BGLI and the glucuronoyl esterase CIPII was increased in the deletion strain.

Many of the cellulases and hemicellulases are represented by several pI isoforms in the 2D gels (Fig. 1). Differences in glycan structures have been shown to contribute to the formation of different pI forms of CBHI (Pakula *et al.*, 2000) and acetylxylan esterase (Harrison *et al.*, 2002). *T. reesei* displays heterogeneous glycan structures on the

Table 1. Plant-cell-wall-degrading enzymes in the secretome analysis of *T. reesei*

CAZy family, Carbohydrate-Active enZymes, http://www.cazy.org/; Protein ID, http://genome.jgi-psf.org/Trire2/Trire2.home.html; Proteome study 1, Herpoël-Gimbert *et al.*, 2008; Proteome study 2, T. M. Pakula and others, unpublished data.

CAZy family	Protein ID	Short name	Description	Proteome study
GH7	123989	Cel7A/CBHI	Cellobiohydrolase	1, 2
GH6	72567	Cel6/CBHII	Cellobiohydrolase	1, 2
GH7	122081	Cel7B/EGI	Endo-1,4-glucanase	1, 2
GH5	120312	Cel5A/EGII	Endo-1,4-glucanase	1, 2
GH12	123232	Cel12A/EGIII	Endo-1,4-glucanase	1, 2
GH61	73643	Cel61A/EGIV	Endo-1,4-glucanase	1, 2
GH61	120961	Cel61B	Endo-1,4-glucanase	2
GH61	27554	–	Endo-1,4-glucanase	1
GH45	49976	Cel45/EGV	Endo-1,4-glucanase	2
GH74	49081	Cel74A	Xyloglucanase	1, 2
GH3	76672	BGLI	β-Glucosidase	1, 2
GH3	121127	BXLI	β-Xylosidase	1, 2
GH11	74223	XYNI	Xylanase	1, 2
GH11	123818	XYNII	Xylanase	1, 2
GH5	111849	XYNIV	Xylanase	1, 2
CE5	73632	AXEI	Acetyl xylan esterase	2
CE5	54219	–	Acetyl xylan esterase	1
GH54	123283	ABFI	α-L-Arabinofuranosidase	1, 2
GH62	76210	ABFII	α-L-Arabinofuranosidase	1, 2
GH54	55319	ABFIII	α-L-Arabinofuranosidase	1
GH5	56996	MANI	β-Mannanase	1, 2
GH27	72632	AGLI	α-Galactosidase	2
GH67	72526	GLRI	α-Glucuronidase	2
GH28	103049		Polygalacturonase	2
	123992	SWOI	Swollenin	1, 2
	73638	CIPI	Cellulose binding	1, 2
	123940	CIPII	Glucuronoyl esterase	1, 2

