Secretory pathway of the filamentous fungus *Trichoderma reesei*

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Abstract

Bottlenecks for overproduction of proteins in filamentous fungi possibly exist within the secretory pathway, therefore better understanding of this pathway is a key to achieving better yields. “Visible” data with high spatial and temporal resolution of morphology of the hyphal compartments, protein localisation, expression and secretion would need to be added to the existing knowledge to help understand protein secretion and to devise strategies for the improvement of protein production. A series of expression plasmids/cassettes containing a gene encoding the fluorescent protein(s) GFP2 and/or VenusYFP alone or fused to the ER-resident folding chaperone Bip1 and the main cellulbiohydrolase I (CBHI) were constructed and introduced into a *Trichoderma reesei* strain Rut C-30. A transformant strain BV47 expressing the Bip1-Venus fusion protein was applied to visualise the endoplasmic reticulum and potential changes in the ER during Bip1-Venus overexpression. A transformant strain CV48 secreting the main cellulbiohydrolase I of *T. reesei* fused with VenusYFP was used to monitor secretion of the CBHI-Venus fusion protein. In order to investigate the potential interaction between the Bip1 and the secretory protein CBHI, a GFP2/VenusYFP FRET pair system was developed. In the developed FRET system, the transformant strains BG29 expressing Bip1-GFP2, CV48 expressing CBHI-Venus, VG15 expressing Venus-GFP2 and BGCV101 coexpressing Bip1-GFP2 served as the donor, the acceptor, the positive FRET control and the FRET sample, respectively.

The ER in the host strain *T. reesei* Rut C-30 was visualised as a typical network of parallel tubular membranes and some punctate-like bodies through the hyphae. The ER structure in the transformant BV47 expressing the Bip1-Venus fusion protein appeared unusual with an abundance of punctate structures and fewer tubular membranes demonstrating modified spatial organisation of the ER, different to what has been seen in other filamentous fungi studied so far. This type of modification of the ER may assist in forming an ER sub-domain to which overproduced and potentially misfolded proteins can be deposited to wait for further processing. The ER structural modifications appeared to have been caused by overproduction of the BiP1-Venus fusion protein. In addition to the changes in the ER morphology in *T. reesei*, it was also noted that BiP1 appeared to have escaped from the ER and become secreted into the culture
medium, possibly due to overloading of the ER retention capability. Light microscopy and immunoelectron microscopy studies confirmed that the Golgi apparatus in *T. reesei* appeared in punctate bodies that were not surrounded with obvious membranes. The Golgi membrane invisibility could be associated with the chemical fixation method used in this study which failed to preserve the delicate Golgi membranes. The morphological characteristics of the Golgi apparatus in *T. reesei* observed in this study were different to those previously reported for *T. reesei* and yeast and were likely due to different methods for sample preparation and observation i.e. immunoelectron microscopy staining or ultrastructural observation without specific staining.

Secretion of the CBHI-Venus fusion protein in the *T. reesei* transformant CV48 was tracked both intracellularly and extracellularly. Intracellular fluorescence of CBHI-Venus in CV48 was detected at the 12 h time point and was in line with the detection of *cbh1-venus* transcript at 12 h. There was a 6 h time lag between the first presence of intracellular fluorescence and the detectable level of CBHI-Venus in the culture supernatant at 12 h. Subcellular localisation of the fusion protein was studied by both light microscopy and immunoelectron microscopy. In addition to association with a typical ER network, the CBHI-Venus protein was found localised in distorted ER membrane structures assumed as ER-derived sub-domains similar to what seen in the BV47 overexpressing BiP1-Venus at both 24 and 48 h. The modification of the ER organisation and formation of ER sub-domains in the CV48 transformant with at least two copies of *venus* gene was assumed as a result of overexpression of the fusion protein. At the early culture stages of 24 and 48 h, CBHI-Venus also localised in the vesicles and Golgi bodies. After 24 h, the protein concentrated into the secretory vesicles for transport. Interestingly, the fusion protein was retained in the cell wall since 72 h, which was possibly the cause for reduction of secretion into the culture medium at the later stages of cultivation. Vacuolation of the hyphae occurred at 120 h. In general, secretion of the fusion protein CBHI-Venus follows the conventional secretory pathway through the ER to Golgi via secretory vesicles.

In this study, a FRET system was developed to analyse the interaction between the ER-resident chaperone BiP1 and the highly secreted protein CBHI using GFP2 and VenusYFP as fusion partners to CBHI and BiP1. The positive control, transformant VG15 expressing *venus-gfp2* demonstrated positive FRET signals although at a considerably low level. However the
transformant BGCV101 coexpressing BiP-GFP2 and CBHI-Venus did not show significant FRET efficiency. This observation could be a result of multiple reasons as discussed in the thesis. The FRET work conducted here will aid in developing an efficient FRET tool for studying protein interactions in living filamentous fungi.
Statement of Candidate

I certify that the work in this thesis entitled “Secretory pathway in the filamentous fungus *Trichoderma reesei*” has not been previously submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

Hong Yu (student No.: 40619249)
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## Abbreviations

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<th>Full name</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>BFP</td>
<td>blue fluorescent protein</td>
</tr>
<tr>
<td>BG transformant</td>
<td><em>T. reesei</em> transformant expressing <em>bip1-gfp2</em></td>
</tr>
<tr>
<td>BGCV transformant</td>
<td><em>T. reesei</em> transformant coexpressing <em>bip1-gfp2/cbh1-venus</em></td>
</tr>
<tr>
<td>BiP</td>
<td>binding protein</td>
</tr>
<tr>
<td><em>bip</em></td>
<td>gene encoding BiP</td>
</tr>
<tr>
<td><em>BiP1-Venus</em></td>
<td>DNA encoding BiP1-Venus</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BV transformant</td>
<td><em>T. reesei</em> transformant expressing <em>bip1-venus</em></td>
</tr>
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<td><em>cbh1</em></td>
<td>gene encoding CBHI</td>
</tr>
<tr>
<td>CBHI</td>
<td>cellobiohydrolase I</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>CLS medium</td>
<td>medium containing minimal salts and 1 % cellobiose, 1 % lactose and 3 % soy hydrolysate</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
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<td>Abbreviation</td>
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<tr>
<td>C-terminal</td>
<td>carboxy-terminal</td>
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<td>CV transformant</td>
<td><em>T. reesei</em> transformant expressing <em>cbh1-venus</em></td>
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<td>electron microscopy</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>fluorescence resonance energy transfer</td>
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<td>green fluorescent protein</td>
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<tr>
<td>h</td>
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<tr>
<td><em>hphB</em></td>
<td>gene encoding hygromycin B phosphotransferase</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IEM</td>
<td>immune electron microscopy/immunoelectron microscopy</td>
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<tr>
<td>Acronym</td>
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<tr>
<td>K/HDEL</td>
<td>lysine/histone-aspartic acid-glutamic acid-leucine motif</td>
</tr>
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<td>kDa</td>
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</tr>
<tr>
<td>kPa</td>
<td>kilopascal</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>M</td>
<td>moles per litre</td>
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<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MM</td>
<td>medium containing minimal salts</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
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<td>mRNA</td>
<td>messenger-ribonucleic acid</td>
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<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
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<td>pBGt</td>
<td>plasmid DNA containing BiP1-encoding sequence (bip1), the GFP2-encoding sequence (gfp2) and truncated cbh1 terminator sequence (tt)</td>
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<td>phosphate buffered saline</td>
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<td>plasmid DNA containing Bip1-encoding sequence (bip1), the Venus-encoding sequence (venus) and truncated cbh1 terminator sequence (tt)</td>
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<tr>
<td>pCVt</td>
<td>plasmid DNA containing CBHI-encoding sequence (cbh1), the Venus-encoding sequence (venus) and truncated cbh1 terminator sequence (tt)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDA</td>
<td>potato dextrose agar</td>
</tr>
<tr>
<td>pDNA</td>
<td>plasmid DNA</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>pki</td>
<td>gene encoding pyruvate kinase</td>
</tr>
<tr>
<td>pVGt</td>
<td>plasmid DNA containing Venus-GFP2-encoding sequence (venus-gfp2) and truncated cbh1 terminator sequence (tt)</td>
</tr>
<tr>
<td>QM6a</td>
<td>wild type strain of <em>T. reesei</em></td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Rut C-30</td>
<td>high protein secreting mutant strain of <em>T. reesei</em></td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SNARE</td>
<td>SNAP receptor</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
</tr>
<tr>
<td>ss</td>
<td>signal sequence</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate-EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>Tris</td>
<td>[2-amino-2-(hydroxymethyl) propane-1,3-diol, (tris)]</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>t-SNARE</td>
<td>SNARE on target membrane</td>
</tr>
<tr>
<td>tt</td>
<td>truncated transcription terminus</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>venus</td>
<td>gene encoding VenusYFP</td>
</tr>
<tr>
<td>VG transformant</td>
<td><em>T. reesei</em> transformant expressing <em>venus-gfp2</em></td>
</tr>
<tr>
<td>v-SNARE</td>
<td>SNARE on vesicles</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
</tbody>
</table>
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Beric Henderson, Dr. David Facey, Dr. Greg Kaplan, Dr. Myth Mok, Mr. Dong Zheng, Dr. Jianhua Wang, Dr. Weiyi Zhang, Ms Yan Sun and many others.

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Chapter 1 Introduction

Filamentous fungi have an excellent capability of secreting high levels of proteins and hydrolytic enzymes, especially cellulases and amylolytic enzymes into the extracellular culture medium (Mandels and Reese, 1957; Bailey and Nevalainen, 1981; Nevalainen and Te'o, 2003). Because of their secretion, purification of the protein products is considered more cost effective, compared to non-secreting E. coli. Hydrolytic enzymes produced by the fungi enable growth of the organism on cheap undefined industrial media and under various conditions making cultivation easy. Furthermore, many industrially exploited strains of filamentous fungi have a GRAS status (Generally Regarded As Safe, by US food and Drug Administration) to be used, for example in food and feed industry. Therefore, filamentous fungi have an established and central role in the industrial production of enzymes for various applications ranging from animal feed manufacture to pulp bleaching.

Filamentous fungi are also considered as potentially feasible host systems for the expression of biotechnologically relevant recombinant proteins originating from other organisms in providing eukaryotic cell machinery for protein processing (Jeenes et al., 1991; Hui et al., 2001; Nevalainen and Te'o, 2003). However, in spite of the gram per litre levels of many homologous fungal proteins (Durand et al., 1988; MacKenzie et al., 1993) products from heterologously-expressed genes have remained much lower than those obtained for homologous proteins (Ward et al., 1990; Contreras et al., 1991; Broekhuijsen et al., 1993; Archer et al., 1994; Gouka et al., 1997b), in particular for commercial exploitation where yields of 30 g/L of a specific protein are not uncommon (Punt et al., 2002; Nevalainen and Te'o, 2003). In many cases, yields of heterologous proteins rarely exceed more than a few milligrams per litre (Gouka et al., 1997a) even though as high as gram levels per litre have been observed for some products (reviewed by Nevalainen et al., 2005). Research so far has shown that foreign proteins expressed in filamentous fungi are lost somewhere in the secretory pathway suggesting the occurrence of secretion bottlenecks (Jeenes et al., 1994; Nyyssönen and Keränen, 1995; Gouka et al., 1996; Nevalainen and Te'o, 2003; Shoji et al., 2008). These bottlenecks in the secretory pathway result in problems of obtaining high secreted yields of heterologous proteins from the expression hosts (MacKenzie et al., 1993). Impediments in protein folding, post-translational ornamentation like
glycosylation, protein sorting and proteolytic processing can have an effect on the production and functional characteristics of a secreted protein (Nevalainen and Te'o, 2003). Therefore, a detailed study into the protein secretion pathway in filamentous fungi would serve as a basis to understand the nature of secretion blocks and eventually lead to the improvement of heterologous protein production in these organisms. A wealth of information already exists on genetic, biochemical and molecular aspects of the secretion pathway in filamentous fungi, while “visible” data on hyphal morphology, protein localisation and function \textit{in vivo} have remained elusive.

\textit{Trichoderma reesei} (\textit{T. reesei}) is one of the most powerful secretors of extracellular proteins in nature and has been extensively used in industry to produce various biocatalysts for over two decades (Nevalainen and Te'o, 2003). Most of the secreted native proteins consist of cellulases, especially cellobiohydrolase I (CBHI). Recently, industrial strains of \textit{Trichoderma} have been reported to achieve total protein production levels of at least 100 g/L (Cherry and Fidantsef, 2003) making \textit{T. reesei} an attractive host for efficient production of recombinant proteins originating from higher eukaryotes (Penttilä, 1988). In this study, the secretory pathway and secretion of the major cellobiohydrolase CBHI in \textit{T. reesei} were studied using molecular biological approaches and different microscopic techniques in order to highlight cell organelles in the secretory pathway and to address the dynamics of protein secretion in the filamentous fungus \textit{T. reesei}.

1.1 Filamentous fungi as cell factories for protein production

Filamentous fungi continue to be investigated for their development as protein expression systems due to their natural ability to secrete large amounts of proteins. Fungal systems are also advantageous over prokaryotic methodologies because fungi possess eukaryotic glycosylation and post-translational modification capabilities, which are especially important if mammalian proteins are to be expressed.

Proteins of interest to be produced in filamentous fungal systems fall roughly into two categories: fungal proteins that are mainly hydrolytic enzymes and non-fungal products of pharmaceutical value originating from higher organisms.
1.1.1 Filamentous fungi in enzyme production

Industrial enzymes produced by filamentous fungi are dominantly applied in the animal feed, detergent, starch, textile, baking, beverage and dairy industries (Saxsena et al., 2004; Guimarães et al., 2006). The main enzymes exploited are amylases, proteases, lipases, xylanases, tannase and phytase and are marketed under product names such as Vinozyme®, Scourzyme®, Denimax®, Plusweet® and Purfect®. These enzymes are predominantly produced by Aspergillus niger var. awamori, Trichoderma reesei, Rhizomucor miehei and Humicola lanuginosa (Nevalainen and Te'o, 2003) and their applications are shown in Table 1-1.
<table>
<thead>
<tr>
<th>Industry</th>
<th>Application</th>
<th>Enzyme</th>
<th>Common source organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent</td>
<td>Removal of protein, starch, fat and oil</td>
<td>Protease, lipase, amylase, cellulase</td>
<td><em>Trichoderma</em> sp., <em>Aspergillus</em> sp.</td>
<td>(Miettinen-Oinonen <em>et al</em>., 2007; Zoller and Sosis, 2008)</td>
</tr>
<tr>
<td>Textiles</td>
<td>Desizing, decolourisation, increased wettability and even dyeing of fabric, bio-polishing, softening of wool and silk</td>
<td>Amylase, pectinase, cellulase, catalase, protease, endoglucanase</td>
<td><em>Trichoderma</em> sp., <em>Aspergillus</em> sp., <em>Thermoascus</em> sp., <em>Fusarium</em> sp.</td>
<td>(Galante <em>et al</em>., 1998; Fang <em>et al</em>., 2004; Raju <em>et al</em>., 2007; Mamma <em>et al</em>., 2009)</td>
</tr>
<tr>
<td>Leather</td>
<td>Hair removal and cleaning of pelt, bathing, increase softness</td>
<td>Lipase, protease, elastase</td>
<td><em>Aspergillus</em> sp., <em>Penicillium</em> sp., <em>Rhizopus</em> sp.</td>
<td>(Rao <em>et al</em>., 1998; Kumar and Takagi, 1999; Hasan <em>et al</em>., 2006)</td>
</tr>
<tr>
<td>Pulp and paper</td>
<td>Removal of lignin and reduction of pitch for increased whiteness, smooth starch coating of paper</td>
<td>Xylanase, cellulase, lipase, amylase</td>
<td><em>Trichoderma</em> sp., <em>Aspergillus</em> sp., <em>Rhizopus</em> sp.</td>
<td>(Buchert <em>et al</em>., 1998; Bajpai, 1999; Savitha <em>et al</em>., 2007)</td>
</tr>
<tr>
<td>Feed</td>
<td>Improved feed utilisation through increased digestibility and accessibility to minerals</td>
<td>Phytase, α-galactosidase, β-glucanase, amylase, mannanase, protease, xylanase, pectinase, cellulase</td>
<td><em>Trichoderma</em> sp., <em>Gliocladium</em> sp., <em>Peniophora</em> sp., <em>Aspergillus</em> sp.</td>
<td>(Galante <em>et al</em>., 1998; Rosgaard <em>et al</em>., 2006; Pontoppidan <em>et al</em>., 2007)</td>
</tr>
<tr>
<td>Ethanol fuel and brewing</td>
<td>Release of protein from grain mash, liquefaction, saccharification, shortened maturation time for beer</td>
<td>Protease, amylase, amylglucosidase, α-acetolactate decarboxylase</td>
<td><em>Aspergillus</em> sp., <em>Fusarium</em> sp.</td>
<td>(Lin and Tanaka, 2006; Fazary and Ju, 2007; Hoskins and Lyons, 2009)</td>
</tr>
<tr>
<td>Category</td>
<td>Description</td>
<td>Enzymes/Contributions</td>
<td>Organisms</td>
<td>Resources</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sweetener production</td>
<td>Saccharification, liquefaction, increased sugar yields from beet, maltodextrin production from starch</td>
<td>Glucoamylase, α-amylase, dextranase, pullulanase, glucose isomerase</td>
<td><em>Aspergillus</em> sp., <em>Rhizopus</em> sp., <em>Endomyces</em> sp.</td>
<td><a href="http://www.codexalimentarius.net/download/report/21/al9512ae.pdf">www.codexalimentarius.net/download/report/21/al9512ae.pdf</a></td>
</tr>
<tr>
<td>Baking</td>
<td>Anti-staling, increased volume of bread, even crumb structure, attractive crusts; increased dough strength and elasticity, dough conditioning and bleaching</td>
<td>Amylase, xylanase glucose oxidase, lipase, lipoxygenase, xylanase, protease, catalase</td>
<td><em>Aspergillus</em> sp., <em>Neurospora</em> sp., <em>Thermomyces lanuginosus</em></td>
<td><a href="https://isibbio.wikispaces.com/Fungal+Amylase+-+Baking+Enzyme">https://isibbio.wikispaces.com/Fungal+Amylase+-+Baking+Enzyme</a> (Schmoll <em>et al</em>., 2005; Yassin and Wheals, 2008)</td>
</tr>
<tr>
<td>Food</td>
<td>Fruit maceration, juice extraction and clarification, vegetable oil extraction; hydrolysis of meat by-products, production of flavour enhancers</td>
<td>Pectin esterase, polygalacturonase, pectin lyase, lactase, glucoamylase, protease</td>
<td><em>Aspergillus</em> sp., <em>Gliocladium</em> sp., <em>Rhizopus</em> sp.</td>
<td>(Galante <em>et al</em>., 1998; Jayani <em>et al</em>., 2005)</td>
</tr>
</tbody>
</table>
Industrial fungal strains have undergone various improvement processes involving traditional mutagenesis and screening and molecular genetic approaches (reviewed by Punt et al., 2002 and Nevalainen and Te’o, 2003).

Among the many T. reesei mutants, Rut C-30 is a widely studied strain (Eveleigh and Montenecourt, 1979). Cellulase production in Rut C-30 is not repressed by glucose to the same extent as in some other strains such as QM9414 (Ilmé n et al., 1996b) and the cellulases produced have improved capabilities (Ilmé n et al., 1997) such as a higher specific filter paper activity (6.2 FPU mg\(^{-1}\) protein) than cellulases produced by other efficient-cellulase-secreting T. reesei mutants, for example VTT-D-79125 (3.6 FPU mg\(^{-1}\) protein) (van den Hombergh et al., 1995; Duff and Murray, 1996; Domingues et al., 2001). The modern industrial production strains are genetically engineered to increase the enzyme yields and to remove the expression of unwanted enzymes, like cellulases during the production of xylanases (Verdoes et al., 1995; Paloheimo et al., 2003). There is a multitude of genetically modified or mutagenised strains, which are used to produce enzyme mixtures tailored for particular industrial applications (Mä ntylä et al., 1998). For example, two T. reesei transformants originating from QM6a and VTT-D-80133, in which carbon catabolite repression regulatory gene cre1 was completely removed, were recently developed and showed enhanced enzyme production with less/no carbon catabolite repression (Nakari-Setälä et al., 2009). For the purpose of screening for strong promoters for gene expression, different versatile reporter systems have been applied including fusions to beta-galactosidase (lacZ), glucuronidase (uidA) (Brakhage and Van den Brulle, 1995; Geisen, 1995) and glucose oxidase (Geisen, 1995).

1.1.2 Fungi as heterologous production hosts

In addition to the production of enzymes for industrial processes, filamentous fungi, such as Trichoderma reesei (Nyyssönen et al., 1993; Joosten et al., 2003) and Aspergillus niger (reviewed by Gouka et al. 1997a and Punt et al., 2002) have been developed as heterologous protein expression hosts for various proteins of interest, including drugs which are conventionally produced by mammalian cell culture (Keränen and Penttilä, 1995). Recently, the potential of A. niger (Nakajima et al., 2006; Karnaukhova et al., 2007) and T. reesei (Joosten et al., 2003) for the production of antibodies has been investigated. These antibodies are not only targeted for the pharmaceutical industry but are to be incorporated into a whole range of other products such as
shampoos and skin care creams with a view of targeting the active ingredients to the specific intended surface, such as hair but not the scalp (www.genencor.com).

There are several examples showing that gene products originating from other fungi can be successfully produced heterologously up to the gram per litre level (Nevalainen et al., 2005) (Table 1-2). However, the levels of foreign gene products which originate from higher organisms, obtained in filamentous fungi, have always been much lower than those for heterologous fungal proteins (Punt et al., 2002). One of the most obvious reasons for the low yields is abundant production of proteases by most of the fungal host strains used for the purpose (Mattern et al., 1992; van den Hombergh et al., 1997). Problems caused by proteases have been partly overcome by engineering protease deficient host strains (van den Hombergh et al., 1995; Idiris et al., 2006), by pH control (i.e. increasing pH from 3 to 6) to minimise extracellular protease activity (Donnella et al., 2001), or by incorporation of the protease inhibitor pepstatin into the culture medium for reduction of protease activity (Ahamed et al., 2006).
Table 1-2 Heterologous products expressed in filamentous fungi.

<table>
<thead>
<tr>
<th>Expression organism</th>
<th>Heterologous protein</th>
<th>Yield (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Products of fungal origin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. oryzae</td>
<td>Glucoamylase</td>
<td>3300</td>
<td>(Ishida et al., 2001)</td>
</tr>
<tr>
<td>T. reesei</td>
<td>Cel45A (Melanocarpus albomyces)</td>
<td>7400</td>
<td>(Haakana et al., 2004)</td>
</tr>
<tr>
<td>T. reesei</td>
<td>Cel7A (Melanocarpus albomyces)</td>
<td>5400</td>
<td>(Haakana et al., 2004)</td>
</tr>
<tr>
<td>T. reesei</td>
<td>Cel7B (Melanocarpus albomyces)</td>
<td>3700</td>
<td>(Haakana et al., 2004)</td>
</tr>
<tr>
<td>T. reesei</td>
<td>Lignin oxidase (Phlebia radiatae)</td>
<td>20</td>
<td>(Saloheimo et al., 1991)</td>
</tr>
<tr>
<td><strong>Products of non-fungal origin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. oryzae</td>
<td>Bovine chymosin</td>
<td>107.9</td>
<td>(Nemoto et al., 2009)</td>
</tr>
<tr>
<td>A. oryzae</td>
<td>Lysozyme</td>
<td>84.4</td>
<td>(Yoon et al., 2009)</td>
</tr>
<tr>
<td>A. awamori</td>
<td>Human lactoferrin</td>
<td>&gt; 2000</td>
<td>(Ward et al., 1995)</td>
</tr>
<tr>
<td>A. niger</td>
<td>Hen egg-white lysozyme</td>
<td>209</td>
<td>(Gheshlaghi et al., 2005)</td>
</tr>
<tr>
<td>A. niger</td>
<td>Human interleukin-6</td>
<td>150</td>
<td>(Punt et al., 2002)</td>
</tr>
<tr>
<td>A. niger</td>
<td>Ig G</td>
<td>900</td>
<td>(Ward et al., 2004)</td>
</tr>
<tr>
<td>A. niger</td>
<td>Human α1-proteinase inhibitor</td>
<td>12</td>
<td>(Karnaukhova et al., 2007)</td>
</tr>
</tbody>
</table>

Gene fusion strategy has been successfully applied in the production of a number of non-fungal heterologous proteins. This involves fusion of a gene encoding a heterologous product to a gene encoding an efficiently secreted endogenous protein. Examples of endogenous secreted proteins applied in fusions are glucoamylase (GLA) from A. niger (Gouka et al., 1996; Gouka et al., 1997b; Wiebe et al., 2001) and cellobiohydrolase I (CBHI) (Nyyssönen and Keränen, 1995; Penttilä, 1998; Bergquist et al., 2004) from T. reesei. It has been proposed that fusion of an endogenous carrier may facilitate translocation of the foreign protein in the secretion pathway and protect the heterologous protein from degradation (Burlingame and Verdoes, 2006). High copy numbers have been also reported to be effective in terms of improving gene product yields (Moralejo et al., 1999; Liu et al., 2003; Retallack et al., 2006).
During the last five years, it has become evident that a variety of intrinsic, metabolic and environmental stresses may have a strong impact on recombinant protein production (Gasser et al., 2006). Several studies have demonstrated that many physiological processes, ranging from stress responses to environmental factors, as well as protein folding/aggregation and secretion are highly relevant in protein production (Gasser et al., 2006). Among the environmental factors influencing protein expression and secretion, pH, osmolarity, oxygen availability and temperature appear to be particularly important (Gasser et al., 2006). These factors may also have an impact on hyphal morphology and thereby productivity (Burlingame and Verdoes, 2006; El-Enshasy et al., 2006). Current commercial fungal fermentation processes for citric acid, glucoamylase, microbial rennet and penicillin produce tens to hundreds of grams per litre of product. Often the highest productivity is observed when the fungus exhibits a specific morphology that has developed under a specific set of physical and chemical conditions.

1.1.3 Enzymes produced by *Trichoderma reesei*

*T. reesei* produces a number of extracellular enzymes (Table 1-3) including two exo-1,4-β-D-glucan cellobiohydrolases (CBH I and II, EC 3.2.1.91), five endo-1,4-β-D-glucan-4-glucanohydrolases (EG I, II, III, IV and V, EC 3.2.1.4), two β-D-glucosidases (BGL I and II, EC 3.2.1.21), at least four endo-1,4-β-xylanases (XYN I, II, III and IV, EC 3.2.1.8) and two β-xylosidases (EC 3.2.1.37) (Bailey et al., 1993b; Zeilinger et al., 1996; Xu et al., 1998; Karlsson et al., 2001; Nogawa et al., 2001). Extracellular cellulases produced by *T. reesei* and the genes encoding them are shown in Table 1-3. Cellobiohydrolase I (CBHI, also named Cel7A) is the major secreted cellulase in *T. reesei*. CBHI accounts for 60% of the total amount of the secreted cellulases by *T. reesei* therefore serving as an excellent model for studying protein secretion in *T. reesei* (Kubicek et al., 1993a; Pakula et al., 2000; Pakula et al., 2003). Thus, the cbh1 gene promoter is used globally for the expression of selected genes in the *T. reesei* system (Nevalainen et al., 2005). Due to the strong protein secretion of *T. reesei*, many of its enzymes are currently produced on a large scale for application in different industrial processes. Commercially available *Trichoderma* enzyme products such as Crystalzyme®, Econase®, Stonenzyme®, Cellubrix®, Ecopulp® and Pulpzyme® (Penttilä et al., 2004) include native hydrolytic enzymes. In addition, *T. reesei* produces β–mannanase (EC 3.2.1.78), β–mannosidase (EC 3.2.1.25), α-L-arabinofuranosidase (EC 3.2.1.55), α–galactosidase (EC 3.2.1.22), acetylxylan esterases (EC
3.1.1.72) and laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) (Stålbrand et al., 1993; Roche et al., 1995; Shabalin et al., 2002; Hakulinen et al., 2003).

Numerous cellulase and semicellulase-encoding genes have been cloned from *T. reesei* and other species, but only a few, *cbh1* and *cbh2* encoding two cellobiohydrolases and *xyn1* and *xyn2* encoding two endoxylanases, have been investigated in detail concerning their expression and regulation (Mach and Zeilinger, 2003). The gene encoding Envoy, a PAS/LOV domain protein which links cellulase induction by cellulose to light signalling has been cloned from *T. reesei* (Schmoll et al., 2005).

Table 1-3 Cellulases secreted by *T. reesei* (adapted from Curach, 2005). The enzyme names are given as the original protein name (e.g. CBHII) as well as the name designated by the nomenclature developed by (Henrissat et al., 1998). The name of the corresponding substrate is abbreviated, e.g. cellulose-CEL and the family to which the enzyme belongs is denoted as a number e.g. CEL7. If an organism produces multiple enzymes of the same family, a letter denotes the particular family member e.g. CEL7A.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>GenBank accession no.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEL7A (CBHI)</td>
<td>cbh1/cel7a</td>
<td>P00725</td>
<td>(Shoemaker et al., 1983; Teeri et al., 1983)</td>
</tr>
<tr>
<td>CEL6A (CBHII)</td>
<td>cbh2/cel6a</td>
<td>M16190</td>
<td>(Teeri et al., 1987)</td>
</tr>
<tr>
<td>CEL7B (EGI)</td>
<td>egl1/cel7b</td>
<td>M15665</td>
<td>(Penttilä et al., 1986)</td>
</tr>
<tr>
<td>CEL5A (EGII)</td>
<td>egl2/cel5a</td>
<td>M19373</td>
<td>(Saloheimo et al., 1988)</td>
</tr>
<tr>
<td>CEL12A (EGIII)</td>
<td>egl3/cel12a</td>
<td>AB003694</td>
<td>(Ward et al., 1993; Okada et al., 1998)</td>
</tr>
<tr>
<td>CEL61A (EGIV)</td>
<td>egl4/cel61a</td>
<td>Y11113</td>
<td>(Saloheimo et al., 1997)</td>
</tr>
<tr>
<td>CEL45A (EGV)</td>
<td>egl5/cel45a</td>
<td>Z33381</td>
<td>(Saloheimo et al., 1994)</td>
</tr>
<tr>
<td>CEL3A (BGLI)</td>
<td>bgll/cel3a</td>
<td>U09580</td>
<td>(Barnett et al., 1991; Mach, 1993)</td>
</tr>
<tr>
<td>CEL1A (BGLII)</td>
<td>bgll2/cel1a</td>
<td>AB003110</td>
<td>(Takashima et al., 1999; Saloheimo et al., 2002)</td>
</tr>
</tbody>
</table>
1.1.3.1 Environmental factors affecting cellulase production

Cellulase genes of *T. reesei* are induced in the presence of cellulose or its derivatives (Aro *et al.*, 2003) therefore, cellulose has usually been considered to be the best inducer for producing a well balanced cellulase preparation (Janas *et al.*, 2002). However, cellulose is expensive and also causes operational and rheological problems in the bioreactor (Janas *et al.*, 2002). The use of a soluble carbon source such as lactose, cellobiose or sorbose allows lower cost of production, greater control of fermentation and simplifies the operation of the process (Janas *et al.*, 2002). Cellulases are also induced by the addition of the glucose disaccharide, sophorose, to the medium containing a neutral carbon source, such as glycerol or the monosaccharide L-sorbose (Aro *et al.*, 2003; Mach and Zeilinger, 2003). In addition, sorbose (Kawamori *et al.*, 1985; Nogawa *et al.*, 2001), lactobionic acid and lactulose (Janas *et al.*, 2002) have been proved to be good inducers for cellulase production in *T. reesei*.

*T. reesei* is an acidophilic fungus and most of its secreted cellulases function optimally at around pH 5 (Boer and Koivula, 2003). Earlier reports described that the production of cellulases was favoured by pH 4 and pH 4.5 (Bailey *et al.*, 1993a; Yang, 2004), as well as a higher pH 6.7 (Wen *et al.*, 2005). However, depending on the *T. reesei* strain and the nature of the carbon source used to induce cellulase production, different initial pH of the cultivation medium may be trialed for maximum cellulase yield (Kadam and Keutzer, 1995; Szengyel and Zacchi, 2000; Juhasz *et al.*, 2004; Gautam and Simon, 2006; Muthuvelayudham and Viruthagiri, 2006). For CBHI production, it is generally accepted that the optimal pH is 5.5 (Mandels and Reese, 1957). Furthermore, the pH value influences cell morphology that may lead to changes in cellulase production (Lejuene *et al.*, 1995; Domingues *et al.*, 2000).

1.2 Protein secretion in the fungal hyphae

1.2.1 The overall passage of protein through a secretory pathway

An overview of the default protein secretory pathway in filamentous fungi is depicted in Fig. 1-1. In this model, protein secretion occurs mostly at the apical and sub-apical regions of the hyphae (Wösten *et al.*, 1991).
Proteins destined for secretion are co-translationally translocated: polypeptide chains under synthesis enter the ER via interaction between their signal peptide and signal recognition particle (SRP) complex. Within the endoplasmic reticulum (ER), the signal sequence is first removed and the preproteins undergo primary glycosylation, folding and proteolytic processing. After that, the proteins are packed and transported in vesicles along the secretory pathway where vesicles bud from the donor membranes and fuse with the acceptor membranes in a highly organised manner. From the ER, the proteins are transported to the Golgi complex or a structure equivalent to Golgi (Shusta et al., 1998; Nuttall et al., 2002), where post-translational modifications, such as further glycosylation and trimming occur. Unfolded proteins are sorted in the ER and targeted to either proteasomes or to vacuoles for degradation (Shoji et al., 2008). Membrane and protein trafficking are bidirectional occurring both outwards (anterograde traffic) and inwards (retrograde traffic). Finally, the secretory proteins are directed to the plasma membrane and further to the cell wall from where they are released to the extracellular environment. In some cases, the proteins will not reach the extracellular space, but are targeted to intracellular compartments such as the vacuole, either to become resident proteins or to undergo proteolytic degradation (Conesa et al., 2001). Many genes are known to be involved in the secretory pathway function in filamentous fungi and are summarised in the Table 1-4.
Table 1-4 Secretion-related genes in filamentous fungi (continued onto next page).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein encoded</th>
<th>Localisation</th>
<th>Function in secretion pathway</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>srpA</td>
<td>Homology to the signal recognition particle (SRP) from yeast</td>
<td>Cytosol/ER membrane</td>
<td>Protein translocation</td>
<td>(Thompson et al., 1995)</td>
</tr>
<tr>
<td>bip1</td>
<td>Binding protein</td>
<td>ER lumen</td>
<td>Protein folding and assembly</td>
<td>(Hijarrubia et al., 1997; Lombraña et al., 2004)</td>
</tr>
<tr>
<td>pdl</td>
<td>Protein disulfide isomerase</td>
<td>ER lumen</td>
<td>Disulphide bond oxidation and isomerisation</td>
<td>(Jeenes et al., 1997; Ngiam et al., 1997; Saloheimo et al., 1999; Conesa et al., 2001; Nigam et al., 2001; Sims et al., 2005)</td>
</tr>
<tr>
<td>prpA</td>
<td>Unfolded protein response (UPR) transcription factors</td>
<td>Nuclear</td>
<td>Transcription factor of unfolded protein response (UPR)</td>
<td>(Saloheimo et al., 2003; Nevalainen et al., 2005)</td>
</tr>
<tr>
<td>ire1</td>
<td>Transmembrane kinase/nuclease</td>
<td>ER membrane</td>
<td>Positive regulator of hac1 splicing</td>
<td>(Conesa et al., 2001; Valkonen et al., 2004)</td>
</tr>
<tr>
<td>ptc2</td>
<td>Protein serine/threonine phosphatase</td>
<td>Cytosolic</td>
<td>Negative regulator of unfolded protein response (UPR)</td>
<td>(Valkonen et al., 2004)</td>
</tr>
<tr>
<td>clxA</td>
<td>Calnexin</td>
<td>ER membrane</td>
<td>Lectin-like chaperone that recognises and assists folding of monoglycosylated glycoproteins</td>
<td>(Wang et al., 2003)</td>
</tr>
<tr>
<td>sarl</td>
<td>GTPase</td>
<td>Cytosolic face of ER membrane</td>
<td>Vesicle budding from ER</td>
<td>(Veldhuisen et al., 1997)</td>
</tr>
<tr>
<td>dpmI</td>
<td>Dolichol phosphate mannose synthase</td>
<td>Cytosolic</td>
<td>Protein core glycosylation</td>
<td>(Kruszewska et al., 2000; Perlinska-Lenart et al., 2006; Deshpande et al., 2008)</td>
</tr>
<tr>
<td>mpg1</td>
<td>Guanylyltransferase</td>
<td>Cytosolic</td>
<td>Protein core glycosylation</td>
<td>(Deshpande et al., 2008; Kruszewska et al., 2008)</td>
</tr>
<tr>
<td>ypt1</td>
<td>GTPase</td>
<td>Golgi membrane</td>
<td>Vesicle fusion at Golgi</td>
<td>(Saloheimo et al., 2004)</td>
</tr>
<tr>
<td>Genotype</td>
<td>Function</td>
<td>Localization</td>
<td>Role</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>----------</td>
<td>--------------</td>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>nsf1</em></td>
<td>ATPase</td>
<td>Cis Golgi/plasma membrane</td>
<td>Disassembly of SNARE complexes</td>
<td>(Saloheimo et al., 2004)</td>
</tr>
<tr>
<td><em>srgA</em></td>
<td>GTPase</td>
<td>Golgi-plasma membrane</td>
<td>Vesicle fusion at plasma membrane</td>
<td>(Punt et al., 2001)</td>
</tr>
<tr>
<td><em>snc1</em></td>
<td>v-SNARE protein</td>
<td>Golgi-plasma membrane</td>
<td>T-SNARE vesicle fusion at plasma membrane</td>
<td>(Valkonen et al., 2007)</td>
</tr>
<tr>
<td><em>rho3</em></td>
<td>Rho-type small GTPase</td>
<td>Golgi-plasma membrane</td>
<td>Small GTP-binding protein, cell signalling</td>
<td>(Vasara et al., 2001)</td>
</tr>
</tbody>
</table>
Genes encoding components of the secretion machinery, such as the GTP-binding protein SARI involved in the ER-Golgi transport (Saloheimo et al., 2003) and the glucosidase II alpha subunit (GIIα) which is the catalytic part of the glucosidase II heterodimeric enzyme involved in the structural modification of N-linked oligosaccharides present on glycoproteins, have been cloned from T. reesei (Geysens et al., 2005). In addition, two genes encoding the Rab protein YPT1/YPTA and the general fusion factor NSFI/NSFA, have been characterised from T. reesei (Saloheimo et al., 2004) with the results suggesting the possibility that the whole secretory pathway of T. reesei could be induced at the transcriptional level by stress responses caused by protein accumulation in the secretory pathway (Saloheimo et al., 2004). In order to elucidate factors involved in the glycosylation of secreted proteins, T. reesei strains which exhibited elevated production and secretion of glycosylated proteins have been obtained by overexpression of the S. cerevisiae DPM1 gene coding for dolichylphosphate mannose synthase (DMP-synthase) (Kruszewska et al., 1999a; Zakrzewska et al., 2003). GDP-mannose (GDPMan) was effectively engaged by mannosyltransferases resulting in hypermannosylation of secreted proteins by both N- and O- glycosylation. These data indicate that the level of cellular GDPMan can play a major regulatory role in protein glycosylation in T. reesei (Zakrzewska et al., 2003). Further studies found that in these transformed cells, an increased level of chitin which localised in the septa rather than in the lateral wall like as seen in yeast, correlated with a decreased level of glucan, suggesting that the cells had turned on the cell wall compensatory mechanism as a reaction to stress conditions (Perlinska-Lenart et al., 2006). Stals et al. reported posttranslational phosphorylation of glycoproteins is not atypical for Trichoderma sp. (Stals et al., 2004b) and that the glycosylation of Cel7A (CBH I) from Trichoderma reesei varies considerably when the fungus is grown under different conditions such as pH, medium, inducer or carbon source (Stals et al., 2004a). Much of the understanding of the secretory pathway in filamentous fungi has been generated from studies in yeast and mammalian neuronal cells. However, the mammalian and yeast models are not entirely applicable due to two features signatory of filamentous fungi: the growth of the mycelium as long branching hyphae and the ability to secrete comparatively copious quantities of protein into the growth medium (Raikhel and Chrispeels, 2000; van Vliet et al., 2003).
1.2.2 Translocation to the ER

The proteins expressed within a cell that are destined for secretion into the surrounding medium are identified by a signal sequence (ss) peptide at the N-terminus which functions as a guide directing the polypeptide to a specific translocation site on the ER membrane called a translocon (Johnson and van Waes, 1999). The signal sequence peptide contains several charged amino acids followed by a hydrophobic core, as demonstrated by the ss of the main cellobiohydrolase (CBHI) of *T. reesei*, MYRKLAVISAFALTARA. The ss of a particular protein can be interchangeable between unlike proteins or between different organisms. For example, the *cbh1* ss has been successfully applied for secretion of various heterologous proteins from *T. reesei* (Penttilä, 1998; Te'o et al., 2000; Paloheimo et al., 2003). Signal sequences of fungal proteins, 34-kDa protein (P34) and glucoamylase, have been used successfully for the secretion of GFP in basidiomycetous fungi *Pseudozyma flocculosa* and *Pseudozyma antarctica* (Cheng et al., 2008) and heterologous xylanase B in *A. nidulans* (Zhang et al., 2008) respectively.

As mRNA is translated on a free ribosome, the emerging ss is recognised by the cytosolic signal recognition particle (SRP), a ribonucleoprotein complex, which pauses further translation until the ribosome is transferred to the ER membrane. The SRP docks the ribosome and emerging protein chain with the SRP receptor on the ER membrane and the translocation complex composed of the proteins Sec66p, Sec67p and Sec63p (Deshaies et al., 1991; Feldheim et al., 1993) before being released. The Sec63p protein of the translocation complex spans the ER membrane and is in direct association with the chaperone, immunoglobulin binding protein (BiP), in the ER lumen (Brodsky and Schekman, 1993). On the transfer of the nascent polypeptide chain and attached ribosome to the Sec61p ER membrane channel, cotranslational translocation into the ER lumen occurs (Pilon et al., 1998) and BiP is released into the ER lumen (Brodsky and Schekman, 1993). In posttranslational protein translocation, the nascent polypeptide is completely translated on soluble ribosomes before being translocated into the ER lumen (Hamman et al., 1998). Cytosolic chaperones in yeast, in particular the 70-kDa heat shock cognate proteins (hsc70s), are required to keep the polypeptide in a translocation-competent form (Chirico et al., 1988; Deshaies et al., 1988). Translocating proteins also interact with luminal chaperones that prevent premature protein folding and aggregation (Gething and Sambrook, 1992). In yeast, the BiP homologue Kar2p is essential for posttranslational protein translocation across the ER membrane (Brodsky et al., 1995; Matlack et al., 1999). Posttranslational
translocation requires the ATPase domain of BiP that undergoes a reaction cycle comprised of ATP binding, hydrolysis, and nucleotide exchange (Awe et al., 2008). This produces energy for BiP to bind to and release Sec63p cyclically (Sanders et al., 1992; Lyman and Schekman, 1997; Hamman et al., 1998). Finally, the ss on the immature protein is cleaved by a signal peptidase upon entering the ER lumen.

1.2.2.1 Protein folding in the ER

A peptide emerges from the translocation channel in an unfolded transition state and begins to fold in the lumen of the ER. The ER-resident protein folding machinery has three components: (i) molecular chaperones assisting other proteins to fold such as BiP, calnexin and calreticulin; (ii) enzymes such as protein disulphide isomerase (PDI); (iii) a ER quality control system including unfolded protein response (UPR) and ER-associated protein degradation (ERAD) (Schröder, 2008). The overall folding process of secretory proteins in the ER is summarised in Fig. 1-2.
Figure 1-2 A simplified diagram of the protein folding process in the ER (adapted from (Dobson, 2003). The newly synthesised polypeptides are translocated into the ER, where they fold to reach their desired conformation with the help of a series of molecular chaperones such as BiP and foldases i.e. PDI. The correctly folded proteins then pass into the Golgi via vesicles and continue through the secretory pathway. In contrast, misfolded proteins are broken down by the quality control system including ER-associated protein degradation (ERAD) and unfolded protein response (UPR).