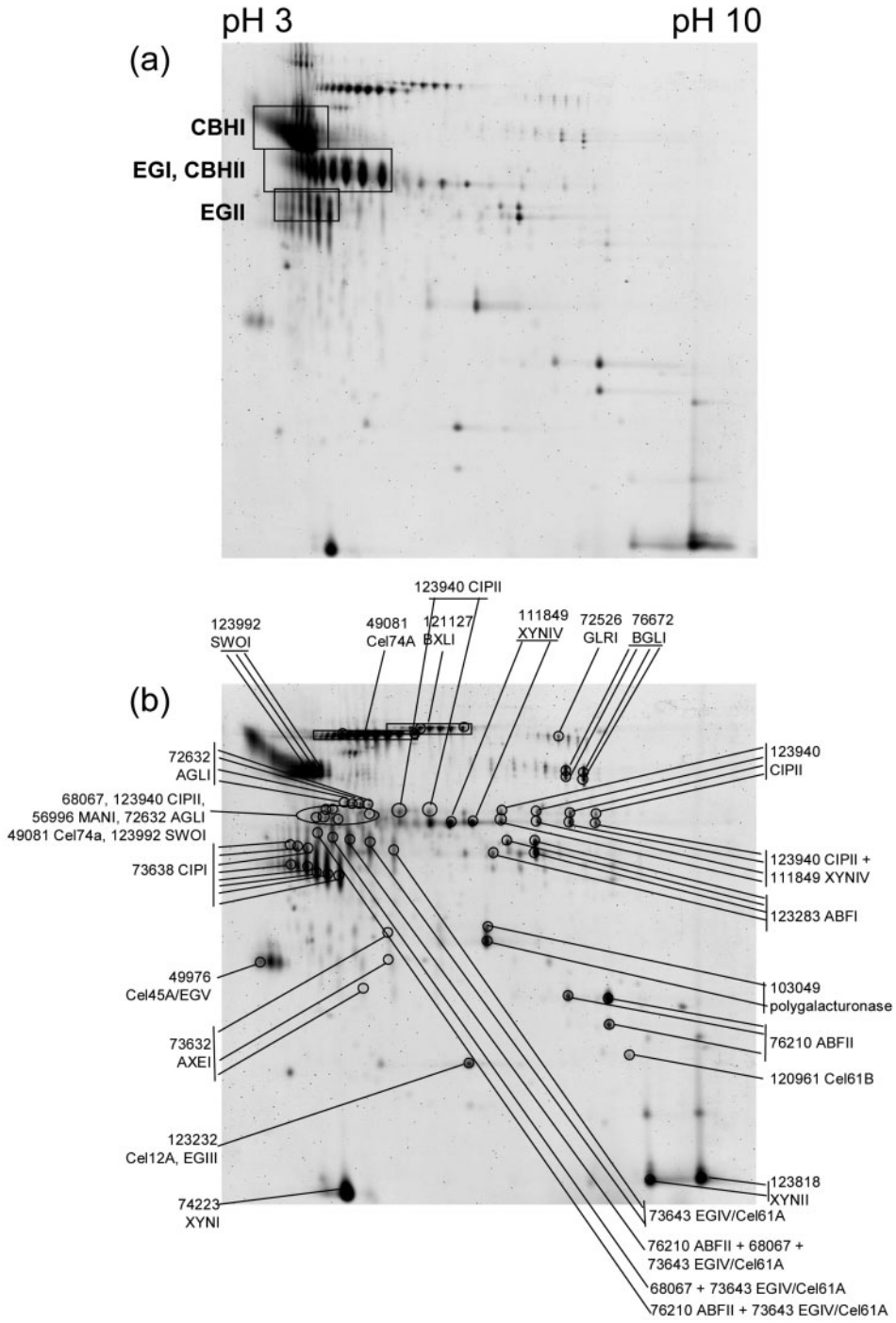

Fig. 1. 2D Gel electrophoresis analysis of the secreted proteins of *T. reesei*. Proteins secreted into the culture medium by a strain with wild-type major cellulase genes (a) and a strain with the major cellulase genes *cbh1*, *cbh2*, *egl1* and *egl2* deleted (b). The protein ID numbers are according to those listed on http://genome.jgi-psf.org/Trire2/Trire2.home.html.

proteins, and glycans modified with mannosyl phosphodieseter residues (Maras *et al.*, 1997; Stals *et al.*, 2004) are particularly likely to alter the pI of the protein. In addition, sulfation has been reported in the linker region of CBHI and acetylxylan esterase (Harrison *et al.*, 1998, 2002) which could also affect the pI.

Overview of protein secretion

Proteins destined to be secreted to the cell exterior are trafficked through the secretory pathway, a part of the endomembrane system of the cell. Along with secreted proteins, proteins destined for other cellular locations such

as the plasma membrane, vacuole/lysosome or the Golgi complex, are also transported by the endomembrane system (for reviews on the secretory pathway see Spang, 2008; Shoji *et al.*, 2008; Sallese *et al.*, 2009; Hutagalung & Novick., 2011; Conesa *et al.*, 2001).

Proteins are targeted to the secretory pathway by the signal sequence at their N terminus. In co-translational translocation, the newly translated signal sequence is recognized by the signal recognition particle (SRP), and translation is paused. The SRP directs the docking of the ribosome–mRNA–peptide ternary complex to the translocation complex on the membrane of the endoplasmic reticulum (ER). This complex is formed by multiple proteins including SEC61 that forms a channel through the ER membrane. When the docking has occurred, translation continues and the nascent polypeptide chain is translocated into the ER lumen simultaneously with the translation. In post-translational translocation the whole polypeptide chain is translated by the ribosome in the cytosol, and it is associated with the translocation complex through interaction with the SEC62–SEC72–SEC73 subcomplex. After this interaction, the polypeptide is translocated through the SEC61 channel into the ER lumen.

The most important function of the ER in the secretory process is the folding of the proteins into their final conformation. Folding is assisted by chaperones that bind to hydrophobic regions of the folding chain and prevent unfavourable interactions between these regions. The ER chaperones include members of the HSP70 family, and the most important one of these is BiP (binding protein). Many secereted proteins have disulphide bridges, and their formation is catalysed by the ER redox transfer system. The foldases interacting with the folding proteins belong to the protein disulphide isomerase (PDI) family or the ERV1 family and have thiol oxidase activity. The prolyl-peptidyl *cis*/*trans* isomerases (PPI) are another foldase group important for the folding of secreted proteins.