Foldases catalyse slow, often rate-limiting, covalent changes, such as disulfide bond formation and proline isomerisation. Molecular chaperones are not regarded as catalysts but as assisting proteins that transiently and non-covalently bind to non-native proteins to prevent non-productive protein-protein interactions and thus promote correct folding (Conesa et al., 2001). Chaperones prevent incorrect intra- and inter-chain connections. Thus, they can increase the amount of correctly folded proteins (Hartl, 1996).
1.2.2.2 ER chaperone BiP and its role in the protein folding

BiP was originally identified independently as the immunoglobulin heavy chain binding protein (Haas and Wabl, 1983) and as the glucose regulated protein in mammals, Grp78 (Pouyssegur et al., 1977), or Kar2 protein in Saccharomyces cerevisiae (Robinson et al., 1996). It is a member of the heat shock 70 protein family of molecular chaperones and localised in the lumen of the ER (Munro and Pelham, 1986).

The BiP protein resides in the ER and is responsible for assisting in the folding and assembly of newly synthesised proteins by recognising unfolded polypeptides and by inhibiting intra- or intermolecular aggregation by interacting with exposed hydrophobic regions of the unfolded protein. These regions are normally located in the interior of the fully-folded protein. The duration of BiP binding may last a few minutes in the case of Ig light chains. Depending on the protein which BiP binds to, early folding intermediates can process immediately or the binding is delayed until the protein reaches a more mature conformation (reviewed by Gething, 1999). BiP is also one of the components responsible for the opening and closing of the translocation pore on the ER membrane and for the transport of aberrant proteins back across the ER membrane for degradation by the proteasome (Plemer et al., 1997; Brodsky et al., 1999a).

The BiP protein has two domains: an N-terminal domain that contains an ATPase binding and a catalytic site and a C-terminal domain that binds to the substrate. There is communication between the two domains such that the rate and duration of binding depends on the rate of hydrolysis of ATP. A protein undergoing folding may undergo several rounds of BiP binding folding and release (reviewed by Gething, 1999). BiP cycles through rounds of adenosine diphosphate (ADP) ATP exchange, which is regulated by cochaperones including DnaJ proteins that accelerate ATP hydrolysis to stabilise the BiP–substrate complex (Awad et al., 2008) (Fig. 1-3).
BiP’s role as a general chaperone in the ER lumen depends on its ability to recognise a wide variety of nascent polypeptides that share no obvious sequence similarity, while accurately discriminating between properly folded and unfolded structures. In vitro, BiP can interact with short synthetic peptides whose binding stimulates its ATPase activity (Flynn et al., 1989; Knarr et al., 1995) and alters its oligomeric state (Blond-Elguindi et al., 1993b; Chevalier et al., 1998). The characteristics of peptides that bind to BiP were defined using affinity screening of large, highly diverse libraries of peptides displayed by fusion to the pIII protein present at one tip of bacteriophage fd particles (Blond-Elguindi et al., 1993; Chevalier et al., 1998). The peptides displayed by BiP-binding bacteriophages showed extensive sequence diversity, consistent with the observed “promiscuity” of BiP’s interaction with a wide variety of unrelated nascent polypeptides and usually exhibit marked hydrophobicity, consistent with the likelihood that BiP interacts with sequences normally located in the interior of a fully-folded protein. The analysis revealed a heptameric motif best described as Hy(W/X)HyXHyXMy, where Hy is a bulky aromatic or hydrophobic residue (most frequently tryptophan, phenylalanine or leucine, but also methionine and isoleucine), W is tryptophan, and X is any amino acid (Gething, 1999). There is a program that was written to predict BiP binding domains within a polypeptide chain (Blond-Elguindi et al., 1993). Such computer program studies suggested that in vivo, BiP regulated the folding and assembly of antibody molecules by binding to hydrophobic surface regions on the
isolated subunits that subsequently participate in interchain contacts. Similar studies with other BiP substrates indicated that binding sites may be located in regions of the polypeptide chain that did not take up their final conformation until late in the folding pathway (Gething et al., 1995). However, BiP-binding sites may also be present within sequences that fold rapidly, precluding their interaction with the chaperone binding sites for Hsp70 molecular chaperones in nature (Hellman et al., 1999). It thus appears that the rate and stability of the folding of a protein determines whether or not a particular site is recognised, with BiP preferentially binding to proteins that fold slowly or are relatively unstable (Hellman et al., 1999).

BiP overexpression has increased heterologous protein secretion for about 50% of some of the studied proteins (Kim et al., 2003; Chung et al., 2004; Damasceno et al., 2007), but can also have negative effects (Punt et al., 1998). In cells overexpressing BiP, one of its co-chaperones, luminal HSP seventy 1 protein (Lhs1p) or ER luminal ATP may have become limiting, converting BiP into a holdase or inactive chaperone. This may have targeted proteins towards ERAD, stalled translocation, converted Lhs1p into a holdase or inhibited Lhs1p. Increased BiP activity may have stalled the GRP94 (an ER paralog of the heat shock protein 90 kDa beta or Hsp90) and/or calnexin-calreticulin chaperone machineries, because of the hierarchy of ER luminal chaperone systems. As a result, the heterologous protein may have been targeted for ERAD. In addition, BiP overexpression attenuates UPR, thus the BiP levels in the wild type and BiP overexpressing cells may not be dramatically different. Further, BiP overexpressing cells may experience an imbalance in their chaperone machineries, because the UPR coordinates expression of several chaperones. Moreover, if overexpression of BiP shifts the folding bottleneck to the GRP94 and calnexin chaperone systems, up-regulation of these chaperone systems by the UPR may be repressed, because of suppression of UPR activation by the elevated BiP levels (Schröder, 2008).

In filamentous fungi, as in other organisms, the BiP-encoding gene has a basal expression level under normal growth conditions and was overexpressed in situations of cellular stress factors such as inhibition of N-glycosylation, presence of calcium ionophores, glucose deprivation, heat shock and conditions typical of the unfolded protein response (Gething, 1999; Ngiam et al., 2000). In the cells with a bottleneck for heterologous protein secretion, the protein accumulated in intracellular aggregates (Schröder and Kaufman, 2005), associated with the molecular chaperone BiP/Kar2p (Schröder and Kaufman, 2005), and induced dilation of the ER. Therefore,
exit of the correctly folded polypeptide chain from the ER is the rate-limiting step for heterologous protein secretion (Schröder, 2008).

1.2.2.3 ER foldase PDI

Protein disulphide isomerase (PDI) is an abundant soluble ER-resident enzyme in eukaryotic cells (Frands et al., 2000). It has an N-terminal signal sequence for translocation and a C-terminal sequence for retention in the ER. PDI is an indispensable enzyme in fungi as a catalyst for generating native disulphide bonds in the ER (Laboissiere et al., 1995) and catalyses the establishment, and isomerisation or reduction of disulphide bonds depending on the substrate. PDI breaks and reforms correct disulfide bonds between the sulphydryl groups of cysteine residues (Freedman et al., 1994). The peptide binding capability of PDI is functionally and physically divergent from the catalytical activity. The substrate binding competence of PDI is localised in a very acidic region at the C-terminus (Noiva et al., 1993). In addition to the role as a foldase during protein maturation, PDI serves as a chaperone via a peptide-binding domain and as a component of prolyl hydroxylase and of the microsomal triglyceride transfer complex (LaMantia and Lennarz, 1993).

PDI overexpression has increased secretion of some heterologous proteins even those containing no disulfide bonds such as Pyrococcus furiosus beta-glucosidase expressed in S. cerevisiae which has only one cysteine per monomer and no disulfide bonds (Powers and Robinson, 2007) possibly due to improved holdase activity or enhanced chaperone aid of PDI in protein folding (Powers and Robinson, 2007; Schröder, 2008). Alternatively, increased rates of disulfide bond formation or isomerisation in PDI overexpressing cells may explain increased heterologous protein secretion (Schröder, 2008). However, overexpression of PDI, even in conjunction of chaperone coexpression, sometimes does not result in increase in the production of heterologous proteins in fungi. For example, cooverexpression of PDI and BiP demonstrated no apparent effect on secretion of a single-chain antibody fragment (A33scFv) in Pichia pastoris (Damasceno et al., 2007).

Similarly, cooverexpressssion of PDI and calnexin or BiP in S. cerevisiae failed to increase the yield of human A2a adenosine receptor (A2a) or G-protein-coupled receptors (GPCRs) and actually decreased total substance P receptor (SPR) (Butz et al., 2003). The substrate selectivity
of PDI may explain why PDI overexpression has failed to increase heterologous protein secretion in some studies (Schröder, 2008). In addition, cooverexpression of PDI possibly leads to the induction of an unfolded protein response (UPR) due to overloading the ER thus resulting in increased protein degradation through ERAD (Damasceno et al., 2007).

### 1.2.2.4 Other folding chaperones in the ER

In addition to the BiP chaperone as described in the above, other chaperones such as calnexin and calreticulin are also involved in protein folding in the ER. The lectin-like chaperone calnexin is an ER-resident integral calcium binding membrane phosphoprotein (Wada et al., 1991). Calnexin has a cytosolic phosphorylatable C-terminus that undergoes phosphorylation and a putative ER retrieval sequence (Wada et al., 1991; Trombetta and Helenius, 1998). The luminal domain of calnexin has sequence similarity to calreticulin, an abundant, soluble ER protein with overlapping but not identical substrate specificity with calnexin (Hebert et al., 1996). Calnexin prevents aggregation of unfolded polypeptides and folding intermediates by attaching to N-glycosylated nascent secretory proteins and/or peptide structures of incompletely folded proteins. In addition, the interaction of some glycoproteins with calnexin was particularly induced by the N-linked oligosaccharides which served as signals of a glycopolypeptide’s folding status (Hammond et al., 1994). Calreticulin, is an ER resident chaperone that buffers Ca2+ and participates in the folding of newly synthesized proteins and glycoproteins (Trombetta, 2003). Calreticulin has been proposed to prefer soluble glycoproteins, whereas calnexin can interact with membrane-attached glycoproteins (Peterson et al., 1995). Calnexin/calreticulin overexpression has generally improved heterologous protein secretion (Conesa et al., 2002; Chung et al., 2004). Further, the elevated calnexin or calreticulin levels not supported by concomitant increases in activities of other calnexin cycle components, may convert these surplus amounts of calnexin and calreticulin into holdase or inactive chaperones (Schröder, 2008).

Together, calnexin, calreticulin and ERp57 (a PDI-like protein resident in the ER) comprise the so-called “calreticulin/calnexin cycle” which is responsible for quality control and folding in newly-synthesised (glyco)proteins (Trombetta, 2003). Calreticulin and calnexin are chaperones that share several functions, including Ca2+ binding, lectin-like activity, and recognition of misfolded proteins. A major distinction between the two proteins, however, is that calnexin is an integral membrane protein whereas calreticulin is a luminal protein, which is able to travel freely
within the ER lumen (Michalak et al., 1999). Therefore, calnexin interacts transiently with its protein-folding intermediates at the stationary phase of the ER membrane, whereas calreticulin interacts with its protein-folding intermediates in the more mobile environment of the lumen. The glycosylation of newly-synthesised proteins acts as a signal for enhanced folding and quality control and/or for the degradation of misfolded proteins (Yoshida et al., 2002). Calnexin and calreticulin bind monoglucosylated carbohydrate on newly-synthesised glycoproteins. The ER and Calnexin/calreticulin cycle also contains a glucosidase II which removes terminal glucose on a UGGT (UDP-glucose:glycoprotein glucosyltransferase) which can re-glucosylate chains that have been glucose-trimmed. This establishes a cycle of de-glucosylation and re-glucosylation. UGGT discriminates between folded and unfolded proteins, adding back a glucose residue to unfolded proteins only. This de-glucosylation-glucosylation cycle may be repeated several times before a newly synthesized glycoprotein is properly folded. If not folded properly they are degraded. Calnexin and calreticulin also affect calcium homeostasis. Calreticulin is a major Ca$^{2+}$ storage protein and calnexin affects function of SERCA2b (http://www.abcam.com).

### 1.2.2.5 ER quality control

A protein quality control system in the ER, consisting of molecular chaperones and proteases, ensures that correctly folded proteins are delivered to subsequent cellular compartments and regulates the folding status of proteins and prevents the aggregation of misfolded proteins by either refolding or degrading aggregation-prone species (Mogk and Bukau, 2006). During severe stress conditions or upon protein overproduction this protection system can be overwhelmed by high substrate load, resulting in the formation of protein aggregates. Rousseau et al. (2006) found that aggregation of a polyglutamine repeat (polyQ)-containing protein tagged with an ER retention sequence KDEL was abolished when its expression was targeted to the ER. Once retrogradely transported outside of the ER by lactacystin, the aggregation-prone polyglutamine containing protein recovers its ability to aggregate (Rousseau et al., 2006).

There are two major cellular mechanisms involved in the ER quality control: the unfolded protein response (UPR), which detects the presence of unfolded proteins in the ER and induces the synthesis of folding enzymes, and the ER-associated protein degradation (ERAD), which degrades those proteins that fail to reach the correct conformation (reviewed by Conesa et al., 2001). The UPR is induced by a variety of factors that alter the function of the ER, such as arrest
of glycosylation by treatment with tunicamycin (Gjymishka et al., 2009; Sugiura et al., 2009), inhibition of disulfide bridge formation by treatment with reducing agents such as dithiothreitol (DTT) (Richie et al., 2009), Ca\(^{2+}\) depletion in the ER (Vander Griend et al., 2009), inhibition of protein glycosylation (Valkonen et al., 2003a), expression of aberrant proteins (Nakatsuksa et al., 2004) and overexpression of normal proteins (Raden et al., 2004). During the overexpression of protein, stress within the ER can activate the unfolded protein response (UPR). The UPR initiates a cascade of events that alter the expression pattern of numerous genes involved in the secretory pathway including \(\text{bip}\) and \(\text{pdi}\) (Pakula et al., 2003; Saloheimo et al., 2003; Mulder et al., 2004; Ogawa and Mori, 2004; Penttilä et al., 2004). The effects of the UPR on protein production continue to be investigated (Pakula et al., 2003; Valkonen et al., 2003b; Al-Sheikh et al., 2004). It has been shown that UPR not only regulates ER-resident chaperones, but many other genes involved in secretion, and particularly those of the ERAD, which indicates that these two processes cooperate in maintaining the folding competence of the secretory pathway (Travers et al., 2000; Guillemette et al., 2007) (Fig. 1-4). Guillemette et al. (2007) have induced ER-associated stress in \(A.\ niger\) by chemical treatment of the wild-type cells with dithiothreitol (DTT) or tunicamycin, and by expressing a human protein, tissue plasminogen activator (tPA). Results showed that all of these treatments triggered UPR. The proteins encoded by most of the up-regulated genes function as part of the secretory system featuring chaperones, foldases, glycosylation enzymes, vesicle transport proteins and ER-associated degradation proteins. Several genes were down-regulated under stress conditions and these included several genes that encode secreted enzymes (Guillemette et al., 2007) (Fig. 1-4).
Figure 1-4 A schematic diagram of the secretory pathway with examples of genes that are transcriptionally induced or repressed by UPR (modified from Guillemette et al., 2007). The genes highlighted in yellow and blue represent the genes upregulated and downregulated by the UPR, respectively. N, nucleus; ER, endoplasmic reticulum; E, endosome; V, vacuole; G, Golgi. ERAD stands for ER-associated degradation.

The ERAD system eliminates slowly folding, incompetently folded or misfolded proteins from chaperone cycles to prevent them from clogging the protein folding machinery. This is achieved by targeting these proteins for retro-translocation into cytosol and degradation by the proteasome in the ERAD process (Yoshida, 2007). Defective proteins are recognised in the ER, possibly by resident chaperones such as calnexin and BiP, retrotranslocated to the cytosol through the Sec61p translocon complex, and targeted to the 26S proteasome by ligation to ubiquitin-conjugating enzymes for proteolytic degradation (Sommer and Wolf, 1997; Brodsky and McCracken, 1999b). One component of the ubiquitin-proteasome machinery that has been characterised in filamentous fungi is the prs12 gene of T. reesei, which is homologous to the gene encoding mouse regulatory subunit 12 of the 26S proteasome (Goller et al., 1998). The prs12 gene was shown to be moderately upregulated by treatments which caused cellular stress and accumulation of unfolded proteins in the ER (Goller et al., 1998), indicating a coordinated regulation of the UPR and ERAD systems, as suggested by Travers and co-workers (Travers et al., 2000) (Fig. 1-5). Very recently, the protein subunits of the catalytic 20S core particle and some of the 19S regulatory particle of the 26S proteasome have been purified from T. reesei and mapped using a
chromatography-based approach followed by 2DE analysis and protein identification by mass spectrometry (Grinyer et al., 2007; Kauto et al., 2009). Proteasome subunits homologues can also be found in the *N. crassa* and *A. nidulans* sequence databases (http://www.genome.ou.edu/fungal.html).

Figure 1-5 Unfolded proteins in the ER activate both ER-associated degradation (ERAD) and the unfolded protein response (UPR) (adapted from (cCracken and Brodsky, 2000). Unfolded proteins are directed to the cytosol, by Sec61p-mediated retro-translocation, for degradation by the proteasome. Unfolded proteins also activate the ER transmembrane kinase/nuclease, Ire1p, which in conjunction with the tRNA ligase, Rlg1p, splices the *HAC1* primary transcript. *HAC1* mRNA passes to the cytoplasm for translation, and the newly synthesised transcription factor, Hac1p, enters the nucleus where it affects transcription of the UPR target genes. Translation of the target gene mRNAs provides proteins for secretory pathway functions and other cellular processes.

1.2.3 Protein glycosylation in the ER

Glycosylation has been found to initiate in the ER in mammalian cells, yeast and some filamentous fungi. N- and O-glycosylation are independent processes and occur autonomously by separate mechanisms (Goochee et al., 1991; Ernst and Prill, 2001; Munro, 2001) (Figure 1-6). During the process of protein translocation, the nascent polypeptide chain are glycosylated in the
lumen of the ER by a multisubunit membrane protein complex called the oligosaccharyl transferase (OT) (Yan and Lennarz, 2005). After a protein has folded into its three-dimensional conformation, glycosylation sites may no longer be exposed (Holst et al., 1996; Holkeri et al., 1998). Upon exit from the ER, for which the native folded structure of proteins appears to be a prerequisite, carbohydrate chains are trimmed and elongated extensively during subsequent transfer through the Golgi apparatus. Thus, core-glycosylation may assist efficient folding, whereas extensive glycosylation of secreted proteins may interfere with protein folding (Kern et al., 1993). Thermal stabilisation of the protein by glycosylation was observed in silico by destabilisation of the unfolded state rather than stabilisation of the folded state via decreasing its entropy or increasing its enthalpy e.g. by breaking some residual structural elements (Shental-Bechor and Levy, 2008). Glycosylation has been reported to affect the rate of protein folding possibly via catalysis by an abundant enzyme located in the ER membrane, the oligosaccharyl transferase complex (Silberstein and Gilmore, 1996). Partial folding of the polypeptide chain would occur and the ubiquitously present N-glycosylation sites would become inaccessible to OT (Chavan and Lennarz, 2006). Replacement of amino acids on the glycosylation sites has been postulated to reduce folding possibilities of a protein in vivo and to induce accumulation of unfolded proteins in the ER. For example, removal of N-glycosylation sites of the yeast acid phosphatase severely affected protein folding and resulted in irreversible retention of misfolded acid phosphatase in the ER (Riederer and Hinnen, 1991).

Figure 1-6 Schematic structures of the N-linked core oligosaccharide and O-linked oligosaccharides in fungi (modified from Nykänen, 2002).
In addition to protein folding, glycosylation may have an effect on protein secretion and many protein characteristics like activity, isoelectric point and thermostability. N-glycosylation of cellulases such as endoglucanases (EG) and cellobiohydrolases (CBH) of *T. reesei* has not appeared to be compulsory for their secretion or activity (Merivuori *et al.*, 1985a; Kubicek *et al.*, 1989), but it affects their thermostability and protects them from proteolysis (Merivuori *et al.*, 1985a; Wang *et al.*, 1996). Protein O-glycosylation has a role in modulating the function of secretory proteins by enhancing their stability and solubility (Goto, 2007). Many observations have shown that O-glycosylation can facilitate the secretion and affect the activity of cellulases in *Trichoderma* (Merivuori *et al.*, 1985a; Merivuori *et al.*, 1985b; Kubicek *et al.*, 1987; Merivuori *et al.*, 1987). The O-glycosylation positions on *T. reesei* cellulases are mostly localised in the linker area connecting the catalytic and cellulose binding domains (Miettinen-Oinonen *et al.*, 1997; Harrison *et al.*, 1998). If the linker were not glycosylated it might become a target for proteolytic degradation. Consequently, unglycosylated cellulases have been exposed to proteolysis (Kubicek *et al.*, 1993b). Incorrectly glycosylated heterologous proteins that seem to function normally in *vitro* are possibly not able to perform correctly in *vivo* (Liu, 1992). This is particularly important to remember when filamentous fungi are planned to be used for the production of heterologous proteins intended to therapeutical practice.

### 1.2.4 Export protein from the ER

Export of cargo from the ER is confined to specific export sites, called transitional ER (tER) (Palade, 1975; Bannykh *et al.*, 1996). These are highly enriched in COPII and COPI transport components and are likely to promote the coupling of anterograde and retrograde transport (Bannykh *et al.*, 1996). These vesicles subsequently fuse with pre-existing post-ER membranes of the ER–Golgi intermediate compartment (Appenzeller-Herzog and Hauri, 2006). The structures of the transitional ER and immediate post-ER compartments up to the ER-Golgi intermediate compartments (ERGIC) are defined as ER exit site (ERES). ERES are distributed at the cell periphery and perinuclear region and are adjacent to the Golgi in many eukaryotes (Aridor *et al.*, 2004; Blumental-Perry *et al.*, 2006). The number and spatial organisation of ERES seems, to some extent, to be correlated with Golgi organisation. This connection becomes clear when the arrangement of ERES in various organisms is analysed (Budnik and Stephens, 2009). In the yeast *Pichia pastoris*, it is believed that ERES are the origin of the Golgi (Glick and Malhotra, 1998). The budding yeast *P. pastoris* shows a clear organisation of the ER exit sites
and the Golgi apparatus of *P. pastoris* is arranged as stacked Golgi cisternae. EM revealed that these cisternae are associated with ER exit sites (Rossanese *et al.*, 1999). A similar organisation is found in the unicellular parasites *Trypanosoma brucei* and *Toxoplasma gondii*, which have one ER exit site adjacent to one stacked Golgi (Hager *et al.*, 1999; He *et al.*, 2004). Recently, it was reported that the ERES for a secretory protein, AnSec23 that was tagged with GFP, are numerous and relatively static foci localising across the entire cell. However, the density of the AnSec23-GFP ERES was greatest near the tip, correlating with predominance of early and trans-Golgi elements in this region (Pantazopoulou and Penalva, 2009).

### 1.2.5 Vesicles and protein transport along the secretory pathway

Protein transfer between organelles is mediated by carrier vesicles that continually bud from one membrane and fuse with another. Vesicles act as carriers that mediate uni- or bi-directional transport between two adjacent membranes in the secretory pathway (Rothman and Wieland, 1996; Schekman and Orci, 1996). Vesicle targeting and docking with acceptor membranes is mediated by a number of proteins, including fibrous “tethering” molecules such as p115 and giantin, which, through specific interactions, provide a first level of specificity for these transport steps (Pfeffer, 1999); v-SNAREs on the vesicles specifically bind t-SNAREs at the target membranes and have been proposed to facilitate delivery of the cargo by fusion (Jahn and Sudhof, 1999; Scales *et al.*, 2000) (Fig. 1-7).

![Figure 1-7](https://www.erin.utoronto.ca/~w3bio315/lecture19.htm)

**Figure 1-7** SNARES and vesicle targeting (adapted from the online lecture provided by Professor Danton O’Day, University of Toronto at Mississaugua [www.erin.utoronto.ca/~w3bio315/lecture19.htm]).
Each vesicle transport reaction can be divided into four essential steps that include vesicle budding, transport, tethering, and fusion (Bonifacino and Glick, 2004). These steps are tightly regulated to ensure that vesicles generated from a donor compartment are delivered to their correct acceptor compartment. The four steps are as follows: (i) Budding: coat proteins are recruited onto the donor membrane to induce the formation of a vesicle. Cargo and SNAREs are incorporated into the budding vesicle by binding to coat subunits; (ii) Movement: the vesicle moves toward the acceptor compartment by diffusion or with the aid of a cytoskeletal track; (iii) Tethering: tethering factors work in conjunction with Rab GTPases to tether the vesicle to their acceptor membrane; (iv) Fusion: the vesicle-associated SNARE and the SNARE on the acceptor membrane assemble into a four-helix bundle (trans-SNARE complex), which drives membrane fusion and the delivery of cargo (Cai et al., 2007b) (Fig. 1-7).

COPI (coat protein complex I) and COPII (coat protein complex II) mediate vesicle transport in the early secretory pathway. COPI primarily involved in the vesicle traffic from the Golgi to the ER and between Golgi cisternae, while COPII mediates traffic from the ER to the Golgi. Driven by the SAR1 GTPase cycle, cytosolic COPII proteins exchange on and off the membrane at specific sites on the ER to regulate cargo exit (Kirk and Ward, 2007). Properly folded and assembled proteins exit the ER in COPII vesicles (Baines and Zhang, 2007). Recruitment of the coat components to donor membranes, sorting of cargo into vesicles and the regulation of coat release at the target membrane are regulated by small GTPases ARF1 (for COPI) and SAR1 (for COPII) (Rothman and Wieland, 1996; Schekman and Orci, 1996; Springer et al., 1999; Wieland and Harter, 1999) (Fig. 1-8). In addition to coat proteins, recent data have indicated that the Ras-like small GTPase Sar1 plays multiple roles, such as COPII coat recruitment, cargo sorting and completion of the final fission (Sato and Nakano, 2007). Recent developments in COPII research by live-cell imaging has revealed that the seemingly static ER exit sites are in fact highly dynamic (Kirk and Ward, 2007). A coat complex involved in transporting only a few cargoes from the Golgi to the plasma membrane in yeast and other fungi is the Chs5/6, or exomer, complex (Sanchatjate and Schekman, 2006; Trautwein et al., 2006; Wang et al., 2006). The SAR1 homologues of A. niger and T. reesei were the first fungal vesicular GTPases to be characterised (Veldhuisen et al., 1997). Other cloned fungal GTPases include the ypt1 homologues of N. crassa (Heintz et al., 1992) and T. reesei and A. niger var awamori (Saloheimo et al., 2000). There are numerous recent reports of proteins being exported by unconventional
secretory routes (Nickel, 2003; Nickel, 2005; Riquelme et al., 2007) including some routes involving multivesicular bodies and chitosome microvesicles that may not be generated by budding from a membranous structure, but rather by self-assembly from 16S subunits (Riquelme et al., 2007).

**Figure 1-8** COP-mediated vesicle transport between the ER and the Golgi (adapted from Roy et al., 2006). ARF1 and SAR1 are activated by binding GTP, which stimulates coat assembly. Activation of SAR1 leads to the recruitment of the components of the coat complex COPII to ER membranes. COPII recruits cargo proteins that are to be delivered to other organelles and packages these into membrane tubules and vesicles. Activation of ARF1 leads to recruitment of the components of the coat complex COPI to early secretory vesicles. On these membranes, exchange of the COPII coat for a COPI coat allows further protein sorting to occur and allows transport of cargo proteins away from the ER. Cells can then assemble a functional Golgi apparatus, and the secretory pathway is fully operational (Roy et al., 2006). The generation of COPII coat requires the minimum coat components Sar1, SEC13/SEC31, SEC23/SEC24. These proteins contribute to the formation of the COPII coat, which is composed of heteromeric protein complexes, comprising SEC23/SEC24 (heterodimer) and SEC13/SEC31 (heterotetramer) and the Sar1 GTPase (Futai et al., 2004). SEC23 acts as a GAP specific for SAR1 (Yoshihisa et al., 1993).
SEC23/SEC24 is responsible for the selection of cargo proteins for packaging, providing binding sites for multiple cargo proteins (Miller et al., 2003; Mossessova et al., 2003). SEC13/31 bridges SEC23/SEC24 molecules bound to cargo proteins to create a coat that envelopes the membrane into a bud (Matsuoka et al., 2001).

From the ER proteins are selected and packaged into vesicles which bud off and are transported to the Golgi via passage along microtubules (Phillipson et al., 2001). In mammalian cells, the transport may be a two-step process where the cargo is first transferred to COPI coated vesicles for transport to the Golgi (Stephens et al., 2000). If there is a backlog in the flow of the secretory pathway, the vesicles fuse to form larger ERGIC where some further sorting occurs and ER-associated proteins can be recycled by retrograde transport.

1.2.6 Further modification of the secretory proteins in the Golgi apparatus

Proteins that are destined for secretion are next delivered to the Golgi apparatus for further modification. Although the classical dictyosome organisation of the Golgi compartment is not commonly seen in filamentous fungi (Markam, 1994), typically Golgi-associated functions are present in the fungal cell, and the term Golgi-like structure is used (Conesa et al., 2001). On reaching the Golgi, the protein may undergo further folding, glycosylation and phosphorylation. The chaperones and enzymes involved in these processes are not uniformly dispersed but show distinct gradients of concentration, which are representative of the progressive stages of protein modification that occurs in the direction from the cis to the trans Golgi network (TGN). Proteins in the TGN have a number of destinations and the sorting of these proteins occurs in response to the specific signals they contain (Keller and Simons, 1997).

Correct glycosylation of a protein within the ER and Golgi is important for its successful secretion and for protein functionality. Consequently, differences between mammalian and fungal glycosylation patterns present a challenge for the production of mammalian proteins in fungi. However, the basic core N-glycan structure in T. reesei is similar to the mammalian-like Man3GlcNAc2 core (reviewed by Nevalainen et al., 2004). This similarity offers the opportunity to build upon this common foundation and replicate mammalian glycosylation in fungi (Maras et al., 1997; Maras et al., 1999).
The Golgi and TGN are also the sites for Kex2-like endoprotease cleavage of protein proregions. In the absence of other signals, the default pathway for soluble proteins in the TGN lumen is for packaging and exocytosis from the cell (Raikhel and Chrispeels, 2000).

Whilst there is a wealth of information on membrane-associated proteins, membrane complexes and the carrier peptides involved in protein secretion in yeast and mammalian cells, the knowledge of the fundamental recognition mechanism upon specific fungal proteins is still lacking (Curach, 2005).

1.2.6.1 Completion of protein glycosylation in the Golgi apparatus

Oligosaccharide chains of glycosylations are trimmed and elongated by membrane proteins residing in the Golgi compartment. This process is the most different one in protein maturation between filamentous fungi and other eukaryotes (Esmon et al., 1981). Differently to mammalian cells, filamentous fungi are not able to add, for example, sialic acid to the glycoproteins, and they have also a tendency to hypermannosylate proteins (Upshall et al., 1987). In the filamentous fungal N-glycans, more variable carbohydrate residues have been detected than in S. cerevisiae. Oligomannose N- and O-glycans are predominant in filamentous fungi, whereas in S. cerevisiae hypermannosylation often occurs (Archer and Peberdy, 1997). In general, it is accepted that in filamentous fungi N-glycans are small, and the glycans added resemble the high-mannose glycans of animal cells more than the glycans in yeast proteins (Garcia et al., 2001). This view is supported by comparative bioinformatic analyses of the glycosylation pathways in various organisms including mammalian cells (Kornfeld and Kornfeld, 1985), yeast (Herscovics, 1999) and filamentous fungi (Maras et al., 1999; Deshpande et al., 2008).

Glycoproteins from T. reesei have significantly diverse carbohydrate structures, depending on the particular protein to be glycosylated, production strain, cultivation medium and culture conditions (Goto, 2007). In T. reesei, the majority of secretory proteins are highly glycosylated with both N- and O-linked glycans (reviewed by Kruszewska et al., 2008). The structures of N-glycans differ distinctively between different T. reesei strains and culture conditions (Miettinen-Oinonen et al., 1997; Bergquist et al., 2004). In the T. reesei Rut C-30 strain, the CBHI linker between the catalytic and the cellulose binding domain is extensively O-glycosylated at threonine and serine residues with di- and tri-saccharides, and in addition some phosphorylated di-saccharides are also
found (Hui et al., 2001). The O-glycans in CBHI are composed of one to four hexoses that are presumably mannose (Salovuori et al., 1987; Miettinen-Oinonen et al., 1997; Harrison et al., 1998). In addition, CBHI has four N-glycosylation sites, all in the catalytic domain (Kruszewska et al., 2008). The prevailing N-glycans of CBHI, \((\text{Man})_{7-8}(\text{glcNac})_{2}\) oligosaccharides, are linked with phosphate and glucose moieties (Maras et al., 1997). In another high cellulase-producing mutant strain of \(T.\ reesei\), ALKO2877, every site of glycosylation of CBHI has been identified and each site in terms of its modifying carbohydrate and site-specific heterogeneity has also been characterised (Harrison et al., 1998). The catalytic core domain comprises three N-linked glycans each of which consists of a single \(N\)-acetylglucosamine residue. Within the glycopeptide linker domain, all eight threonines are variably glycosylated with between at least one, and up to three, mannose residues per site. All serines in this domain are at least partially glycosylated with a single mannose residue (Harrison et al., 1998).

### 1.2.6.2 Proteolytic processing

In addition to folding and glycosylation, protein maturation involves proteolytic processing that occurs intracellularly within ER, Golgi and lysosomes or on secretion by membrane associated proteases. The protease profile for \(A.\ niger\) has been partially characterised. These proteases include aspartyl-, serine- and carboxy proteases (reviewed by van den Hombergh et al., 1997) but the profile can vary according to the strain and growth medium. Similar proteases are also present in \(T.\ reesei\) (Kubicek and Harman, 1998). Classical mutagenesis and screening has generated some protease-deficient strains of \(A.\ niger\) (Mattern et al., 1992) and \(T.\ reesei\) (Mäntylä et al., 1994) which have been beneficial for heterologous protein expression (Broekhuijsen et al., 1993; de Faria et al., 2002). Isolation of protease genes or genes involved in their regulation would allow for the specific genetic manipulation and inactivation of these loci (reviewed by Berka et al., 1990; Nevalainen et al., 2004). For example, deletion of the gene encoding the aspartic proteinase, \(Trichoderma\) pepsin from \(T.\ reesei\) resulted in a 94 % decrease in acid protease activity (Mäntylä et al., 1998). There are some additional recent papers as well on \(Aspergillus\). In these studies, double disruption of the protease genes (\(tppA\) and \(pepE\)) improved heterologous protein (human lysozyme) production by \(A.\ oryzae\) (Maruyama and Kitamoto, 2008) and further quintuple protease gene disruptants of five protease genes (\(tppA, pepE, nptB, dppIV,\) and \(dppV\)) showed the maximum production level of bovine chymosin (CHY) that was 34 % higher than those of the double protease gene disruptant of \(tppA\) and \(pepE\) in \(A.\ oryzae\) (Yoon et al., 2009).
Proteolytic activity can have a major effect on protein production in fungi especially when concerning heterologous products. Intracellular proteases have been shown to be responsible for the low amounts of human IL-6 produced in *A. awamori* (Gouka *et al.*, 1996). Degradation of human IL-6 has been suggested to happen either immediately after translation or beyond Golgi (Gouka *et al.*, 1997b). In a study with *S cerevisiae*, expression of recombinant insulin resulted in an incorrect insulin precursor. This problem was resolved by adding a removable spacer peptide between Kex2p cleavage site and the mature insulin N-terminus (Kjeldsen *et al.*, 1996). A Golgi-located peptidase (Kex2p) activity has been characterised in *T. reesei* (Goller *et al.*, 1998), *A. niger* (Jalving *et al.*, 2000; Svetina *et al.*, 2000), *Neurospora crassa* (Rasmussen-Wilson *et al.*, 1997) and *Cryphonectria* (McCabe and Van Alfen, 1999); furthermore, the gene of the *A. niger* homologue (KEXB) has been cloned and characterised (Jalving *et al.*, 2000).

1.2.7 The post-Golgi sorting and trafficking

The *trans*-Golgi network (TGN), the last station of the Golgi complex, plays a vital role in sorting proteins in the secretory pathway to the appropriate cellular destination (Traub and Kornfeld, 1997). Clathrin and the adaptor protein complexes (APs), i.e. AP1, AP2 and AP3, interact with each other, with membranes, and with the sorting signals found on cargo molecules (Schmid, 1997). Clathrin-coated vesicles (CCVs) mediate the endocytosis of plasma membrane receptors and the sorting of transmembrane proteins in the TGN for transport to endosomes (Mellman, 1996; Schmid, 1997; Traub and Kornfeld, 1997). The AP1 and AP2 of CCVs function as sorters in TGN-to-endosomal transport and endocytosis, respectively (Mellman, 1996). AP3 functions in the transport of proteins to different destinations and mediates vesicle traffic from the late Golgi to the vacuolar lysosome (Angers and Merz, 2009). Sorting by AP1 and AP3 takes place independently (Le Borgne and Hoflack, 1998). Vps1p is required for early events in vacuolar sorting (Vater *et al.*, 1992; Stack *et al.*, 1995), presumably during clathrin-mediated vesicle budding (Schmid, 1997). Two complexes that play a role in tethering events that lead to endosome-vacuole (lysosomes in mammals) biogenesis in both yeast and mammalian cells (Cai *et al.*, 2007b), CORVET (late Golgi-endosome) and HOPS (endosome-vacuole) are required for vacuolar protein sorting *in vivo* (Conibear *et al.*, 2003; Peplowska *et al.*, 2007). The HOPS complex is also required for the homotypic fusion of yeast vacuoles and the fusion of transport vesicles to the vacuole (Price *et al.*, 2000). In addition to its role in secretion to the cell surface,
the TGN is also regarded as the starting point of the transport route that leads to the lytic vacuole (Schellmann and Pimpl, 2009). In this process, two protein complexes, retromer and ESCRT, are coat/adapter combinations that are crucial for endosomal and vacular transport (Schellmann and Pimpl, 2009). Retromer mediates recycling of sorting receptors back to the Golgi apparatus, ESCRT is needed for sorting of transmembrane cargo to the vacuole (Schellmann and Pimpl, 2009).

Two models have been proposed to explain sorting into secretory granules. The first model suggests that proteins destined for regulated secretion would bind via a conformation-dependent motif to a sorting receptor in the TGN that would mediate incorporation of either individual proteins or of aggregates of proteins into immature secretory granules. This model predicts that signals are required for entry into the secretory pathway, and that proteins lacking these signals would be excluded (Keller and Simons, 1997). The second model proposes that entry into the forming granules is not selective, and that aggregated regulated proteins would be selectively condensed and retained, while others would be progressively removed via CCVs (Dittie et al., 1996) during maturation into dense core secretory granules (Castle et al., 1997; Kuliawat et al., 1997). In yeast, exocytic cargoes are packed in two populations of post-Golgi secretory vesicles to be directed to the plasma membrane. These vesicles were separated into two species based on their different densities, light and high density vesicles (Harsay and Bretscher, 1995).

The post-Golgi transport events are facilitated by cytoskeletal elements, particularly microtubules and their associated motor proteins (Cole and Lippincott-Schwartz, 1995; Lafont and Simons, 1996). Microtubules are essential for delivery and fusion of post-Golgi vesicles which move along the microtubules until they exocytose (Schmoranzer and Simon, 2003). The long-distance transport of post-Golgi organelles to the tips of the developing hyphal extensions in Neurospora crassa shows an absolute requirement for microtubules and microtubule-associated motors (Seiler et al., 1997). It is believed that actin organisation is responsible for establishing the directionality of secretion and secretory vesicles are transported along actin cables (Bretscher et al., 1994). In addition, kinesin and dynamin are required for post-Golgi transport (Kreitzer et al., 2000).
1.2.8 Secretion of protein from the mycelium into the extracellular environment

The final stage in the secretion of a protein occurs via two alternative pathways. The constitutive secretory pathway is a result of a steady rate of packaging of proteins into vesicles from the TGN and the exocytosis of their contents. The regulated secretory pathway involves the packaging of extracellular proteins into vesicles that accumulate intracellularly until exocytosis is induced by an external stimulus.

Many of the details of this final transport step in the secretory pathway are not clearly understood in filamentous fungi. Microtubules facilitate the transport of secretory vesicles from the TGN to the cell membrane in mammalian cells (Schmaranzer and Simon, 2003) and actin filaments are involved in the transport of glucoamylase to the cell wall in A. niger (Gordon et al., 2000b). The Rho family of small GTPases has been implicated in vesicle trafficking and cell polarisation in yeast (Takai et al., 2001) and the rho3 gene of T. reesei is important for growth and total protein secretion (Vasara et al., 2001). The involvement of the cytoskeleton indicates that the passage of secretory vesicles to hyphal tips is not via a passive mechanism.

1.2.8.1 The site of protein secretion from the mycelium

For a long time, the general consensus was that all secretion from the mycelium occurred only from the apical cells or sub-apical regions of hyphae (Wösten et al., 1991). This concept was supported by the bulk flow of protoplasm toward the tips and the dynamic nature of the cell wall in this region (Wösten et al., 1991; Wessels, 1994). In addition, the distribution of a GFP-glucoamylase fusion protein in A. niger showed that secretion occurred dominantly from the hyphal apex, even though some fluorescence accumulated in the cell wall of the hyphae (Gordon et al., 2000b). However, Nykänen’s data suggest that the site of secretion is variable depending on the protein. Immunohistochemical labelling for CBHI localised the protein throughout the cytoplasm and within the cell wall of mature hyphae, indicating that active secretion was also occurring away from the hyphal tips (Nykänen, 2002).

Evidence is also emerging to suggest that there is a spatial relationship between the region of transcription and the site of protein secretion from the cell. For example, the distribution of CBHI protein throughout the mycelium of T. reesei mostly mirrored the distribution of cbh1 transcripts (Nykänen, 2002) and the distribution of barley endopeptidase B (EPB) protein and epb transcripts
were colocalised to the apical and sub-apical compartments of hyphae at the edge of the colony (Nykänen et al., 1997). As localisation of mRNAs is a post-transcriptional mechanism for spatial and temporal regulation of protein production (Lipshitz and Smibert, 2000; Palacios and St Johnston, 2001) allowing specific proteins to be synthesised in the subcellular regions, this colocalisation of transcripts and protein suggests that transcription and secretion occur locally in the apical and sub-apical sites of hyphae (Nykänen et al., 1997).

Localised transcription and secretion implies differential promoter activity throughout the mycelium. For example, glucoamylase (GLA) is secreted by only some hyphae at the colony periphery in *A. niger* (Wösten et al., 1991). The expression of GFP under the *A. niger* glaA promoter revealed that hyphal branches displayed significantly lower levels of fluorescence than the leading hyphae but expression of GFP under the *gpdA* promoter demonstrated a more even distribution of fluorescence across the mycelium. The authors suggested that the differential distribution of fluorescence was an indication of spatial differences in transcriptional activity of the *glaA* promoter across the mycelium, which resulted in the observed localised secretion of glucoamylase (Vink et al., 2004).

The information at hand indicates that the mycelium cannot be considered as a uniform mass, but more as a dynamic multicellular organism which displays spatial regulation of transcription and secretion throughout (Curach, 2005).

1.3 **Research methods for studying the secretory pathway**

Traditionally, the secretory pathway has been studied using a combination of genetic, microscopic (both light and electron microscopic) and biochemical approaches (Presley, 2005). The information achieved from the integration of these techniques has led to our current understanding of the molecular mechanisms controlling this complex biochemical pathway (Pimpl and Denecke, 2002). Here, focus is directed to the aspects related to molecular genetic and microscopic approaches which were the main techniques used in this work to study the secretory pathway in the industrially-exploited filamentous fungus *T. reesei*.

1.3.1 **Introduction of DNA-based fluorescent reporters into filamentous fungi**

The use of fluorescent reporter proteins commenced with the identification of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and cloning of the gene encoding
GFP (Prasher et al., 1992). Fluorescent reporters have the advantage over other detection systems in that they require no addition of cofactors and can be imaged in situ. The fluorescent reporters are also available with a range of spectral properties to allow multi-labelling within one living system. The number of mutants of the fluorescent markers available and the number of applications have been steadily increasing.

1.3.1.1 Overview of GFP
Wild type GFP contains 238 amino acids, folded into a series of 6 alpha helices and 11 beta strands, connected by loops. The strands form a classical beta barrel, a cylindrical beta sheet with anti-parallel strands. Inside this beta-structure there is an alpha-helix (light blue), in the middle of which is the chromophore (yellow) (Fig. 1-8) (Yang et al., 1996). It is remarkable that the principal fluorophore is derived from a triplet of adjacent amino acids: serine, tyrosine and glycine residues at locations 65, 66 and 67 (referred to as Ser65, Tyr66 and Gly67). Wild type GFP from the jellyfish has two excitation peaks, a major one at 395 nm and a minor one at 475 nm with an emission peak at 509 nm over the lower green portion of the visible spectrum. In a wide range of pH, increasing pH can lead to a reduction in the fluorescence at 395 nm excitation and an increased sensitivity to 475 nm excitation (Kain and Kitts, 1997).