Simultaneously with the translocation and folding, many of the newly synthesized secreted proteins also become glycosylated. The core *N*-glycan is attached to an Asn residue by the oligosaccharyltransferase complex residing in the ER membrane. In lower eukaryotes the *O*-glycans are attached to the proteins in the ER. The ER has a sophisticated quality control system that monitors the folding state of the cargo proteins and only permits exit for fully folded ones. Misfolded or aggregated proteins are removed from the ER and degraded by the ER-associated protein degradation (ERAD) system (Bernasconi & Molinari, 2011).

After folding and core glycosylation of the secreted protein, its trafficking towards the cell exterior begins. This trafficking occurs via two vesicle transport steps; in the first one the protein is transferred from the ER to the Golgi complex and in the second one from the Golgi to the plasma membrane. In both of the transport steps the cargo protein is packaged inside membrane vesicles coated by specific protein coats. These vesicles are then transported to their destinations in a cytoskeleton-assisted process, and eventually the vesicles dock and fuse with the target membrane and release their contents to the Golgi matrix or outside the plasma membrane (Spang, 2008). The vesicle formation, transport and fusion processes are controlled by a high number of proteins and protein complexes. A part of these are general for all the membrane trafficking steps of the cell and others are specific for just one trafficking step. The general factors include the SEC17 and SEC18/NSF proteins. The trafficking-step-specific ones include, for example, the SAR1/ARF1 type small GTPases involved in vesicle budding, the Rab-type small GTPases involved in vesicle fusion (Hutagalung & Novick, 2011) and the v-SNARE- and t-SNARE-type proteins on the vesicle and on the target membrane, respectively. The SNARE proteins recognize and bind each other during the vesicle docking stage and provide specificity for each vesicle fusion event (Malsam *et al.*, 2008; Kienle *et al.*, 2009). Concerning the transport to the plasma membrane, a large protein complex called the exocyst is important for the localization of the fusion event on the membrane. This complex has multiple proteins including SEC3, SEC5, SEC6, SEC8, SEC10 and SEC15 (reviewed by He & Guo, 2009). A schematic view of the fungal secretion pathway and the components studied in *T. reesei* is shown in Fig. 2.

Protein secretion in *T. reesei*

Imaging and kinetic analysis of secretion

In the early stages of development of *T. reesei* as an enzyme production cell factory, improved production strains were created by random mutagenesis and selection programs. One of the most interesting strains obtained in this way at Rutgers University is Rut-C30 (Montenecourt & Eveleigh, 1979). Probably the first study on the protein secretory pathway of *T. reesei* was the electron microscopic analysis of the ER structures in Rut-C30 and the wild-type isolate QM6a performed by Ghosh *et al.* (1982). These authors discovered that ER was more abundant and more developed in Rut-C30 than in QM6a, and suggested that this would be one of the reasons for the better enzyme production capacity of Rut-C30.

The secretion of barley cysteine endopeptidase (EPB), the native CBHI, calf chymosin and CBHI–chymosin fusion protein has been investigated with immunofluorescence and immunoelectron microscopy. It was discovered that both the expression and secretion of the foreign protein EPB was different from the native CBHI (Nykänen *et al.*, 1997, 2002). EPB was detected only in apical compartments of the young hyphae, whereas CBHI could be visualized in all hyphae of a colony. In ultrastructural analysis of strains expressing calf chymosin, CBHI or chymosin fused with CBHI as a carrier, it was observed that a low percentage (4 %) of chymosin labelling was associated with the cell wall compared with labelling in secretory pathway structures inside the cell. The corresponding percentage was 83 % for CBHI and 49 % for the CBHI–chymosin