**Figure 1-9** The structure of GFP and its association into dimers (adapted from (Yang et al., 1996). Two monomers are associated into a dimer. The monomer has 11 strands (numbered from 1 to 11) of β-sheet which form the walls of a cylinder. Short segments of α-helices (blue) cap the top and bottom of the “β-can” and also provide a scaffold for the fluorophore which is near geometric centre of the can.
Due to the potential for widespread usage and the evolving needs of research, many different mutants of GFP have been engineered (Shaner et al., 2005). The first major improvement was a single point mutation (S65T) reported in 1995 in Nature (Heim et al., 1995). This mutation dramatically improved the spectral characteristics of GFP, resulting in increased fluorescence, photostability and a shift of the major excitation peak to 488 nm with the peak emission kept at 509 nm. This matched the spectral characteristics of commonly available FITC filter sets, increasing the practicality of general use. A 37 °C folding efficiency (F64L) point mutant to this scaffold yielding enhanced GFP (EGFP) was discovered in 1995 by the lab of Ole Thastrup (Thastrup et al., 1995). Thereafter, many other mutations have been made, including color mutants; in particular blue fluorescent proteins (EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein (ECFP, Cerulean, CyPet) and yellow fluorescent protein derivatives (YFP, Citrine, Venus, YPet).

1.3.1.2 GFP-based genetic fusion techniques

Virtually any protein can be tagged with GFP. The resulting chimeras are easily expressed within cells, are bright and can be imaged repetitively without loss of fluorescence. The GFP tag also does not usually alter the target protein’s transport or function; therefore the chimeras can be used, for example, to track proteins through intracellular pathways. To minimise potential interference by the GFP tag with its fusion partner, it is desirable and sometimes necessary to incorporate a peptide linker to allow sufficient spatial separation of the two protein moieties to assure fusion protein stability and functionality (Su, 2005). Flexible linkers lacking large bulky hydrophobic residues e.g. GSAGSAAGSGEF (Waldo et al., 1999) are commonly used, while hydrophilic helix-forming linker peptides have been reported to be superior to flexible linkers in some cases (Arai et al., 2001). To allow removal of the GFP tag, an enzymatic cleavage site (e.g. enterokinase or Factor Xa cleavage site) can be engineered into the linker (Su, 2005). It is preferred to fuse the GFP/linker to the N-terminus of the target protein, provided such fusion does not impair function and stability of the target protein. In most of these fusion constructions, the GFP has been treated as an indivisible entity, usually appended to the amino or carboxyl terminus of the host protein, occasionally inserted within the host sequence where the GFP-coding sequence still has been kept essentially intact (Siegel and Isacoff, 1997; Biondi et al., 1998; Baird et al., 1999). With the majority of the enzymes commonly used for tag removal, this fusion orientation enables elimination of the tag without leaving extraneous amino acid residues.
on the target protein after cleavage. Alternatively, chemical cleavage based on cyanogen bromide, formic acid, or hydroxylamine may be considered, provided the target proteins are not susceptible to cutting by these chemical agents (Su, 2005). Further information of tag removal can be found in a comprehensive review by (Hearn and Acosta, 2001).

Although deleterious effects on GFP folding and fluorescence was observed by inserting random peptides up to 20 residues in length at several locations within loops of GFP (Abedi et al., 1998), evidence showed that GFP can remain fluorescent using strategies of circular permutation by rearrangement of GFP gene sequence and insertion of foreign proteins (Heinemann and Hahn, 1995; Baird et al., 1999) suggesting certain locations within GFP can tolerate insertion of entire proteins. Examples included insertions of calmodulin or a zinc finger domain in place of Tyr-145 of a yellow mutant (EYFP) of GFP resulted in several fold enhanced fluorescence (Baird et al., 1999). So far, it has been possible to translate the permissive sites identified in green GFP variants to a variety of Aequorea GFP-derived colour shifted variants including YFP (Baird et al., 1999; Chiang et al., 2006), CFP (Baird et al., 1999), T-Sapphire (Zapata-Hommer and Griesbeck, 2003), mCherry red fluorescent protein (Li et al., 2008). Thus, insertion of receptor proteins of interest into sequences of GFP family FPs has offered a new strategy for generating genetically encoded indicators for important biochemical and physiological signals (Baird et al., 1999).

1.3.1.3 GFP applications involving protein secretion in filamentous fungi

Tagging target proteins with DNA encoding either the entire fluorescent protein or part of the protein in a fungal host allows producing a reporter to facilitate studies of protein secretion. Genetic approaches using transformed organisms coexpressing GFP reporters have opened up new ways to study the protein-protein interactions in the secretory pathway (Pimpl and Denecke, 2002). Fluorescent reporters are now widely employed as biomarkers and in studies into the secretory pathway and protein secretion in filamentous fungi (Table 1-5).
Table 1-5 Examples of the uses of fluorescent reporter proteins in fungal protein secretion (continued onto next page).

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<th>Transformation method</th>
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<th>Reference</th>
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<td>Glucoamylase (GLA)</td>
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<tr>
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<tr>
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<td>α-coat protein (CopA)</td>
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</tr>
<tr>
<td>A. niger</td>
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<td>Glucoamylase (GLA)</td>
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<tr>
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<td>A. oryzae</td>
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<td>A. oryzae</td>
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<td>amyB</td>
<td>Binding protein (BiPA)</td>
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<td>A. oryzae</td>
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<tr>
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<tr>
<td><em>A. oryzae</em></td>
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<tr>
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<td>mRFP1</td>
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<td>Putative uric acid-xanthine permase AoUapC</td>
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<td>Nuclear and mitochondrial Localisation</td>
<td>Higuchi et al., 2006</td>
</tr>
<tr>
<td><em>A. oryzae</em></td>
<td>mRFP1</td>
<td>AouapC</td>
<td>Putative uric acid-xanthine permase AoUapC</td>
<td>Protoplast</td>
<td>Nuclear and mitochondrial Localisation</td>
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</tr>
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<td>Putative uric acid-xanthine permase AoUapC</td>
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<td><em>T. reesei</em></td>
<td>mCerulean VenusYFP</td>
<td>SSOI</td>
<td>SSOI</td>
<td>N/A</td>
<td>Localisation and interaction between SNARE proteins</td>
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<tr>
<td><em>T. reesei</em></td>
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<td>cbh1</td>
<td>SSOI</td>
<td>N/A</td>
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<td><em>U. maydis</em></td>
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<td>ER retention signal (HDEL)</td>
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<td>Visualisation/localisation of the ER; the role of the tubulin cytoskeleton in ER organisation and dynamics</td>
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</tbody>
</table>

Note:

*alcA* promoter: the promoter of the alcohol dehydrogenase I gene (*ALC)*

*gpdA* promoter: the promoter sequence of the gene for glyceraldehyde-3-phosphate dehydrogenase A (*GPD)*

*gluA* promoter: the promoter sequence of the gene encoding glucoamylase (*GLA)*

*amyB* promoter: the promoter of the Taka-amylase A gene

*pgkA* promoter: the promoter of the gene encoding 3-phosphoglycerate kinase gene (*PGK)*

*AouapC* promoter: the promoter of the gene expressing acid-xanthine permease AoUapC

*cbh1* promoter: the promoter of the gene encoding the protein cellobiohydrolase I (*CBHI)*

*nar* promoter: the promoter of the gene encoding nitrate reductase (*NAR*)

N/A: not applicable
First of all, fluorescent reporters can be targeted to specific organelles by fusion with an appropriate and necessary localisation signal (De Giorgi et al., 1996; Takeuchi and Ozawa, 2007), therefore serving as excellent markers for subcellular organelles in the secretory pathway. So far there are commercially available fluorescent reporters targeted to the ER, Golgi, cell membrane, nucleus, vacuoles, cytoplasm, peroxisomes, mitochondria and cytoskeleton (www.bdbiosciences.com/clontech/). The only limitation to intracellular localisation of fluorescent reporter proteins seems to be the identification of gene sequences for organelle-specific marker molecules.

GFP also has been used as an indicator for the protein localisation and secretion in yeast, plant cells and filamentous fungi (Gordon et al., 2000b; Jones et al., 2004; Surribas et al., 2006). A glucoamylase-green fluorescent protein fusion (GLA-sGFP) was used as an in vivo reporter of protein secretion in A. niger. Expression of GLA-sGFP revealed that fluorescence was localised in the hyphal cell walls and septa, and that fluorescence was most intense at hyphal apices (Gordon et al., 2000b). The effect of various treatments that block protein secretion has been visualised in A. niger using glucoamylase-GFP fusion protein (Khalaj et al., 2001). Secretion blockers causing retention and aggregation of secretory proteins inside the hyphae or cell wall, have also been used in combination with green fluorescent protein fusions to visualise unfolded proteins and locate potential bottlenecks (Gordon et al., 2000b).

Furthermore, when coupled to modern microscopy techniques such as fluorescence recovery after photobleaching (FRAP), fluorescent resonance energy transfer (FRET) and/or fluorescence lifetime imaging microscopy (FLIM), GFPs have played a crucial role in the studies of cellular events involved in secretion. For example, FRAP analysis was conducted to monitor the flow of GFP-labelled chitin synthases (CHS-GFP) towards the Spitzenkörper (the equivalent of a multi-vesicular bodies at the very tip of fungal hyphae) in N. crassa transformants NMR3 and strain NMR6 (Riquelme et al., 2007). FRAP analysis indicated that the fluorescence of the Spitzenkörper originated from the finely dispersed fluorescence in the proximal sub-apex therefore supporting the prediction that the Spitzenkörper was directly involved in guiding the cell wall formation (Bartnicki-García, 2002). Valkonen et al. (2007) quantified the interactions between SNARE proteins in Trichoderma reesei and showed that the site of the ternary SNARE
complex formation between SNCI (a functional homologue of vertebrate synaptic vesicle-associated membrane proteins or synaptobrevins proteins from the yeast *Saccharomyces cerevisiae*) and SSOI (a t-SNARE coiled-coil homology domain profile at position 234-296 in the *T. reesei*) or SSOII (a t-SNARE coiled-coil homology domain profile at position 233-295 in the *T. reesei*), respectively, was spatially segregated. SNARE complex formation could be detected between SNCI and SSOI in sub-apical hyphal compartments along the plasma membrane, but surprisingly not in growing hyphal tips, previously thought to be the main site of exocytosis. In contrast, SNCI-SSOII complexes were found exclusively in growing apical hyphal compartments. These findings demonstrated spatially distinct sites of plasma membrane SNARE complex formation in fungi and the existence of multiple exocytic SNAREs which are functionally and spatially segregated (Valkonen *et al.*, 2007).

1.3.1.4 The cbh1 gene promoter of *Trichoderma reesei*

A fluorescence marker in a fungus must be expressed under a fungal promoter. Examples of frequently used promoters for expression of proteins in filamentous fungi include the inducible *T. reesei* cellobiohydrolase I (*cbh1*) gene promoter, the inducible *A. awamori* glucoamylase A (*glaA*) gene promoter and the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*pgk1*) gene promoter (reviewed by (Curach, 2005).

The *T. reesei cbh1* promoter is one of the strongest inducible promoters known in fungi and consequently, the promoter is used extensively in the production of recombinant industrial enzymes (reviewed by (Nevalainen *et al.*, 2005). The *cbh1* promoter has been used for the expression of heterologous genes with varying degrees of success. Expression of proteins fused to the CBHI core has been used to improve extracellular yields of foreign proteins such as human antibody fragments by 150 fold and of calf chymosin by fivefold (Nykänen, 2002). In general, the *cbh1* promoter is strongly induced by sophorose and cellulose and by oligosaccharides and disaccharides derived from cellulose (Rautio *et al.*, 2007). Also several other saccharides such as lactose induce *cbh1* expression in some strains (Ilmén *et al.*, 1997). However, when grown on crystalline cellulose, the actual mechanism of *cbh1* induction is dependent upon an inducer produced by the activity of CBHII and EGII enzymes (Seiboth *et al.*, 1997). Alternatively, the *cbh1* promoter may also be induced by the soluble carbon source lactose, and galactokinase was
regarded as a key enzyme for cellulase induction during growth on lactose (Seiboth et al., 2003). Evidence thus suggests that the cbh1 promoter has more than one mechanism of induction and one of these mechanisms involves the transcription enhancer ACEII (a main and universal transcriptional activator) (Aro et al., 2003). The cbh1 promoter is strongly repressed by glucose via the action of the transcriptional repressor, CREI. Glucose repression has been relieved by mutation of a single CREI binding site in the cbh1 promoter in the T. reesei QM9414 (Ilmén et al., 1996a). However, an additional eight CREI binding sites were discovered in the cbh1 promoter using electrophoretic mobility shift assays and DNase I footprinting assays (Takashima et al., 1996) which suggested that the multiple CREI binding sites have an additional role in the regulation of the cbh1 promoter. Recently, a series of artificial cellobiohydrolase I gene (cbh1) promoters has been constructed to improve heterologous gene expression in T. reesei (Liu et al., 2008). In this study, the region from -677 to -724 with three potential glucose repressor binding sites was deleted. Then the region from -620 to -820 of the modified cbh1 promoter, including the CCAAT box and the Ace2 binding site, was repeatedly inserted into the modified cbh1 promoter, obtaining promoters with copy numbers of 2, 4, and 6 of the 200 bp DNA fragment (−620 to −820 region) containing the CCAAT box and Ace2 binding sites. The results showed that the glucose repression effects were abolished and the expression level of the glucuronidase (gus) reporter gene regulated by these modified multi-copy cbh1 promoters was markedly enhanced as the copy number increased (Liu et al., 2008).

1.3.1.5 Transformation of filamentous fungi
Since the identification of the first amino acid biosynthesis pathway genes by complementation of mutants with the transforming DNA, transformation has continued to play a critical role in the elucidation of gene function and regulation (Bradshaw, 2007). To express heterologous genes such as those encoding fluorescent proteins in filamentous fungi, the plasmid-mediated expression construct must be introduced into cells of the fungal host. In fungi, there are three major gene-transfer strategies: treating fungal cells with lytic enzymes to create protoplasts, biolistic bombardment, and electroporation (reviewed by (Fincham, 1989).

Protoplast transformation
The preparation of protoplasts from fungal cells using various cell-wall degrading enzyme remains the most common method for preparation of cells for transformation (Ruiz-Diez, 2002).
The starting cells can be germinated asexual spores (Rossier et al., 1985), young hyphae (Buxton and Radford, 1983) or basidiospores (Zapanta et al., 1998). The choice of enzyme for digesting the cell wall is a key factor and the optimum timing of the cell wall digestion has to be determined for each batch of enzyme (Ruiz-Diez, 2002). An example of the enzyme mixture used for protoplast making in filamentous fungi is Novozyme 234 (Riach and Kinghorn, 1996; Gordon et al., 2000b; Khalaj et al., 2001) now called L1412 Sigma lysing enzymes from Trichoderma harzianum. All protoplast preparations have to be protected by the presence of an osmotic stabiliser such as sodium chloride, magnesium sulphate, mannitol or most commonly sorbitol or sucrose. Sorbitol, at concentrations between 0.8 and 1.2 mol l\(^{-1}\) has been most commonly used and seems to be satisfactory for the majority of fungi (Fincham, 1989). Sometimes lower concentration, i.e. 0.2 mol l\(^{-1}\) sucrose or sorbitol in the plates and 1 mol l\(^{-1}\) sucrose and sorbitol in the top agar, promotes a more rapid growth of colonies than 1 mol l\(^{-1}\) of the stabiliser in both the plates and the top agar (May, 1992). This is presumably because high concentration the stabiliser is inhibitory to the regeneration of the protoplasts (Ruiz-Diez, 2002). Uptake of DNA (usually either linear or double-stranded circular) by protoplasts is carried out in the presence of calcium ions, followed by the addition of polyethylene glycol (PEG) (Ruiz-Diez, 2002).

**Biolistic transformation**

Biolistic transformation was introduced in 1987 (Klein et al., 1987) and has been developed as a method for incorporation of exogenous DNA into intact, thick-walled fungal cells. Typically, it results in the integration of several copies of the transforming plasmid into the fungal genome as well as in increased mitotic stability of the transformants (Lorito et al., 1993; Herzog, 1996). This technique involves tungsten or gold particles coated with the transforming DNA, which are accelerated at a high velocity directly into the fungal spores or cells. Transformation by bombardment does not require protoplast isolation thus providing a fast method for transformation of intact cells (Sunagawa et al., 2007) compared to transformation using protoplasts. Biolistic transformations of the different target cell types require optimisation of the physical parametres used in bombardment (Sanford et al., 1993). The parametres which have the greatest effect on transformation efficiency include the vacuum in the bombardment chamber, the distance travelled by the microparticles before hitting the target cells, the size and density of the particles used in bombardment, and the driving power source (Te'o et al., 2002). Each of these
interacting factors has to be addressed systematically in order to produce the best conditions for the particular target cells of interest. Biolistic transformation has been applied to a number of filamentous fungi such as *N. crassa*, *Magnaporthe grisea*, *T. harzianum*, *A. nidulans* (Riach and Kinghorn, 1996), and *T. reesei* (Hazell *et al*., 2000; Te'o *et al*., 2002).

This technique is based on reversible permeabilisation of biomembranes induced by short duration, high amplitude electric fields. The membrane changes that occur during an electric pulse permit the uptake of recombinant DNA, which, in turn, can result in molecular transformation (Ruiz-Diez, 2002). There are basically three options for the electroporation of filamentous fungi (Ruiz-Diez, 2002): (i) electroporation of conidial preparations treated with a cell wall weakening agent (Chakraborty *et al*., 1991; Ozeki *et al*., 1994); (ii) electroporation of protoplasts (Kothe and Free, 1996), and (iii) electroporation of germinated conidia. The last option, especially applied for *A. nidulans*, circumvents the requirement for enzymatic treatment of the organism (Sánchez and Aguirre, 1996). Electroporation systems have been developed for a large number of filamentous fungi including *N. crassa*, *Penicillium urticae*, *Leptopheria maculans*, *A. oryzae* (Chakraborty *et al*., 1991), *Scedosporium prolificans* (Ruiz-Diez and Martinez-Suarez, 1999), *A. niger* (Ozeki *et al*., 1994), *Wangiella dermatitidis*, *A. fumigatus* (Kwon-Chung *et al*., 1998) and *T. harzianum* (Goldman *et al*., 1990).

**1.3.2 Confocal laser scanning microscopy**
Together with the development of a wide range of fluorescent probes for e.g. cell organelles and new computer software, confocal laser scanning microscopy (CLSM) is having a profound impact on the experimental analysis of the secretory pathway by providing direct and visible data for better understanding of the protein secretion processes. Currently, CLSM is being extensively applied in studies into visualisation of subcellular structures (reviewed by Hickey *et al*., 2004), dynamic processes of exocytosis and endocytosis (Bi *et al*., 1997; Atkinson *et al*., 2002; Higuchi *et al*., 2006), trafficking of vesicles and vacuoles (Fischer-Parton *et al*., 2000; Dijksterhuis, 2003; Shoji *et al*., 2006), fungal tip growth and fusion protein localisation (Hickey *et al*., 2002) and interaction (Kuratsu *et al*., 2007).
1.3.2.1 Sample preparation for CLSM study of living filamentous fungi

The growth of the cylindrical hyphae of filamentous fungi is by unidirectional extension from the hyphal tip at an extremely fast growth rate (> 1 μm/s) and highly elongated form (Seiler and Plamann, 2003). In addition to the growth by the extension of the hyphal tips, branches develop from lateral walls to expand the area colonised by the mycelium and to access more nutrients from the environment (Yarden, 2004). Optimal live cell imaging with CLSM requires hyphae staying in the same plane of focus (especially important when imaging at low magnification), and reduction of spherical aberration to a minimum (and thus have sharply in-focus images) (Hickey et al., 2004). For this purpose, different sample preparation methods have been developed. For example, an “inverted agar block” (~20 mm², 5 mm thick) was adopted to image samples for 1 h or more without apparent deleterious effects on growing hyphae (e.g. from O₂ deprivation) (Hickey et al., 2004). It was noted that this technique was limited to long time observation of the fungal growth from conidia to hyphae due to difficulties in inoculation of conidia into agar medium and keeping it sterile over time as well as O₂ deprivation (Hickey et al., 2004). Adding a stain directly to the agar block containing the mycelial sample was also applied for membrane staining of T. reesei (Fischer-Parton et al., 2000; Valkonen et al., 2007). However, it takes extra time for the stain to reach the hyphae inside the agar. Alternatively, fungal cultures can be grown first on an agar plate and then excised (agar piece size: 5 mm × 2 mm × 2 mm) and sandwiched between two pieces of cellophane films before imaging (Chapman et al., 1983; Cole et al., 1997). For the treatment of hyphae with fluorochromes, large sectors (maximum width of 5 mm) of mycelium can be excised and placed directly in fluorochrome solution (Cole et al., 1997).

Similarly, a thin agarose layer with fungi grown inside was sandwiched between two perforated polycarbonate membranes allowing free diffusion of gases, water, nutrients and protein as well as colonies growing nearly two-dimensionally (Wösten et al., 1991). For staining and imaging of the fungal cultures sampled from liquid medium, Aspergillus oryzae cells were cultivated in liquid medium on the coverslip for at least 24 h and then transferred to the dye solution for staining (Maruyama et al., 2006). The coverslip technique allows easy manipulation of washing steps after staining but handling with washes must be with special care as filamentous fungi tend to drop off from the glass along with the washing solutions.
1.3.2.2 Application of fluorescent probes for studies of subcellular compartments in filamentous fungi

Filamentous fungi grow as hyphae that may branch to create a new growing axis. In this highly polarised morphology, organelles are positioned in the appropriate regions of the hyphae (Maruyama and Kitamoto, 2007) and a wide range of fluorescent markers can be used for staining of fungal organelles to allow visualisation of them by CLSM. According to the nature of staining/binding, fluorescent probes for imaging the cellular structures and their function, and interactions of molecules can be divided into three categories (see below).

(i) Synthetic chemical stains that directly attach to specific sites on small cellular molecules (the chemical structures of commonly-used organelle dyes are shown in Fig. 1-10). Cellular structures such as the nucleus, plasma membrane and cytoskeleton can be visualised with chemical reagents that bind to the biomolecules specific to these particular structures. When these reagents are fluorescent or are coupled with fluorochromes, the cellular structures can be seen by fluorescence microscopy. One can choose suitable dyes for experiments depending on the excitation and emission wavelength properties of the probe, photobleaching speed, and permeability in the cellular membrane (Suzuki et al., 2007).
(ii) Antibodies conjugated with fluorescent dyes which recognise organelle-specific antigens. Immunofluorescence microscopy is a versatile procedure and is able to detect any biomolecule in the cell provided that specific antibodies are available for the target. To stain intracellular biomolecules, permeabilisation of the plasma membrane/cell envelope or thin-sectioning of samples is required for the easy access of the antibody molecules to the target molecules in the cell. The technique generally requires fixation of tissues and cells prior to staining to preserve the cell structures and antigens, which makes it unsuitable for live-imaging. There are two strategies used for the immunofluorescence detection of antigens in cells, the direct method (Fig. 1-11A) and the indirect method (Fig. 1-11B). The direct method is a one-step staining method, and involves a fluorescently-labelled antibody reacting directly with the antigen in cells. This
technique utilises only one antibody and the procedure is therefore simple and rapid. However, it can suffer problems with sensitivity due to little signal amplification and is in less common use than indirect methods (Javois, 1999). The indirect method involves an unlabeled primary antibody which reacts with organelle-specific antigen, and a fluorescently-labelled secondary antibody which reacts with the primary antibody. The secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody.

![Figure 1-7](modified from the images published on [www.answers.com/topic/immunohistochemistry](http://www.answers.com/topic/immunohistochemistry)). A, Direct immunofluorescence staining method. B, Indirect immunofluorescence staining method.

(iii) Fluorescent proteins such as green fluorescent protein (GFP) expressed in the target structures that allow immediate visualisation under fluorescent microscopy without any further preparation. Filamentous fungi can be transformed with the DNA construct encoding GFP by integrating the DNA construct in the host chromosome. Chromosomal integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell (Ole et al., 2004). Particular gene products can be labelled directly by fluorescent proteins (FPs) (refer to Section 1.3.1.2 for details). They can also be tagged with certain amino acid sequences that specifically bind to the particular cell-permeable fluorescent dyes (Suzuki et al., 2007). FP-fusion gene products are detected after the transformation of the cells with DNA encoding the fusion partners. Real-time monitoring of fluorescence emitted from FP markers genetically tagged to the organelle-located proteins is an important tool to allow in situ visualisation of dynamics of the subcellular structures such as differential distribution of the ER.
by using a BiPA-EGFP fusion (Maruyama et al., 2006) and mobility of the Golgi using a GFP-COP1 fusion in living Aspergillus oryzae hyphae (Hickey and Read, 2009).

**Probes for the ER**

The secretion process is initiated as proteins enter the ER. Morphologically, the ER is a particularly prominent feature of cells that specialises in protein export (Preuss et al., 1991). The ER is a continuum of the nuclear envelope and consists of a network of tubules that extend from the cell centre to the periphery (Maruyama et al., 2006). In filamentous fungi, the ER locates throughout the hyphal cell, its membranes exhibit a smooth profile, and the cisternae are generally parallel to each other and usually orientated parallel to the long axis of the hyphae (Cole et al., 2000).

The most widely used chemical stains are DIOC₆(3) and hexylrhodamine B. DiOC₅(3) is used less often. These dyes have been used to identify ER in a variety of cell types including cultured human NK cells (Lim and Liu, 2001), fungi (Kamisaka et al., 1999), and plant cells (Cantrill et al., 1999). DIOC₆(3) and hexylrhodamine B are amphipathic and have lipophilic cations with moderate-sized conjugated systems. The moderately lipophilic character permits probe uptake by passive diffusion without non-specific accumulation in biomembranes. The moderately amphipathic character favours uptake into the ER, perhaps owing to its high concentration of zwitterionic lipid head-groups (Colston et al., 2003). At low concentrations, DIOC₆(3) accumulates in mitochondria, while at higher concentrations it labels the ER, which may be identified by its tubular morphology (http://www.ihcworld.com/_protocols/immunofluorescence/cellular_structure.htm). Subsequently, the ER is stained as DIOC₆(3) concentration is raised or when exposure to the probe is increased (Colston et al., 2003). Similar to DIOC₆(3), hexylrhodamine B also results in the staining of mitochondria and sometimes these probes are used for this purpose (Varghese et al., 2001). Under some circumstances, staining of additional membranous structures with these two dyes may occur, such as endosomal, Golgi and plasmalemmal membranes (Terasaki, 1989; Terasaki, 1993), and the nuclear envelope in cell-free preparations (Zhang et al., 2002). DIOC₆(3) and hexylrhodamine B are generally considered to be non-specific stains for the ER and therefore not suitable for differentiating the ER from other reticulate organelles (Terasaki and
Reese, 1992). Another fluorochrome, ER-Tracker™ series including three colour options of blue (ER-Tracker™ Blue-White DPX), green and red (ER-Tracker™ Green and Red, respectively) has been introduced as a highly selective probe for the ER in live animal cells (Haugland, 1996). ER-Tracker™ Blue-White DPX is a member of Dapoxyl™ (DPX) dye family which binds to phospholipid bilayer membranes therefore not very specific for the ER although it does not stain mitochondria as DiOC₆(3) does. It appeared to be able to stain punctate structures tentatively identified as Golgi apparatus in addition to staining of the ER which was clearly distinct from mitochondria and the tubular vacuole system in the hyphae of *Pisolithus tinctorius* (Cole *et al*., 2000). ER-Tracker™ Green and ER-Tracker™ Red dyes are conjugates of glibenclamide, a commonly-used anti-diabetes drug, and fluorophores BODIPY® FL and BODIPY® TR, respectively. Glibenclamide binds to the sulphonylurea receptors of ATP-sensitive K⁺ channels which are prominent on the ER therefore selective for staining the ER (http://probes.invitrogen.com).

In addition, it is possible to use antibodies against putative ER-specific antigens to visualise the ER by immunofluorescence microscopy (Louvard *et al*., 1982) (refer to Fig. 1-11 for the principle of immunofluorescence microscopy). A general approach to the visualisation of the ER by immunofluorescence microscopy should employ markers that have been firmly demonstrated to be specifically located in the ER (Koch *et al*., 1987). The anti-HDEL antibodies have been used to label the ER in the oomycete *Phytophthora cinnamomi* (Hardham and Mitchell, 1998). Other ER marker proteins such as Dol-P-Man synthase (Preuss *et al*., 1991) and the yeast BiP homolog the Kar2 protein (Rose *et al*., 1989) were localised in yeast by indirect immunofluorescence microscopy. The antibodies against BiP (or GRP 78) have been used to label the ER in the unicellular yeast *Saccharomyces cerevisiae* (Sagt *et al*., 1998) and the multicellular filamentous fungus *Aspergillus awamori* (Lombaño *et al*., 2004).

Visualisation of the secretory pathway *in vivo* using green fluorescent protein (GFP) has permitted a range of experimental approaches that are based on living cells and their manipulation while under microscopical observation (Pimpl and Denecke, 2002). In filamentous fungi, the localisation and dynamics of the ER network have been studied by expression of GFP-fused proteins (Fernández-Ábalos *et al*., 1998; Wedlich-Söldner *et al*., 2002). A tubular network
of green fluorescence was observed by expression of the BiPA-GFP fusion protein in *A. oryzae* (Maruyama et al., 2006) which resembled the ER structure observed in the hyphae of *A. nidulans* (Fernández-Ábalos et al., 1998) and *U. maydis* (Wedlich-Söldner et al., 2002). Interestingly, the localisation pattern of the BiPA-GFP fusion proteins around the nuclei in *A. oryzae* slightly differed from that when expressed in *S. cerevisiae* according to the observations where the ER network visualised by the BiPA–GFP fusion protein only partially surrounds the nuclei in *A. oryzae*, whereas the expression of the same construct in *S. cerevisiae* labelled complete ring structures of the nuclear envelope (Maruyama et al., 2006; Maruyama and Kitamoto, 2007). Evidence based on similar BiPA-GFP fusion technology has demonstrated the highly differentiated localisation of the ER network in various regions of the hyphae: the gradient distribution of the ER network in the apical compartment; localisation along the septum; differential distribution in adjacent compartments, and the dynamic morphological change during septum formation (Maruyama and Kitamoto, 2007). Recently, four ER-resident SNARE proteins, including AoUfe1p, AoUse1p, AoSec20p, and AoSec22p, were fused to EGFP respectively to visualise the ER (Kuratsu et al., 2007). Generally speaking, the EGFP fluorescence in the strains expressing these fusion proteins was primarily observed as a ring-like structure, while a part of the fluorescence was observed as tubular or diffused structures in the cytoplasm (Kuratsu et al., 2007). Moreover, the overall subcellular distributions of these four SNAREs are essentially consistent with localisation and gradient distribution of the BiPA protein from the apical region in *A. oryzae* (Maruyama et al., 2006; Kuratsu et al., 2007).

**Probes for the Golgi apparatus**

The Golgi is a “mystery” compartment in filamentous fungi due to limited data about its morphology. The term Golgi-like structures, Golgi equivalents, or dictyosomes, is normally used as the classical ordered stack of flattened cisternae architecture of the Golgi apparatus is not commonly seen in filamentous fungi (Markam, 1994). Golgi in the baker’s *S. cerevisiae* was shown to be composed of several networks of individual membrane tubules distributed throughout the cytoplasm (Preuss et al., 1991; Rambourg et al., 1993). By contrast, the methylotrophic yeast *Pichia pastoris* had stacked Golgi organelles (Gould et al., 1992). In filamentous fungi, the Golgi apparatus have been described at the electron microscopic level as punctate structures that are often found at apical regions of hyphae (Cole et al., 2000; Rida et al.,
2006), and consist of individual smooth membrane cisternae (Howard, 1981). Similar punctate bodies were visualised in A. oryzae (Kuratsu et al., 2007; Hickey and Read, 2009). In T. reesei Rut C-30, the Golgi–equivalents emerged as semicircles in the hyphae (Nykänen, 2002). However, no characteristic Golgi structures, as seen in both QM6a and Rut C-30 strains by Ghosh et al., (1990) were observed. Immunolabelling experiments also failed to provide a clue to their morphology (Kurzatkowski et al., 1993) in T. reesei. Although morphology and biochemistry of the Golgi complex in filamentous fungi are poorly understood, functions typically associated with the Golgi are present in the fungal cell (Conesa et al., 2001). In T. reesei, electron-dense vesicles seen by Kurzatkowski et al. (1993) supposed to fulfil the processing role of the Golgi complex.

Classical chemical fluorescent stains for the Golgi apparatus include NBD C₆-Ceramide and NBD C₆-Sphingomyelin as well as BODIPY® Ceramides and BODIPY® Sphingomyelin (http://probes.invitrogen.com/handbook/sections/1204.html). The fluorescent ceramides including NBD C₆-Ceramide and BODIPY® Ceramides, are metabolised to fluorescent sphingomyelin and glucosylerceramide, and accumulate in the Golgi therefore resulting in staining of the Golgi. The accumulation of ceramides and ceramide metabolites was suggested due to their interaction with endogenous Golgi lipids (Pagano et al., 1989). These stains have been used to label the Golgi bodies in animal and plant cells to observe the morphology of the Golgi apparatus (Lipsky and Pagano, 1985; Pagano et al., 1991). However, these dyes have not been proven successful as probes for the Golgi in the filamentous fungus Pisolithus tinctorius (Cole et al., 2000).

Immunofluorescence microscopy studies of the Golgi morphology have mainly focused on the yeasts S. cerevisiae and P. pastoris. Antibodies that recognise yeast proteins functionally associated with the Golgi complex, typically stain several punctate structures when viewed with indirect immunofluorescence microscopy; examples of such proteins include YPT1 (Segev et al., 1988), SEC7 (Franzusoff et al., 1991; Rossanese et al., 1999), KEX2 (Redding et al., 1991), SEC14 (Cleves et al., 1991) and RHO1 (McCaffrey et al., 1991). In double labelling studies, some of these proteins, e.g. Kex2p and Sec 14p (Cleves et al., 1991), Och1p-HA and Sec7p (Rossanese et al., 1999) have been shown to colocalise. As many as 10 Kexp staining structures
per mother cell have been observed (Redding et al., 1991) suggesting that yeast cells may contain multiple Golgi compartments. In *P. pastoris*, immunofluorescence microscopy investigations indicated that the distribution of the Golgi marker protein OCH1-HA showed a dotty pattern in the cis-Golgi apparatus (Rossanese et al., 1999; Callewaert et al., 2001). By immunofluorescence microscopy using the anti-HA antibody against a Golgi-resident protein CaVrg4-HA, Golgi appeared as rod-like spots and its localisation was polarised at the distal regions of the hyphae of *Candida albicans* (Rida et al., 2006).

Recently, GFP has been increasingly used as a marker for the fungal Golgi apparatus. By fusion of EGFP with Golgi-resident SNAREs in *A. oryzae*, the Golgi apparatus was observed as punctate structures at hyphal apices or round structures in the proximal regions (Kuratsu et al., 2007). When a Sed5 homologue and EGFP were fused and expressed, dot-like fluorescence was observed in *A. oryzae* (http://park.itc.u-tokyo.ac.jp/Lab_Microbiology/Englishfile/studiesEfile/PTrans-E.html). A GFP tagged CopA protein in *A. nidulans* localised to distinct hollow “doughnut”-like spherical and elongated structures that were concentrated at the tips of growing hyphae and branches at a short region directly behind the apex (Breakspear et al., 2007; Hickey and Read, 2009). In sub-apical regions, fluorescence was dramatically reduced compared with hyphal tips (Breakspear et al., 2007). Apical accumulation of the Golgi apparatus seems consistent with the gradient distribution of ER from the apical compartment and suggests its central role in the secretion in hyphal tips (Shoji et al., 2006). In addition, the elongated Golgi structures shown by a COPA:GFP fusion (Breakspear et al., 2007) were similar to the Golgi morphology observed in *A. oryzae* by expression of a GFP fusion of 1,2-α-mannosidase (Akao et al., 2006).

**General membrane-selective probes for the endomembrane system**

Most of the internal membranes of filamentous fungi comprise the surrounding membranes of the majority of organelles including the ER, Golgi apparatus, vacuoles and vesicle system. It has been demonstrated that some organelles are physically connected by the membrane network through direct linking between the outer nuclear envelope and the rough ER (reviewed by (Bourett et al., 2007; Lebiedzinska et al., 2009). The ER also appears to be in direct contact (although not necessarily actually linked) to the membranes of many other organelles such as
nuclei, vesicles and vacuoles (Markam, 1994). However, in most cases, the endomembrane system is discontinuous, such that organelles are separated within the cytoplasm, but are kept in dynamic contact with other organelles via the continuous traffic of cytoplasmic vesicles which flows between them. Thus, membrane components, as well as any molecules which may be carried in the lumen of these vesicles, may pass through several separate organelles and ultimately merge with the plasma membrane itself, in the form of apical vesicles or microvesicles during the process of hyphal tip extension (Markam, 1994).

The amphiphilic styryl dyes, FM® 4-64 and FM® 1-43, are membrane-selective probes which stain the plasma membrane and most organelle membranes in fungal cells (Fischer-Parton et al., 2000; Read and Hickey, 2001). The FM-dyes only fluoresce strongly when located in a hydrophobic environment (e.g. a membrane) and thus, advantageously, do not need to be washed out from the medium in which the fungi are growing in (Yu et al., 2005). The FM-dyes enter fungal cells primarily by endocytosis and become distributed to different organelle membranes via the vesicle trafficking network and thus components of secretory pathways can become labelled (Atkinson et al., 2002; Dijksterhuis, 2003; Hickey et al., 2004). Much of this intracellular movement of the dye seems to be via the vesicle trafficking network, and through physical continuity between different membranes of the endomembrane system, but other mechanisms of dye distribution may also exist (Fischer-Parton et al., 2000). In the time-course experiment of FM® 4-64 in Sclerotinia sclerotiorum, the first fluorescent organelles appeared as punctate, roughly spherical organelles and were assumed to be endosomes. Thereafter, vesicles presumed to be primarily secretory in nature, stained up within the Spitzenkörper/apical vesicle cluster indicating that endocytic pathway(s) are connected to the secretory pathway(s). In N. crassa, vacuole membranes have also been successfully labelled; the large spherical vacuoles became stained before those of the tubular vacuolar network (Read and Hickey, 2001). Interestingly, mitochondrial membranes were also stained up with FM4-64 and FM1-43 (Fischer-Parton et al., 2000). FM® 4-64 was found to stain Golgi in tobacco BY-2 cells by colocalisation of FM® 4-64 with a GFP-tagged Golgi protein (Bolte et al., 2004). However, observations in plant cells suggest that the ER and nuclear envelope are not stained by FM® 4-64 (Bolte et al., 2004). One exception is a study on Fucus zygotes indicating possible staining of ER membranes by the dye (Belanger and Quatrano, 2000). In A. oryzae, fusion of vacuolar carboxypeptidase Y
(CPY) with enhanced GFP (EGFP) has been applied to visualise the vacuole system and results suggest presence of peculiar ring- or tube-like structures distinct from normal spherical-shaped vacuoles (Ohneda et al., 2002). In the study where an EGFP-fused plasma membrane protein was used for visualisation of the endocytic pathway in A. oryzae, the endosomal compartments were also discovered appearing upon the induction of endocytosis and moving in a microtubule-dependent manner (Higuchi et al., 2006).

### 1.3.2.3 Confocal fluorescence resonance energy transfer (FRET) microscopy for protein-protein interaction studies

Protein-protein interactions play an essential role in protein secretion processes including translocation, protein folding, post-translational modification and transport (Phizicky and Fields, 1995). For instance, BiP is an ER-resident chaperone responsible for assisting in protein folding. Thus, if the difference in the interactions between BiP and native secreted proteins and between BiP and foreign proteins can be investigated, this may contribute to better understanding of the generally accepted concept that the protein folding mechanism is likely related to the less abundant production of heterogenous proteins in contrast to native proteins in the host organisms. Protein interactions along the secretory pathway are summarised in the Table 1-6.

In order to directly visualise the protein interactions in the process of protein secretion, a microscopy technique, the FRET, can be applied to investigate the interactions between proteins with high spatial resolution in living cells. For example, Valkonen et al. (2007) quantified the interactions between SNARE proteins with high spatial resolution in living T. reesei using FRET analysis and fluorescence lifetime imaging microscopy (FLIM).
Table 1-6 Protein interactions in the secretory pathway (adapted from the review by Pimpl and Denecke, 2002).

<table>
<thead>
<tr>
<th>Protein interaction</th>
<th>Secretory stage of occurrence</th>
<th>Place of occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding of the signal recognition particle (SRP) to the signal peptide of the nascent polypeptide</td>
<td>Starting point of the secretory pathway</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Binding of the ribosome-mRNA-nascent chain complex to the translocation pore</td>
<td>Translocation</td>
<td>Cytosolic face of ER</td>
</tr>
<tr>
<td>Interaction of the nascent protein chain with chaperones such as BiP</td>
<td>Translocation, protein folding</td>
<td>Luminal face of ER</td>
</tr>
<tr>
<td>Interaction of cargo proteins to coat/sorting receptors on the target membranes</td>
<td>Transport, exocytosis, protein secretion</td>
<td>Vesicles, Golgi, plasma membrane</td>
</tr>
<tr>
<td>Interactions between the actin cytoskeleton and the secretory machinery</td>
<td>Polarised growth</td>
<td>Cytoplasm</td>
</tr>
</tbody>
</table>

**FRET principle**

FRET is a quantum mechanical process involving the radiation free transfer of energy from a donor fluorophore to an appropriately designed acceptor fluorophore over a very limited distance with subsequent fluorescent emission by the acceptor fluorophore (illustrated in Fig. 1-11A). The mechanism of FRET involves a donor fluorophore in an excited electronic state, which may transfer its excitation energy to a nearby acceptor chromophore in a non-radiative fashion through long-range dipole-dipole interactions. The theory supporting energy transfer is based on the concept of treating an excited fluorophore as an oscillating dipole that can undergo an energy exchange with a second dipole having a similar resonance frequency. Typically FRET is measured between different donor and acceptor molecules, but homotransfer between fluorophores can also occur for some molecules such as fluorescein (Kenworthy, 2001). Several basic conditions listed below must be fulfilled for FRET to occur between a donor fluorophore and an acceptor fluorophore (Day et al., 2001).

(i) The donor emission spectrum must significantly overlap (>70%) the absorption spectrum of the acceptor (Fig. 1-11B);
(ii) The distance between the donor and acceptor fluorophores must fall within the range of 1-10 nm (in general < 7 nm);
(iii) The donor and acceptor emission dipole moment, the acceptor absorption dipole moment and their separation vectors must be in favourable mutual orientation;
(iv) The donor should have a high quantum yield;
(v) The donor should be saturated by the acceptor;
(vi) The fluorescence lifetime of the donor molecule must be of sufficient duration to permit the event to occur.

**Figure 1-8** Principles of FRET. A, schematic model of protein-protein interaction by FRET. Proteins A and B are labelled with donor and acceptor fluorophores. If A interacts with B, the donor and acceptor are brought close in proximity (e.g. < 10 nm) by the interaction. The energy emitted by the excited donor fluorophore can be absorbed by the neighbouring fluorescent acceptor and re-emitted at acceptor’s wavelength resulting in non-radiative energy transfer FRET. B, Donor emission and acceptor excitation spectra and overlap integral between them. Overlap between donor emission and acceptor absorption (shaded region) leads to efficient FRET interaction.

**FRET pairs**
Real-time imaging of protein-protein interactions with FRET and *in vivo* kinetic studies in living cells require proteins of interest to be genetically tagged with an appropriately-selected pair of fluorescent proteins (donor and acceptor) (Hink *et al.*, 2002). Intrinsic fluorescent proteins are especially suitable for this purpose since biologically functional translational fusions have been made in many cases. For example, the protein environment within the fluorescent proteins allows some rotational freedom of the chromophore (Zimmermann *et al.*, 2002), which may ease constraints on the dipole orientations necessary for efficient FRET (Periasamy and Day, 2005). In addition to the requirement s of spectra, distance, orientation, fluorescence quantum yield of the
FRET pairs, fluorescent proteins are also required to form oligomers or monomers (Baird et al., 2000) to avoid effects of the bulky groups on the activity of its host protein. For example, interactions between proteins binding to large fluorescent complexes/molecules might be difficult to observe resulting in false negative result since the maximum distance tbFRET can take place is about 7 nm (Hink et al., 2002). In addition to smaller tags, challenges still remain in the search for promising fluorescent probes that mature quickly to allow immediate recording of a FRET interaction. Towards this end, new GFP variants and novel combinations of the already available GFP variants are being made available (Table 1-7). In particular, the extensive mutagenesis of GFP has yielded fluorescent protein variants with different spectral properties and enhanced fluorescence (Tsien, 1998).