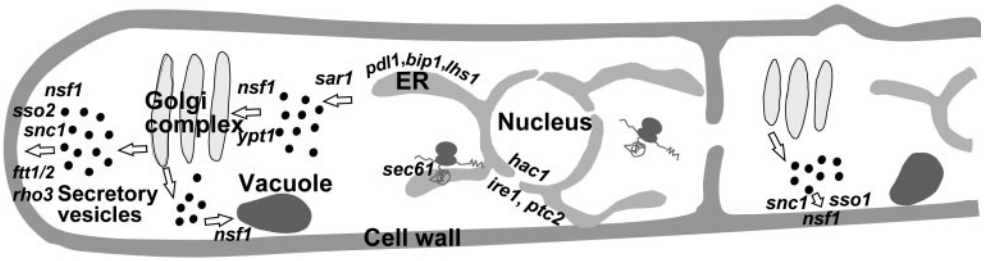

Fig. 2. A schematic view of the secretion pathway in filamentous fungi. The pathway components characterized from *T. reesei* at the genetic level are indicated. *hac1*, transcription factor of the UPR; *ire1*, sensor of the UPR; *ptc2*, phosphatase acting as a negative regulator of UPR; *sec61*, major component of the protein translocation complex in the ER membrane; *pdi1*, protein disulphide isomerase; *bip1*, *lhs1*, HSP70 family chaperones involved in protein folding in the ER; *sar1*, small GTPase involved in vesicle budding from the ER; *ypt1*, ras-type small GTPase involved in vesicle fusion into the Golgi; *nsf1*, general fusion factor involved in multiple vesicle fusion steps; *snc1*, v-SNARE protein involved in vesicle fusion to the plasma membrane; *sso1/2*, t-SNARE proteins involved in vesicle fusion to the plasma membrane; *ftt1/2*, 14-3-3 type proteins involved in the last step of the secretory pathway; *rho3*, ras-type small GTPase involved in cell polarity and vesicle fusion with the plasma membrane.

fusion protein (Nykänen, 2002). These studies would thus suggest that there are spatial restrictions in secretion of foreign proteins whereas native proteins are secreted abundantly from all parts of the mycelium, and fusion of the foreign protein with native CBHI can greatly enhance its secretion out of the cells.

The kinetics of protein secretion and processing in the secretory pathway has been addressed by *in vivo* labelling experiments by Pakula *et al.* (2000). According to this study, the average synthesis time of the CBHI cellobiohydrolase is 4 min, and its average secretion time is 11 min. The secretion time is actually longer than that measured with similar methodology in *Saccharomyces cerevisiae* and another filamentous fungus. Thus, it was concluded that the reason for the good secretion capacity of *T. reesei* is not the speed of secretion; it may rather be the large capacity of the secretion pathway. The formation of the different pI forms of CBHI was also studied by Pakula *et al.* (2000). It was observed that the least acidic pI form is formed first, and as it was the only form present in cells treated with DTT, a folding inhibitor, it was concluded to be the ER form of CBHI. More acidic pI forms appear after the ER form, and deglycosylation experiments showed that these pI forms originate from modifications in the glycan structures of CBHI. Protein production efficiency at different specific growth rates in chemostat cultures has also been studied (Pakula *et al.*, 2005). This study indicated that both the synthesis and the secretion of CBHI and other secreted proteins were most efficient at relatively low growth rates around 0.03 h^{-1}. However, at low growth rates, the secretion rate became the limiting factor for CBHI production. In accordance with this, the genes involved in protein folding in the ER, *pdi1* and *bip1* had higher transcript levels at low growth rates. It was thus suggested that the cell needs to adjust its ER folding capacity according to the growth rate.

Molecular characterization of secretion pathway components

The gene encoding the *T. reesei* protein disulphide isomerase (*pdi1*) was cloned by degenerate PCR and characterized (Saloheimo *et al.*, 1999). The sequence has the typical signatures of protein disulphide isomerases, a signal sequence, an ER retention signal and two canonical WCGHCK active site sequences. It was shown that the *pdi1* cDNA can complement disruption of the *S. cerevisiae PDI1* gene. The transcriptional regulation of the *pdi1* gene was investigated in this study. It was shown that the gene is induced in cells treated with the folding inhibitor dithiothreitol (DTT) and the glycosylation inhibitor tunicamycin, and in cells expressing antibody Fab fragments. These findings indicated that the gene is under regulation of the unfolded protein response (UPR; discussed below), in accordance with the finding of putative canonical UPR regulatory elements in the promoter sequence. Interestingly, it was also discovered that *T. reesei pdi1* is regulated according to the available carbon source, being highly expressed in media where cellulase and hemicellulase genes are strongly induced. A similar result was also obtained with the help of cDNA arrays by Foreman *et al.* (2003), who observed that *pdi1* was induced by lactose and sophorose, while 12 other genes involved in folding, glycosylation and vesicle transport were induced only by sophorose.

SAR1 is a small GTP-binding protein involved in budding of transport vesicles from the ER membrane (Nakańo & Muramatsu, 1989). The *T. reesei sar1* gene was cloned by hybridization with the *Aspergillus niger sarA* gene (Veldhuisen *et al.*, 1997). It was shown in this study that the *T. reesei* and *A. niger sar1/A* genes can complement the *S. cerevisiae sar1* mutation. Yeast *SAR1* was originally cloned as a multicopy suppressor of the gene encoding its guanine-nucleotide exchange factor, SEC12. It was shown by Veldhuisen *et al.* (1997) that the *T. reesei* and *A. niger sar1/*

A genes can also suppress the yeast *sec12* mutation. *T. reesei sar1* was recently found to be a good choice for normalization of gene expression data obtained by quantitative PCR (Steiger *et al.*, 2010), and thus it can be considered to be constitutively expressed in many conditions.

In another study, the *T. reesei* and *A. niger* var. *awamori ypt1/A* and *nsf1/A* genes were examined (Saloheimo *et al.*, 2004). YPT1 is a small GTP-binding protein belonging to the Rab family. It functions in the fusion step of ER-derived transport vesicles with the Golgi membrane. It promotes the formation of a large protein complex including the v-SNARE and t-SNARE proteins that provides specificity to the fusion of the vesicles with the correct target membrane (Søgaard *et al.*, 1994). The *nsf1/A* gene and the *S. cerevisiae* counterpart *SEC18*, on the other hand, encode a general membrane fusion factor acting in all vesicle fusion steps in the cell (Wilson *et al.*, 1989). It is an ATPase that promotes the fusion of the vesicle and target membranes after the recognition step where the Rab proteins are active. The *T. reesei ypt1* gene was isolated by heterologous hybridization with *S. cerevisiae SEC4* as a probe. SEC4 is a Rab protein functional in the fusion of secretory vesicles with the plasma membrane, and is highly similar to the YPT1 proteins. The *ypt1* of *T. reesei* and *yptA* of *A. niger* var. *awamori* were also cloned independently in complementation screenings in a yeast *sso2* temperature-sensitive mutant strain, and were able to rescue the temperature-sensitive phenotype. Sso2 is a t-SNARE protein involved in the fusion of vesicles with the plasma membrane (Aalto *et al.*, 1993) and thus it was unexpected that *ypt1/A* genes could complement it. The identity of the *T. reesei ypt1* was verified by showing that it can complement the depletion of the *S. cerevisiae YPT1* gene. The *T. reesei nsf1* gene was isolated by heterologous hybridization with the *S. cerevisiae SEC18* probe. The *A. niger* var. *awamori nsfA* gene was subsequently isolated by hybridization with the *T. reesei nsf1*. Complementation of the yeast *SEC18* temperature-sensitive mutant with *T. reesei nsf1* cDNA was attempted but was not successful. This is not unexpected, since the sequence identity between the yeast and *T. reesei* amino acid sequences is only 50 %. The *T. reesei snc1* gene encoding the v-SNARE involved in vesicle fusion was cloned in the same complementation screening as the *ypt1/A* genes, where it could suppress the temperature-sensitive mutation of the yeast t-SNARE gene *SSO2* (Valkonen, 2003; Valkonen *et al.*, 2007). In this case, the suppression was expected, because SNC and SSO proteins work in the same fusion event. The *T. reesei snc1* gene was shown to complement the yeast SNC protein depletion (Valkonen *et al.*, 2007).

The transcriptional regulation of the *T. reesei ypt1*, *sar1*, *nsf1* and *snc1* genes involved in vesicle trafficking has been investigated by Northern blotting (Valkonen, 2003; Saloheimo *et al.*, 2004). It was shown that a part of the trafficking genes is induced in cells treated with the folding inhibitor DTT. This induction was relatively low in the Rut-C30 strain and was absent in the case of the *ypt1* and *sar1* genes. All the examined genes showed induction with DTT in the QM9414 strain, along with *pdi1*, that was induced more strongly by far. When cells were treated with Brefeldin A, an agent blocking vesicle traffic at the level of the Golgi complex, all the trafficking genes were induced to a large extent, even in the Rut-C30 strain. These results are consistent with findings made in yeast that a part of the trafficking machinery is induced by the UPR (Travers *et al.*, 2000). However, the *T. reesei* results suggest that there might be a second stress response pathway regulating the membrane trafficking machinery, triggered in this case by the BFA treatment.

The spatial distribution and interactions of the t-SNARE proteins SSO1 and SSO2 and the v-SNARE protein SNC1 were studied with live cell imaging by Valkonen *et al.* (2007). They constructed *T. reesei* strains expressing fusion proteins of these SNAREs with fluorescent marker proteins and studied them using advanced fluorescence microscopy methods. The SSO1 and SNC1 proteins co-localized and formed a complex on the plasma membrane in subapical but not in apical cells, and SNC1 was also found in vesicle clusters of the Spitzenkörper (Fig. 3). SSO2–SNC1 complexes were only found in the growing apical hyphal compartments. The results indicate distinct localizations and roles for the two t-SNARE proteins SSO1 and SSO2 in *T. reesei*. Interestingly, Hayakawa *et al.* (2011) have observed that part of the *A. oryzae* α-amylase was transported to the septa and secreted there to the periplasmic space. This supports the idea that secretion also occurs in other regions besides the hyphal tip (Read, 2011).