**Table 1-7 Fluorescent protein examples for FRET donor-acceptor pairs.**

<table>
<thead>
<tr>
<th>Donor (Excitation)</th>
<th>Acceptor (Emission)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFP (477 nm)</td>
<td>YFP (524 nm)</td>
<td>(Truong and Ikura, 2001)</td>
</tr>
<tr>
<td>GFP (505 nm)</td>
<td>DsRed (558 nm)</td>
<td>(Tramier et al., 2002)</td>
</tr>
<tr>
<td>GFP (505 nm)</td>
<td>Cy3 (554 nm)</td>
<td>(Theis-Febvre et al., 2005)</td>
</tr>
<tr>
<td>CFP (477 nm)</td>
<td>DsRed (558 nm)</td>
<td>(Elangovan et al., 2003)</td>
</tr>
<tr>
<td>EGFP (508 nm)</td>
<td>Ds-Red (558 nm)</td>
<td>(Kinoshita et al., 2001)</td>
</tr>
<tr>
<td>YFP (514 nm)</td>
<td>Ds-Red (558 nm)</td>
<td>(Kawai et al., 2005)</td>
</tr>
<tr>
<td>EGFP (508 nm)</td>
<td>Cy3 (554 nm)</td>
<td>(Calleja et al., 2007)</td>
</tr>
<tr>
<td>EGFP (508 nm)</td>
<td>YFP (524 nm)</td>
<td>(Dumas et al., 2004)</td>
</tr>
<tr>
<td>BFP (475 nm)</td>
<td>YFP (524 nm)</td>
<td>(Day et al., 2003)</td>
</tr>
<tr>
<td>BFP (475 nm)</td>
<td>EGFP (488 nm)</td>
<td>(Day, 1998)</td>
</tr>
<tr>
<td>BFP (475 nm)</td>
<td>GFP (396 nm)</td>
<td>(Periasamy and Day, 1999)</td>
</tr>
<tr>
<td>GFP2 (508 nm)</td>
<td>YFP (524 nm)</td>
<td>(Zimmermann et al., 2002)</td>
</tr>
<tr>
<td>CFP (477 nm)</td>
<td>VenusYFP (514 nm)</td>
<td>(Evanko and Haydon, 2005)</td>
</tr>
<tr>
<td>Cerulean CFP (505 nm)</td>
<td>YFP (524 nm)</td>
<td>(Rizzo et al., 2004)</td>
</tr>
<tr>
<td>mCFP (485 nm)</td>
<td>VenusYFP (524 nm)</td>
<td>(Rizzo and Piston, 2005)</td>
</tr>
</tbody>
</table>
Two fluorescent proteins GFP2 and Venus YFP and their applications in FRET studies

The GFP2 mutant has been obtained by substitution of the phenylalanine residue at position 64 for leucine (F64L, GFP2) (Rekas et al., 2002). This mutant retains the 400 nm excitation peak and results in a significant increase of the intensity of the fluorescent signal from cells expressing the mutated GFP and incubated at 30°C or above (preferably at about 37°C), compared to the previous GFP variants. Due to the high degree of brightness of GFP2, the emitted light can be detected even after excitation with low energy light sources. Detectable fluorescence may be obtained faster due to shorter maturation time of the chromophore, higher emission intensity, or a more stable protein or a combination thereof (Ole et al., 2004). Due to lack of a commercial source of GFP2, its application has been limited. GFP2 has been mainly used as a molecular report for subcellular localisation of proteins in different cell systems. Examples include localisation of a peroxisomal citrate synthase Cit2p in S. cerevisiae (Lee et al., 2006) and an actin-like protein 1 (ALP1) in Toxoplasma gondii (Gordon et al., 2008). Thanks to the UV-excitation peak (400 nm), GFP2 can be therefore coupled as an effective FRET donor with YFP (Zimmermann et al., 2002; Torvinen et al., 2005; Carriba et al., 2008; Megias et al., 2009). It has been suggested that the GFP2-YFP pair works more efficiently than the CFP-YFP pair in the FRET studies because GFP2 has higher quantum efficiency compared to CFP and a larger overlap integral with YFP acceptor (Zimmermann et al., 2002) (see Fig. 1-8 for excitation and emission properties of GFP2, YFP and CFP).

![Figure 1-13 Normalised spectra of the excitation and the emission of GFP2, CFP and YFP](image)

Figure 1-13 Normalised spectra of the excitation and the emission of GFP2, CFP and YFP (adapted from Zimmermann et al., 2002). GFP2: green, CFP: blue, YFP: yellow. Dashed lines and solid lines represent excitation and emission, respectively.
Among *Aequorea* GFP variants, YFP is relatively acid-sensitive, and uniquely quenched by chloride ion (Cl\(^{-}\)). VenusYFP is an improved engineered YFP containing a novel mutation, F46L, which at 37°C greatly accelerates oxidation of the chromophore (a rate-limiting step in chromophore maturation). As a result of other introduced mutations, F46L/M153T/V163A/S175G, VenusYFP folds well and is relatively tolerant of exposure to acidosis and Cl\(^{-}\). VenusYFP’s maturation at 37°C is 1.5 min as measured *in vitro* and is much faster than previous YFP variants (about 0.5-2 h) (Nagai *et al.*, 2002). Shortly after the development of this novel YFP, the crystal structure of VenusYFP was solved at 2.2 Å resolution (Rekas *et al.*, 2002). The structural studies revealed that rearrangement of several side chains near the chromophore, initiated by the F64L mutation improved maturation at 37°C by removing steric and energetic constraints, which may hinder folding of the polypeptide chain, and by accelerating the oxidation of the Cδ-Cβ bond of Tyr66 during chromophore formation (Rekas *et al.*, 2002). VenusYFP’s internal residues oxidise to produce its fluorophore with excitation/emission maxima of 515/528nm (Nagai *et al.*, 2002). When fused to β-gal, the β-galactosidase quickly cleaved the substrate DDAO-gal allowing the corresponding fluorescence creation, thus the fluorescence from VenusYFP was served as a timer for *in vivo* for measuring VenusYFP maturation (Frieda *et al.*, 2003). Due to fast maturation, VenusYFP has been used for monitoring gene expression by observing real-time production of single protein molecules in living individual *Escherichia coli* cells (Yu *et al.*, 2006). VenusYFP has also contributed to FRET work as an acceptor together with the donor CFP (Nagai *et al.*, 2002; Sheridan and Hughes, 2004; Shimozono and Miyawaki, 2008). It was noted that VenusYFP photobleached easily under high power excitation (Frieda *et al.*, 2003), therefore special attention must be paid during imaging of VenusYFP to minimise photobleaching.

1.3.3 Application of transmission electron microscopy (TEM) in fungal protein secretion studies

High resolution of TEM has allowed the production of images with resolution below 50 picometres at magnifications above 50 million times (Erni *et al.*, 2009). This enables directly visualising not only ultrastructure of cell organelles but also protein complexes such as cellulose synthase rosettes in secretory pathway (Pimpl and Denecke, 2002). Enhanced by
immunocytotoxic techniques i.e. immunogold labelling of antigen, TEM has been widely applied in fungal protein secretion studies.

1.3.3.1 Introduction to the general principle of immunogold labelling and sample preparation for TEM

The principle of immunogold labelling is similar to that of conventional immunofluorescence techniques with the difference that colloidal gold particles are electrostatically adsorbed to antibodies instead of fluorophores are tagged to antibodies in immunofluorescence analysis. However, immunogold electron microscopy (EM) allows an added dimension over the usual fluorescence assays, as the localisation and distribution of macromolecules can be clearly observed at an ultrastructural level, at high magnifications, with high resolution. The theory of the immunostaining is based on specific antigen-antibody interaction. There are mainly two different techniques normally used for immunogold labelling, dependent on the type of sample submitted and the location of the protein or macromolecule of interest: (i) pre-embedding immunogold labelling, which refers to immunolabelling is done prior to embedding. This method is used primarily for the detection of proteins/antigens of interest, that are located on the surface or the exterior of biological specimens, and (ii) post-embedding labelling, which refers to immunolabelling is done on embedded sections. It is used exclusively for the detection of proteins/antigens of interest that are located in the interior or intracellular regions of biological specimens. (http://www.virusys.com/custom-services/electron-microscopy/electron-microscopy-%11-immunogold-labelling/) (Fig. 1-13). It is possible to localise two proteins/antigens in the same section by TEM when primary antibodies raised in different species are available and the secondary antisera are conjugated with two different-sized gold nanoparticles, i.e. 10 nm and 20nm gold particles (Fig. 1-3B). However, the efficiency of this simultaneous/double staining technique is lower when compared to that of the single labelling method (Fig. 1-3A) due to the inherent problems: (i) spatial competition (steric hindrance) between primary antibodies and/or gold probes, and (ii) the necessity to obtain a proper exposure of all antigens to be immunostained at the surfaces on the section (Hacker and Gu, 2002).
A central issue for sample preparation for immunolocalisation at the EM level is to preserve the epitope of interest and to allow accessibility to antibodies while minimising the loss of ultrastructural details (Follet-Gueye et al., 2003). There are several strategies for the preparation of biological samples destined for immunogold labelling of proteins: (i) chemical fixation with aldehyde, i.e. 4 % (w/v) paraformaldehyde and 0.25-2 % (w/v) glutaraldehyde eventually followed by a postfixation with 1 % (w/v) osmium tetroxide, before progressive dehyddration and embedding under low-temperature conditions (Carlemalm et al., 1982); (ii) a brief chemical fixation with aldehyde followed by cryofixation and cryosectioning (Liou et al., 1996), and (iii) cryofixation techniques followed by freeze-substitution or freeze drying and embedding at low temperatures (Tokuyasu, 1973). There is a general agreement that high-pressure freezing and freeze substitution provides the best preservation of cellular ultrastructures and antigenicity (Staehelin et al., 1990; Driouich et al., 1997). Organelle ultrastructures treated by freeze-substitution and chemical fixation have been studied in the basidiospores of Gymnosporangium Juniperi-virginianae (Mims et al., 1988) and in the hyphae of the basidiomycete Laetisaria arvalis (Hoch and Howard, 1980) at the EM level. Compared to low-temperature embedding,
improved cellular structures were visualised in *Ustilago avenae* (Hippe and Hermanns, 1986). The above studies showed that freeze-substitution yielded information of Golgi bodies, vesicles, Spitzenkörper, multivesicles, vacuoles, and microtubules that was not obtainable from chemically fixed samples. Most of the immunogold studies into protein secretion in filamentous fungi have been carried out using cryofixation or freeze-substitution (Beckett *et al*., 1974; Kurtz *et al*., 1994; Driouich *et al*., 1997; Nykänen *et al*., 1997; Cole *et al*., 2000; Nykänen *et al*., 2002; Kaminskyj and Boire, 2004; Uchida *et al*., 2004).

### 1.3.3.2 Ultrastructural studies into secretory organelles at the EM level

The ultrastructural organisation of cell organelles is below the resolution of CLSM and EM and/or immunoEM (IEM) allows the mapping of the secretory compartments. TEM has been instrumental in studying the fungal secretory pathway morphologically (Pimpl and Denecke, 2002).

The ultrastructure of the ER in filamentous fungi is contiguous with the nuclear membrane and forms an interconnected network therefore easily to be morphologically discriminated from other organelles by EM (Kavanagh, 2005). For example, the ER membrane exhibited a smooth profile, and the cisternae were generally parallel to each other and to the long axis of the hyphae (Cole *et al*., 2000). In *Pisolithus tinctorius*, the ER was shown to be located throughout hyphae up to the hyphal tip. The Golgi apparatus differs morphologically in different fungi. In most fungi, such as *Aspergillus nidulans*, *A. fumigatus* and *N. crassa*, Golgi bodies imaged with TEM have single pleiomorphic cisternae (Beckett *et al*., 1974; Kurtz *et al*., 1994; Kaminskyj and Boire, 2004; Uchida *et al*., 2004). In *Pisolithus tinctorius*, EM has revealed that the Golgi bodies consist of a cisternum composed of several cisternae of varying shapes and electron opacities with associated vesicles agreeing well with the punctate structures seen at the light microscopy level (Cole *et al*., 2000). Although no characteristic Golgi structures have been observed in *T. reesei* Rut C-30 (Kurzatkowski *et al*., 1993), an electron dense Golgi equivalent appeared as a semicircle of size of approximately 60-90 nm at the cell periphery of sub-apical regions in the *T. reesei* transformant ALKO3713 producing both the endogenous CBHI and recombinant barley cysteine endopeptidase B (EPB) in the studies of Nykänen (2002). With the high resolution, TEM has allowed the detection of the smallest entities of the secretory pathway, the COPI and COPII.
coated vesicles and their irregular protein coat (Orci et al., 1986). Some other organelles such as secretory vesicles and mitochondria were visualised in the sub-apical region of N. crassa hyphae (Uchida et al., 2004). EM ultrastructural studies have also demonstrated that the structure called Spitzenkörper at the very tip of fungal hyphae is a complex, multicomponent structure composed of vesicles (reviewed by (Harris et al., 2005)).

Interestingly, changes in the ultrastructural morphology of filamentous fungi upon genetic transformation have been observed by EM. For example, increased cell wall permeability and a clearly flocculous structure on the cell wall were found in a T. reesei transformant overexpressing the S. cerevisiae mannosylphosphodolichol synthase-encoding gene (DPM1) (Kruszewska et al., 1999b; Lenart et al., 2003). In A. nidulans, significant changes in ultrastructure were observed in the secretory pathway especially in the number of vacuoles and secretory vesicles, which were doubled after transformation with the yeast DPM1 gene encoding dolichylphosphate mannose synthase (DPMS) (Perlinska-Lenart et al., 2005). The ER of the mutant strain Rut C-30 of T. reesei is remarkably different from the wild type QM6a. The ER of the high cellulase-producing mutant Rut C-30 features long membranes with multiple foldings and parallel stacks, while in QM6a this stacking is not found. The ER membrane of an individual stack layer in Rut C-30 appears always rough with significant cisternal regions of the ER indicating an increased surface area compared to QM6a (Ghosh et al., 1984). It has been suggested that the increased amount of ER observed in the high-secreting mutant of T. reesei Rut C-30 contributed to the increased cellulase production (Ghosh et al., 1984).

Insight into the secretory pathway in the filamentous fungus P. tinctorius has been achieved by studies of the ultrastructure in the hyphae using the fungal metabolite Brefeldin A (BFA) which blocks protein transport from the ER to Golgi (Cole et al., 2000). The results showed that the BFA treatment resulted in disruption of the Spitzenkörper, reduction in the number of apical vesicles, redistribution and mild dilation of the ER, and increased size and complexity of the Golgi bodies (Cole et al., 2000).

EM has also been extensively used for investigation of hyphal growth and morphogenesis (reviewed by (Harris et al., 2005). Fungal hyphae grow and maintain their characteristic shape
through cell extension at their tips (Uchida et al., 2004). TEM images have illustrated that in the apical dome, microtubules can traverse the Spitzenkörper, some terminating at the plasma membrane at the extreme hyphal apex (Howard and Aist, 1977; Roos and Turian, 1977; Roberson and Fuller, 1988; Barja et al., 1991; Vargas et al., 1993; Roberson and Vargas, 1994) and that actin microfilaments are abundant in and around the Spitzenkörper. Furthermore, secretory vesicles associated with the Spitzenkörper were often in a close association with both microtubules and microfilaments (Howard and Aist, 1980; Howard, 1981; Roberson and Fuller, 1988). These cytoskeletal features and the associated secretory machinery are likely to be involved in establishing the point of branching behind the tip (Bartnicki-García, 2002; Riquelme and Bartnicki-Garcia, 2004).

1.3.3.3 IEM studies into protein secretion in the secretory pathway

Immunocytochemistry using colloidal gold labelling of antigens at the EM allows the localisation of secreted proteins to compartments of the secretory pathway therefore adding valuable information to the understanding of protein secretion in filamentous fungi (Pimpl and Denecke, 2002). ER and Golgi markers have been visualised by IEM allowing identification and localisation of the ER and Golgi structures in the unicellular yeast S. cerevisiae (Preuss et al., 1992). The use of IEM has also facilitated the localisation of native secreted proteins and heterologous proteins. Early IEM studies in T. reesei showed occurrence of native β-glucosidases in the outermost exopolysaccharide layer and in the plasma membrane, and to a lesser degree, carbohydrate portions of the compact cell wall also accommodated β-glucosidases (Sprey, 1988). Glucoamylase secretion was seen only at the tips of growing hyphae in A. niger (Wöstten et al., 1991). A native β-1,4 endoglucanase and the exoglucanase cellobiohydrolase I (CBHI) were localised within the cisternae of the ER and within the membrane complexes of T. reesei hyphae (Chapman et al., 1983). In addition, an endogenous xylanase enzyme XYN II was immunologically detected in the ER, electron dense vesicles, large vacuoles, especially the tonoplast, plasma membrane, and also in the cell wall of T. reesei (Kurzatkowski et al., 1993; Kurzatkowski et al., 1996). Endogenous CBHI enzyme and the recombinant protein barley cysteine endopeptidase EPB were both localised in the ER and vesicles in a Rut C-30 transformant by IEM (Nykänen et al., 1997). A difference in the secretion pattern between EPB and CBHI was that the main secretion of the heterologous EPB took place in the apical or sub-
apical cells while that of the endogenous CBHI occurred throughout the *T. reesei* hyphae, suggesting a complementary mechanism for fungal protein secretion in addition to the bulk flow associated with the polarised apical growth of hyphae (Nykänen et al., 1997). Comparison of subcellular localisation of a heterologous protein calf chymosin and the endogenous protein CBHI in *T. reesei* was carried out also by IEM (Nykänen, 2002). Both investigated proteins were localised in the ER and various endomembraneous structures and close to the putative Golgi-like structures. Interestingly, the concentration of chymosin was significantly higher in the cell wall than in the cytoplasm in all transformants. On the other hand, the CBHI concentration was higher in the cytoplasm than in the cell wall implying that trapping to the cell could be the reason for lower secretion of the heterologous calf chymosin into the extracellular medium (Nykänen, 2002).

IEM also permits the colocalisation of two different molecules to the same organelle (Pimpl and Denecke, 2002). For example, the cause of the low level secretion of a hydrophobic mutant cutinase by *S. cerevisiae* was identified by investigation into interaction of a hydrophobic mutant cutinase with BiP during the secretion process by immunogold colocalisation at the EM level (Sagt et al., 1998). In this study, double labelling with the anti-cutinase (6 nm-diametre gold) and anti-BiP (10 nm-diametres gold) was carried out. The finding that more gold-labelled cutinase was found in the transformant producing a hydrophobic mutant cutinase (CY028) than in the mutant secreting a wild-type cutinase (CY000), suggested that cutinase aggregates in CY028 were too large to be transported through the secretion pathway and were therefore retained in the ER-derived structures. Furthermore, BiP is part of a control system which prevents proteins with exposed hydrophobic residues from leaving the ER and the mutant cutinase CY028 had two exposed hydrophobic stretches which contributed to formation of a more hydrophobic surface than that of the wild-type, therefore resulting in a higher affinity for BiP which might cause the retention of this mutant cutinase in the ER (Sagt et al., 1998).

In addition, IEM has been applied to confirm the light microscopic localisation of GFP-tagged proteins within a specific organelle, such as the ER and the Golgi apparatus. Studies related to the EM immunogold localisation of GFP-tagged proteins have been reported in plant (Satiat-
Jeunemaitrea et al., 1999), mammalian cells (Paupard et al., 2001; Bjorkoy et al., 2005) and yeast (Shen et al., 2009).

1.3.4 Correlative light microscopy (LM) and electron microscopy (EM)

The visualisation of fluorescent proteins in living cells provides the overview and orientation of the protein(s) of interest in vivo, therefore is a powerful approach to study intracellular biology and dynamics (Schwarz and Humbel, 2007). A limitation of fluorescence imaging, however, is that it lacks fine structural information; a fluorescent spot could represent an entire organelle, an organelle sub-domain or even aggregates of proteins or membranes (van Rijnsoever et al., 2008). These limitations can be overcome by immunoelectron microscopy (IEM), which uniquely combines protein detection with ultrastructural detail. EM, however, requires fixation of the cells, resulting in static images with only limited field of view inherent to high resolution (Schwarz and Humbel, 2007). To bridge the gap between live-cell imaging and EM, techniques for correlative light-electron microscopy (CLEM) combining live cell imaging by light microscopy and immunostaining by TEM have been developed which has been helpful to correlate light and electron microscopic observations in, for example, research into the secretory pathway (Polischchuk et al., 2000; Mironov et al., 2003; Polishchuk et al., 2003; Polishchuk et al., 2006; Lučić et al., 2007). Correlative light and electron microscopy is particularly useful for the interpretation of light microscopic data because the low resolution often limits the conclusions that can be drawn from light microscopic investigations alone (Griffiths et al., 1993; Muhlfeld et al., 2007).

In CLEM, fluorescently tagged proteins are first imaged by light microscopy and then visualised via EM by immunolabelling (Polischchuk et al., 2000; Polishchuk et al., 2003; Polishchuk et al., 2006). The fluorescence labelling probes used for CLEM include fluorescence derivatives of nanogold, such as FluoroNanoGold (Takizawa and Robinson, 2000), tetracysteine-ReAsH system (Giepmans, 2008) and Qdots (Giepmans et al., 2005). The major advantage of FluoroNanoGold is that smaller reagents yield enhanced labeling efficiency. However, these reagents rely on silver enhancement to amplify the nanogold signal and have been limited to fixed cells (Cortese et al., 2009). The tetracysteine-tags are EM-visible and bind to membrane-permeable biarsenical compounds. After exposing the cells to a membrane-permeant, non-
fluorescent, biarsenical derivative of fluorescein, FlAsH–1,2-ethanedithiol (EDT)2 and ReAsH-EDT2, the presence of the tetracysteine motif causes the two dyes to emit green/yellow and red fluoresce respectively (Giepmans, 2008). Qdots provide a wide range of potential emission wavelengths (Delehanty et al., 2009) and are electron-dense and therefore directly visible by EM, allowing straightforward switching between LM and EM (Delehanty et al., 2009). In addition, photooxidation of the peroxidase substrate DAB has been used for CLEM studies. DAB photooxidation generates an electron-dense, insoluble polymer clearly visible by conventional EM upon strong illumination of a fluorescent molecule in conventional EM, i.e., Lucifer Yellow (Maranto, 1982). Furthermore, photooxidation and photoconversion of different fluorochromes GFP has been recently optimised by methodological adaptations of the DAB photooxidation step, providing high-quality ultrastructural localisation, 3D tomographic analysis of complex cellular architectures, and correlative microscopy of consecutive ultrathin sections (Meiblitzer-Ruppitsch et al., 2008).