The system maintaining cell polarity is closely connected with the secretory pathway and with the localization of secretion on the plasma membrane. The site of secretion is regulated in *S. cerevisiae* by the exocyst complex which has many components, including Sec15p (He & Guo, 2009). When a *T. reesei* cDNA library was screened in a temperature-sensitive *sec15* mutant yeast strain, the *rho3* and *ftt1* genes were cloned as suppressors of the mutation (Vasara *et al.*, 2001a, 2002). RHO3 is a Ras superfamily small GTP-binding protein involved in regulation of actin cytoskeleton functions and is associated with the exocyst complex (Matsui & Toh-E, 1992). *ftt1* is a member of the 14-3-3 family of proteins that have been implicated in many cellular functions including secretion. It was shown that the *T. reesei* RHO3, FTT1 and FTT2 proteins can suppress a number of other yeast secretory mutants in addition to *sec15* (Vasara *et al.*, 2001b, 2002). Disruption of the *rho3* gene from *T. reesei* caused a clear reduction in the amount of secreted protein production, demonstrating its role in the secretory process (Vasara *et al.*, 2001b).

Analysis of the secretion pathway components in the genome sequence of the *T. reesei* natural isolate strain QM6a has shown that most of the genes are present in this species as single copies (Martinez *et al.*, 2008). The few exceptions to this rule include the protein disulphide isomerase (PDI), for which there are three homologues,

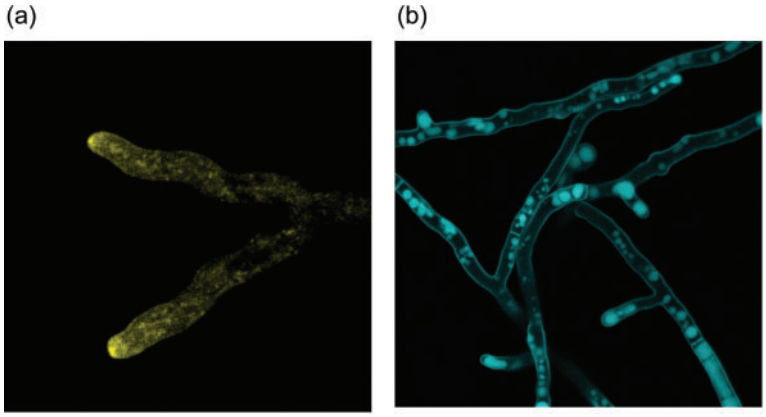

Fig. 3. Localization studies of the v-SNARE protein SNC1 and the t-SNARE protein SSO1 using fluorescent marker protein fusions. (a) Expression of the SNCI-Venus fusion protein in *T. reesei* cells shows that localization of the fusion protein is more pronounced in structures that are possible transport vesicles on the plasma membrane. (b) The SSOI-mCerulean fusion protein shows a uniform localization over the plasma membrane in the older parts of the hyphae. In actively growing hyphal tips, the expression of the fusion protein was either not observed or was seen at very low levels. The figure was kindly provided by Mari Valkonen (VTT Technical Research Centre).

and the *der1* and *ufd1* components of the ERAD pathway that are present in two copies. *T. reesei* has orthologues for the mammalian Rab2, Rab4, Arf6 and Arf10 small GTP binding proteins that are absent in *S. cerevisiae*. As described above, there are also two homologues for the t-SNARE SSO1, and these appear to have distinct functions. Analysis of the genome sequence can provide very little insight into the excellent secretion capacity of *T. reesei*. However, having several PDI copies is consistent with the fact that *T. reesei* is able to secrete massive amounts of proteins with multiple disulphide bridges.

Stress responses of the secretory pathway

As mentioned above, the cells respond to improper folding of secreted proteins in the ER by activating the UPR pathway (reviewed by Hetz & Glimcher, 2009; Malhotra & Kaufman, 2007). This means that when misfolded proteins accumulate in this compartment, the genes encoding ER chaperones and foldases, the ER-associated degradation pathway and a part of the trafficking and glycosylation machineries are induced. The mechanism of UPR activation has been studied in *S. cerevisiae* and mammalian cells, and it has been shown to be quite unlike other signalling pathways of the cell. The UPR pathway and its activation mechanism has been investigated in *T. reesei*, and it has been shown to have both similar and different features compared with other organisms.

When unfolded proteins accumulate in the ER, they bind the ER chaperone BiP, and less BiP can be bound to the IRE1 protein residing in the ER membrane. This activates the UPR pathway. The *ire1* gene was cloned from *T. reesei* and shown to be able to complement the *IRE1* gene disruption in *S. cerevisiae* (Valkonen *et al.*, 2004). The IRE1 protein was expressed in *Escherichia coli* and shown to be phosphorylated in an autophosphorylation assay. Phosphorylation is necessary for the activation of IRE1. In an IRE1 overexpression *T. reesei* strain, the transcript levels of a number of target genes were increased, including *pdi1*, the ER chaperone genes *bip1* and *lhs1*, the glycosylation pathway gene *pmi40*, the translocation channel gene *sec61* and the lipid biosynthesis gene *ino1*. This demonstrated the wide regulatory range of the UPR. The *ire1* gene was overexpressed in a strain expressing the *Phlebia radiata* laccase, but this overexpression did not alter the amount of laccase produced. On the other hand, improved heterologous protein production by UPR activation could be demonstrated in *S. cerevisiae* (Valkonen *et al.*, 2003a) and *Aspergillus niger* var. *awamori* (Valkonen *et al.*, 2003b). The *T. reesei ptc2* gene encoding a phophatase that dephosphorylates IRE1 and reduced UPR, was also cloned and shown to complement the yeast *PTC2* deletion (Valkonen *et al.*, 2004).

The transcription factor mediating the UPR at promoter level is HAC1 (Cox & Walter, 1996). It has been shown to be activated in *S. cerevisiae* through a unique mechanism; a 250 bp intron is spliced off from its mRNA upon UPR activation. The intron borders are cleaved by the activated IRE1 protein with its RNase activity, and the exons are ligated back together by the tRNA ligase (Cox & Walter, 1996; Sidrauski *et al.*, 1996). When the intron is in the mRNA, it attenuates translation of this transcript. Removal of the intron releases the mRNA from this attenuation and also changes the open reading frame and the spliced mRNA encodes the active transcription factor. The *T. reesei hac1* and *Aspergillus nidulans hacA* genes were cloned, and it was discovered that upon UPR activation in *T. reesei* and *A. nidulans*, an intron of 20 nt is spliced from the *hac1/A* mRNA (Saloheimo *et al.*, 2003). In addition to this, the *hac1/A* mRNA is truncated by about 200 nt at the 5′ flanking region. When this occurs, an upstream open reading frame (uORF) is left out from the mRNA. These results suggest that *hac1/A* induction in *T. reesei* and *A. nidulans* occurs through a dual mechanism including intron splicing that restores an active open reading frame and use of an alternative transcription start site which omits a uORF that inhibits the use of the correct translation start site. In mammals, the UPR transcription

factor corresponding to HAC1 is XBP-1. Its activation involves splicing of a short intron but not truncation at the 5′ flanking region (Yoshida *et al.*, 2001). It was further shown in this study that *T. reesei hac1* can complement the yeast *HAC1* and *IRE1* deletions, and that yeast Ire1p is able to cleave the *T. reesei hac1* mRNA at the intron site, but the exon ligation reaction is not working for it (Fig. 4).

While studying the effects of folding and secretion inhibitors on *T. reesei*, a novel type of secretion stress response was discovered (Pakula *et al.*, 2003). When cells were treated with DTT, Brefeldin A or A23187 that perturbs the Ca^{2+} homeostasis and inhibits protein folding and/or exit from the ER, the transcript levels of the major cellulase and xylanase genes declined rapidly, resulting in reduced synthesis of these proteins. DTT treatment was also done on strains carrying a marker gene either under the full-length *cbh1* promoter or a truncated *cbh1* promoter where regions upstream of position −161 had been deleted. The level of the marker gene mRNA did not decrease upon DTT treatment in the case of the truncated promoter, suggesting that the downregulation of the mRNA took place at the transcriptional level and was mediated by the region upstream from −161. This kind of secretion stress response has not been discovered in other organisms except *A. niger* (Al-Sheikh *et al.*, 2004), and thus it may be specific to filamentous fungi. The novel pathway was named REpression under Secretion Stress (RESS). It can be seen as an additional way for the cells to deal with having unfolded proteins in the ER, by shutting down the synthesis of new protein into the ER at the transcriptional level.

The regulatory range of secretion stress responses has been investigated in *T. reesei* cells treated with DTT and in a strain expressing the human tissue plasminogen activator and a strain overexpressing the *ire1* UPR sensor protein (Arvas *et al.*, 2006; Valkonen *et al.*, 2004). This study was performed before the *T. reesei* genome sequence was available, and therefore it was done by cDNA subtraction and cDNA AFLP methodologies. Over 400 genes induced under secretion stress conditions were identified, including the expected folding-related genes and some of the vesicle trafficking genes. In addition, the *cpc1* gene implicated in amino acid synthesis regulation, a number of genes involved in amino acid and glutathione metabolism and histone genes in yeast were found to be induced by secretion stress. Thus, the regulatory range of secretion stress responses has both similar and distinct features compared with *S. cerevisiae*.

The regulation of genes encoding the major cellulases and the folding-related genes were studied by Collén *et al.* (2005) in a two-phase fermentation where cells were first grown on glucose and after its exhaustion the cellulase expression was induced by adding a batch of lactose to the medium. After the medium change, the major cellulase genes were induced to high levels and declined again when lactose in the culture was running out. The *pdi1* and *bip1* gene transcript levels followed closely the pattern of the cellulase gene expression. Interestingly, during the induction phase, the induced, truncated form of the *hac1* mRNA was transiently observed in the Northern blots. This study showed that folding and secretion capacity of the ER is tightly regulated according to the protein load in the ER, and that UPR is one of the regulatory pathways mediating this regulation.

Conclusions

We have discussed the extracellular enzymes and other proteins produced by *T. reesei* and the secretion machinery in this review. Most studies of the produced proteins have concentrated on the lignocellulose degradation enzyme system. Compared with most other fungi, it is less versatile in *T. reesei*, including fewer cellulase and hemicellulase enzymes. Our previously unpublished results of 2D gel extracellular proteomics analysis showed that when the major cellulase enzyme genes are deleted, a number of other enzymes are produced at higher levels. Future work on the secreted enzymes of *T. reesei* will likely include the discovery and investigation of novel minor enzymes for the biorefinery as well as other application fields.

The studies on the *T. reesei* secretory pathway have included imaging and kinetics analysis of secretion,

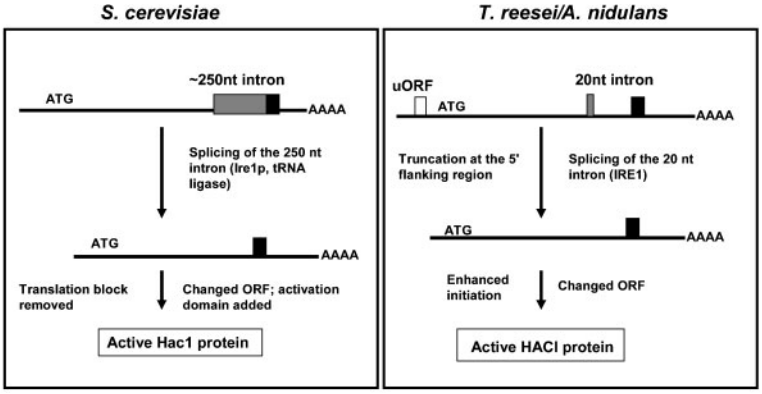

Fig. 4. A schematic presentation of the activation mechanisms of the UPR transcription factor HAC1 in yeast and in *T. reesei* and *A. nidulans*. The unconventional intron is shown as a grey box, the activation domain as a black box and the upstream ORF as an open box.

molecular characterization of the secretory pathway components and analysis of their transcriptional regulation. The individual secretion pathway components generally show good conservation with other organisms, and a number of them can complement their counterparts in *S. cerevisiae*. Kinetics analysis has indicated that the secretion event is not faster in *T. reesei* than in other species, but it is likely that the capacity of the secretion machinery is a reason for the good secretion capability. The studies on the transcriptional regulation of secretion have revealed interesting new mechanisms and a novel secretion stress response, RESS. It has been shown that *T. reesei* can adjust the efficiency of folding and protein traffic in the secretion pathway, and it can also limit the amount of cargo protein synthesis if folding or secretion capacity is limited. Future directions in the field might include more delicate analysis of the secretion event and secretory pathway components by imaging and further elucidation of the transcriptional regulation mechanisms. An interesting line would be to connect the research of secretion more closely with the protein production bioprocess.

Acknowledgements

We thank Terhi Puranen and Jari Vehmaanperä (Roal Oy, Rajamäki, Finland) for collaboration in the secretome analysis of the cellulase deletion strain. LC-MS/MS identification of the proteins was done by Heini Koivistoinen, Aili Grundström is acknowledged for excellent technical assistance and we thank Mari Valkonen for kindly providing Fig. 3 (all VTT Technical Research Centre).

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