Studying of the secretory pathway using correlative microscopy has been mainly conducted in mammalian cells but not in filamentous fungi to date. Complicated cellular landscapes in mature neurons have been visualised by application of correlative LM and tomography EM in order to provide new insights into the structure-function relationship of supramolecular organisation in neurons (Lučić et al., 2007). Using correlative video/light electron microscopy and tomography EM, the dynamics and structural features of the carriers mediating protein transport between the ER and the Golgi in mammalian cells was examined during and after their formation from the ER (Mironov et al., 2003). By combination of the green fluorescent protein technology and correlative light-electron microscopy, Polishchuk et al. (2000) investigated the structure of transport intermediates operating from the Golgi apparatus to the plasma membrane in Hela cells. Further, the vesicular transport carriers involved in the delivery of mannose 6-phosphate receptors from the trans-Golgi network (TGN) to the endosomal system was revealed by correlative microscopy (Polishchuk et al., 2006).

1.4 Aims of the study
The overall aim of this thesis was to map out the secretory pathway in the industrially-exploited filamentous fungus *Trichoderma reesei*. This involved visualisation of the main organelles in the
secretory pathway (Chapter 6), tracking secretion of the main cellulase, cellobiohydrolase I (CBHI) through the secretory pathway (Chapter 7) and investigating potential interaction of the CBHI protein with the ER-resident molecular chaperone BiP (Chapter 8). The techniques used in the work included application of chemical dyes to visualise secretory organelles involved in protein secretion, molecular biology procedures for the construction of the various plasmids for the study, combination of CLSM and EM to map the secretory pathway and track secretion of CBHI, and Fluorescence Resonance Transfer (FRET) microscopy to explore CBHI-BiP interaction.

The work started with visualisation of the intracellular secretory landscape by developing methods for the tagging of membranes and the ER and the Golgi apparatus. Strain construction was started parallel with the chemical organelle tagging, the emphasis being in successful expression of the fluorescent proteins GFP and VenusYFP in T. reesei as part of the expression vector. The early focus was in the construction of vectors and strains suitable for FRET studies, which would require a relatively strong fluorescent signal (and thus multicopy transformants) to be effective. In addition to attempting FRET, selected transformants constructed during the work were used for visualisation of the ER (Bip1-Venus) in live T. reesei hyphae and visualisation and tracking progression of the CBHI enzyme (CBHI-Venus) in the secretory pathway against the intracellular landscape.

It is anticipated that the results obtained from this study will contribute to the advancement of knowledge on the cellular basis for protein secretion in filamentous fungi and facilitate unravelling of the secretion bottlenecks that affect protein yields.
Chapter 2 Materials and Methods

2.1 Frequently used reagents and solutions

All chemicals used in this work were of analytical, biochemical or molecular grade and were purchased from Sigma (Australia) unless otherwise stated. Agarose and SDS were purchased from Amresco (USA). The reagents, solutions and media used throughout this work were prepared using H₂O filtered through the Millipore Milli-Q Academic filtration system and sterilized by autoclaving at 121 °C for 30 min. Some of the commonly used solutions are listed in Table 2-1. Throughout this chapter, the quantities provided as a percentage are in w/v unless otherwise stated.

Table 2-1 Solutions frequently used throughout experimental work in this thesis.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase buffer</td>
<td>15.8 g Tris-HCl and 5.8 g NaCl in 1 L H₂O, pH 9.5</td>
</tr>
<tr>
<td>Blocking solution</td>
<td>1 % blocking reagent (Roche, Germany) in maleic acid buffer</td>
</tr>
<tr>
<td>LB</td>
<td>1 % tryptone, 0.5 % yeast extract and 1 % NaCl, pH 7.0</td>
</tr>
<tr>
<td>Extraction buffer</td>
<td>625 mM Tris buffer (pH 7.4) containing 1 mM dithiothreitol (DTT), 1 µM phenylmethylsulfonyl fluoride (PMSF), and 8 µL protease inhibitor cocktail (Sigma, USA)</td>
</tr>
<tr>
<td>Maleic acid buffer</td>
<td>11.6 g maleic acid and 8.8 g NaCl in 1 L H₂O, pH adjusted to 7.5 using NaOH pellets</td>
</tr>
<tr>
<td>MgSO₄-sodium phosphate buffer</td>
<td>1.2 M MgSO₄, 10 mM NaH₂PO₄ solution was added to 1.2 M MgSO₄, 10 mM Na₂HPO₄ solution, until the pH reached 5.8</td>
</tr>
<tr>
<td>PBS (20×)</td>
<td>160 g NaCl, 4 g KCl, 28.8 g Na₂HPO₄ and 4.8 g KH₂PO₄ in 1 L H₂O, pH 7.4</td>
</tr>
<tr>
<td>SSC (20×)</td>
<td>175.3 g NaCl and 88.2 g sodium citrate in 1 L H₂O, pH 7.0</td>
</tr>
<tr>
<td>TBE (10×)</td>
<td>90 mM Tris-HCl, 1.25 mM EDTA, 90 mM Boric acid, pH 8.4</td>
</tr>
<tr>
<td>TBS (1×)</td>
<td>50 mM Tris base and 150 mM NaCl, pH 8.0</td>
</tr>
<tr>
<td>TE (10×)</td>
<td>100 mM Tris-HCl and 10 mM EDTA, pH 8.0</td>
</tr>
<tr>
<td>Washing buffer</td>
<td>Maleic acid buffer with 0.3 % Tween 20 (BDH)</td>
</tr>
</tbody>
</table>
2.1.1 Antibiotics and reagents for the selection of recombinants
Recombinant *E. coli* colonies were identified based on blue and white selection from the inclusion of X-Gal (Progen, Australia) at 20 g/mL and IPTG (Progen, Australia) at 0.4 g/mL, in the growth medium. Ampicillin was used at a concentration of 100 μg/mL to screen for recombinants. Hygromycin B (Calbiochem, USA) was used at 60 U/mL for selection of fungal recombinants.

2.2 Fungal growth media
2.2.1 Minimal medium
The preparation of minimal salt medium (MM) was as described by Penttilä *et al.* (1987). To make 1 L, 15 g KH₂PO₄, 5 g (NH₄)₂SO₄, 10 mL 100 × trace elements (50 mg FeSO₄·7H₂O, 16 mg MnSO₄·H₂O, 14 mg ZnSO₄·7H₂O, and 20 mg CoCl in 100 mL dH₂O) were added to 892 mL H₂O and the pH was adjusted as required with 5 M KOH. This minimal salt solution was sterilised by autoclaving at 121 °C for 30 min before adding 2.4 mL 1 M MgSO₄ and 5.4 mL 1 M CaCl₂, which were sterilised separately by autoclaving. For solid medium, agar (Spectrum Chemicals, USA) was added to the minimal salt solution to a final concentration of 2 %.

2.2.2 Cellobiose/lactose/soy hydrolysate medium
Cellobiose/lactose/soy hydrolysate (CLS) medium has been described previously (Lim *et al.*, 2001). This medium is based upon MM salts supplemented with a soluble carbon source that induces *cbh1* expression. The components of the minimal salt solution were prepared as in 2.2.1 with the addition of D-(+)-cellobiose (1 %). After sterilisation, lactose (1 %) and soy bean flour extract (3 %) were added. The soy bean flour extract was pre-prepared by mixing 18 g of soy bean flour with 600 mL of H₂O and autoclaving at 121 °C for 30 min. Insoluble components were then removed by centrifugation at 10, 000 rpm for 20 min.

2.2.3 Protoplast regeneration medium
Protoplast regeneration medium was prepared similarly to MM and supplemented with a final concentration of 1 M sorbitol as an osmotic stabiliser. To make solid medium plates, bacteriological grade agar (Oxoid, UK) was added to a final concentration of 1.8 %; for the overlay agar the concentration was 2.5 %.
2.3 Fungal strains and culture conditions

2.3.1 Fungal strains

*Trichoderma reesei* strain Rut C-30 (Eveleigh and Montenecourt, 1979) was used as a host strain for recombinant gene expression. *Escherichia coli* DH-5α served as a host for plasmid amplification.

2.3.2 Culture conditions

Fungal cultures were maintained on Potato Dextrose Agar (PDA) plates (Oxoid, UK). Inoculated plates were incubated at 28 °C in the dark for 3-4 days, until the mycelium had covered the plate, and then moved to room temperature and day light to encourage conidiation. After 7-10 days, conidia were collected by flooding the plate with 8 mL of 0.9 % NaCl, 0.01 % Tween 80 (BDH Chemicals Ltd., England) solution and gently scrubbed with a glass rod spreader. The conidial suspension was filtered through absorbent cotton wool to remove any hyphae and the quantity of conidia within the suspension was counted using a haemocytometre. Conidial suspensions were used immediately or frozen at –20 °C for medium term storage until required.

All liquid cultures were inoculated with freshly harvested conidia. A standard inoculum of 1×10⁸/50 mL (unless otherwise stated) was used in 50 mL liquid medium (CLS medium) in 250 mL flat bottom conical flasks. The cultures were incubated at 28 °C with shaking at 250 rpm for different times as required with no pH regulation or feeding during culture. The initial medium pH was defined, in this study, as the pH of the minimal medium before adding cellobiose, lactose, and hydrolysate. The pH value was measured using an ISFET pH metre (Shindengen, Camarillo, CA). Cultures destined for fluorescence analysis were grown in the dark to limit photobleaching of the fluorescent reporter protein.

2.4 General molecular techniques

DNA manipulations were as described by (Sambrook and Russell, 2001). All plasmids were propagated in *E. coli* strain DH-5α cultured at 37 °C. Plasmids were extracted using the Qiagen QIAprep spin miniprep kit (Germany) following the manufacturers’ instructions. The Qiagen QIAfilter plasmid maxi kit was used for large-scale plasmid preparations.
PCR products and restriction enzyme digest products were purified using the Qiagen QIAquick PCR purification kit when appropriate. DNA bands excised from agarose gels were purified using the Qiagen QIAquick gel extraction kit as directed by the manufacturer.

2.4.1 Creating the *cbh1* truncated terminator (tt) DNA fragments with 5’ or 3’ overhangs

The enzymes *AflIII* and T4 DNA polymerase required for DNA manipulations were supplied by Fermentas (USA) and Roche (Germany), respectively. The reaction for creating blunt-ended DNA fragments with incompatible overhangs contained 400 ng of cut plasmid, 2.4 U *AflIII*, 1.25 U T4 DNA polymerase, 1 mM ATP, 0.2 mM dNTP mix (BioLine, USA), 1 × end repair buffer (33 mM Tris-acetate [pH 7.8], 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT) and H2O to 50 µL. The reaction mix was incubated at 37 °C for 1 h. The blunt-ended DNA was purified by a phenol/chloroform extraction (Sambrook and Russell, 2001), precipitated and re-suspended in 10 µL H2O.

2.4.2 Restriction endonuclease digestion

All restriction enzymes were obtained from Fermentas (USA) unless otherwise stated. Restriction digests of DNA were carried using commercially provided buffers and following the manufacturer’s instructions.

2.4.3 Ligations

Ligation reactions were carried out using the Fast-Link DNA ligation kit (Epicentre Technologies, USA) as instructed by the manufacturer.

2.4.4 Phosphatase treatment of DNA fragments

Linearised plasmids were treated with Shrimp Alkaline Phosphatase (Boehringer, Germany) to prevent self-ligation, when required. Reactions were carried out in accordance with the manufacturer’s instructions in the buffer supplied. The reaction was terminated by incubation at 70 °C for 10 min.

2.4.5 Polymerase chain reaction (PCR)

PCR amplifications were performed directly from either bacterial colonies or from genomic DNAs throughout this work. PCR was carried out on the Perkin Elmer Gene Amp 2400 PCR System.
A standard PCR reaction contained 1 unit of AmpliTaq Gold polymerase (Applied Biosystems, USA), 10 pM of each primer, 3 mM MgCl₂, 12.5 mM dNTPs, approximately 10 ng of template DNA and H₂O to 50 µL. Standard reaction conditions were 94 °C, 10 min, 35 × (94 °C, 30 s; 55-70 °C, 30 s; 72 °C, 1 min), 72 °C, 5 min. PCR products were separated on agarose (Amresco, USA) gels containing 0.5 g/mL ethidium bromide and were run in 1 x TBE which also contained ethidium bromide (0.05 g/mL), unless otherwise stated. PCR products which required isolation were excised from the agarose gel and eluted using the Qiagen QIAquick gel extraction kit (Section 2.4).

2.4.5.1 Primers for PCR

All primers were synthesised by Sigma-Genosys (Sydney, Australia). The primers used in PCR throughout this work are shown in Table 2-2.
Table 2-2 Summary of the primers used in PCR analysis (continued onto next page). Features such as the primer sequence, inclusion of restriction enzyme recognition site, the target of the PCR and the annealing temperature used, are also provided. *using QIAGEN one-step reverse transcription PCR kit without Q solution.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
<th>PCR use</th>
<th>Expected size of PCR product (bp)</th>
<th>Annealing T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>actin.fwdpr</td>
<td>TCGTACCTGACATGAGGACGTACGTC</td>
<td>Amplification of actin probe</td>
<td>actin: 1600</td>
<td>56</td>
</tr>
<tr>
<td>actin revpr</td>
<td>CTGTATCCCATGCTCTTTAGAGCAGCTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>actinf</td>
<td>AAAGGAGCTGACACGTCAACA</td>
<td>Amplification of actin cDNA in real time PCR</td>
<td>actin mRNA: 146</td>
<td>60</td>
</tr>
<tr>
<td>actinr</td>
<td>ACTTCGGTCTCAGAAACA</td>
<td></td>
<td>actin DNA: 222</td>
<td></td>
</tr>
<tr>
<td>biporf.seqpr</td>
<td>GTGGGCCCTGAGAGAACGCGCG</td>
<td>Screening of BV, BG, or BGCV transformants</td>
<td>bip1-gfp2: 1365</td>
<td>60</td>
</tr>
<tr>
<td>pki.revpr</td>
<td>GAGTGGACCGCTAATCGAGG</td>
<td></td>
<td>bip1-venus: 1365</td>
<td></td>
</tr>
<tr>
<td>bipf</td>
<td>CAA GTTCGGAGAGCTCAACA</td>
<td>Amplification of bip1 cDNA in real time PCR</td>
<td>bip1: 149</td>
<td>60</td>
</tr>
<tr>
<td>bipr</td>
<td>ACCTCGCTTCTTTTCTGTTCGT</td>
<td></td>
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<tr>
<td>Bip1-Venusf</td>
<td>CTACCCCATACACCTCAAAG</td>
<td>Amplification of bip1-venus cDNA in real time PCR</td>
<td>bip1-venus: 160</td>
<td>60</td>
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<tr>
<td>Bip1-Venusr</td>
<td>GCTGAACCTTGGCGGCGGCGCGGTGG</td>
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<td></td>
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<tr>
<td>cbh1f</td>
<td>CTGCTACGAAGCAGACGTCCGTC</td>
<td>Amplification of cbh1 fragment cDNA in real time PCR</td>
<td>cbh1 fragment: 76</td>
<td>60</td>
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<tr>
<td>cbh1r</td>
<td>GTCCAGCAGACAGCAGTTCTTCG</td>
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<tr>
<td>cbh1Fdseq.pr</td>
<td>GTCAACCGGCGACTGCGCATC</td>
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<td></td>
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<tr>
<td>venusAflBamrevpr</td>
<td>CTCGAGGATCCCTTAAGTTACTTTGTACAGCTCGTC</td>
<td>Reverse transcription PCR</td>
<td>product: 2400</td>
<td>60</td>
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80
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequences</th>
<th>Description</th>
<th>Product Size</th>
<th>Temp</th>
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<tr>
<td><code>cbh1prom.seq.pr</code></td>
<td>TAAAGGTTCGAGGTCCGTGC</td>
<td>Screening of VG transformants</td>
<td>venus-gfp2: 2085</td>
<td>60</td>
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<tr>
<td><code>pki.revpr</code></td>
<td>GAGTTGAGCGCTAACTGAGG</td>
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<tr>
<td><code>cbh1coreprob.fw.pr</code></td>
<td>CATCAACCGA TACTATGTCC</td>
<td>Screening of CV and/or BGCV transformants</td>
<td>cbh1-venus: 1685</td>
<td>60</td>
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<tr>
<td><code>pki.revpr</code></td>
<td>GAGTTGAGCGCTAACTGAGG</td>
<td></td>
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</tr>
<tr>
<td><code>cbh1tffwd.pr</code></td>
<td>TACGTACTTAAGTAAAGCTCCGTGGCG</td>
<td>Amplification of <code>cbh1 tt</code> DNA</td>
<td>cbh1 tt: 300</td>
<td>65</td>
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<tr>
<td><code>cbhlttrevpr</code></td>
<td>GGATCCCTAAAGCCATCTGGCGGAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><code>cbh1-venusf</code></td>
<td>ACATCAAGTTCCGGACCATT</td>
<td>Amplification of <code>cbh1-venus</code> cDNA in real time PCR</td>
<td>cbh1-venus: 136</td>
<td>60</td>
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<td><code>cbh1-venusr</code></td>
<td>CTGAACCTTG GTGCGCCGTTAC</td>
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<td></td>
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<tr>
<td><code>gfp2EcoSnabfwd.pr</code></td>
<td>CCCACCGGAATTCTACGTAGTGAGCAAGGG CGAGGAG</td>
<td>Amplification of the <code>gfp2</code> probe</td>
<td>gfp2: 720</td>
<td>60</td>
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<td><code>gfp2AflBamrev.pr</code></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><code>venusEcoSnabfwd.pr</code></td>
<td>ATAGAAGAATTCTACGTAGTGAGCAAGGGGC GAGGAG</td>
<td>Amplification of <code>venus</code> probe</td>
<td>venus: 720</td>
<td>60</td>
</tr>
<tr>
<td><code>venusAflBamrev.pr</code></td>
<td>CTCGAGGGATCCCTAAGTTACTCTGTACAGCTCGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><code>hph4.fw.pr</code></td>
<td>CCCGGAGCTTGCGAGGATCGC</td>
<td>Confirmation of homologous integration of <code>gfp2</code> or <code>venus</code> into the genome of T. reesei</td>
<td>product: 1500</td>
<td>55</td>
</tr>
<tr>
<td><code>cbh1intnew.revpr</code></td>
<td>GACTTTCGAC TC GACCGCGA GAAGGGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><code>hphspe1.revpr</code></td>
<td>CGGGGATCCACTAGTCATGCACTTATTC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4.6 DNA sequencing
DNA sequencing was carried out using the ABI Prism 377 (Applied Biosystems, USA) DNA sequencing facility at Macquarie University, Australia. DNA sequences were analysed and manipulated using programmes from the University of Wisconsin Genetics Computer Group (GCG) (Devereux et al., 1984) and the BioManager web interface supported by the Australian National Genomic Information Service (ANGIS).

2.5 Expression vectors
The plasmids (Fig. 2-1) containing expression cassettes bip1-venus (pBV), bip1-gfp2 (pBG), cbh1-venus (pCV), cbh1-gfp2 (pCG), venus-gfp2 (pVG) were previously constructed by Dr. Natalie Curach (Department of Chemistry and Biomolecular Sciences, Macquarie University). For the current work, modifications were carried out by the insertion of a truncated form of the cbh1 gene terminator (tt) directly after the venus or gfp2 to ensure transcription termination and the final plasmids were renamed as pBVt, pBGt, pCVt, pCGt, and pVGt correspondingly. The venus gene was kindly provided by Dr. Atsushi Miyawaki (Brain Science Institute, Japan). The gfp2 gene was amplified from the pGFP2-N vector supplied from Perkin Elmer (Germany).

Figure 2-1 Schematic representation of the vectors designed for expression of the different fusion proteins (see next page). All the plasmids contained the T. reesei cellobiohydrolase I (cbh1) promoter to drive expression of the fusion proteins upon induction in the CLS medium; a full cbh1 terminator, and a 300 bp truncated cbh1 terminator (tt) placed directly after the gene fusion to ensure transcription termination. The plasmids also contained the bacterial hygromycin B phosphotransferase (hphB) gene expressed under the constitutive T. reesei pyruvate kinase (pki) promoter for the selection of transformants based on hygromycin. pCVt: the plasmid was made by fusing venus to the cbh1 core gene with a 15 bp linker in between with a cbh1 signal sequence(ss) followed the cbh1 promoter to facilitate secretion. pCGt: cbh1 core gene and the GFP2 encoding sequence (gfp2) were separated by a short 15 bp linker region with a cbh1 signal sequence(ss) followed the cbh1 promoter to facilitate secretion. pBVt: BiP1 encoding sequence (bip1) and the VenusYFP encoding sequence (venus) were separated by a short 15 bp linker region. An ER retention signal HDEL was placed after the venus gene to reinforce localisation of the resulting protein into ER. pBGt: BiP1 encoding sequence (bip1) and the GFP2 encoding sequence (gfp2) were separated by a short 15 bp linker region. The plasmid also features an ER retention signal HDEL placed after the gfp2 gene to reinforce localisation of the resulting protein into ER. pVGt: VenusYFP encoding sequence (venus) and the GFP2 encoding sequence (gfp2) were separated by a short 15 bp linker region. The plasmid has an ER retention signal HDEL placed after the gfp2 gene to reinforce localisation of the resulting protein into ER.
2.6 Fungal transformation

Fungal transformation was conducted based on two standard methodologies as described below. The generated transformants were further subjected to screening the expression of the desired proteins (Section 2.5).

Table 2-3 Strains/transformants and expression vectors. *T. reesei* transformant strains BG29, BV47, CV48, BGCV101 and VG15 were obtained from the high-secreting strain Rut C-30.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vector</th>
<th>Main use</th>
<th>Refer to Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>pBG (bip1-gfp2)</td>
<td>For plasmid amplification and purification</td>
<td>2, 3</td>
</tr>
<tr>
<td></td>
<td>pBV (bip1-venus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCV (cbh1-venus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pBGCV (bip1-gfp2/cbh1-venus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pVG (venus-gfp2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. reesei Rut C-30</em></td>
<td>None</td>
<td>As a expression host</td>
<td>2- 7</td>
</tr>
<tr>
<td><em>T. reesei BG29</em></td>
<td>pBGt (bip1-gfp2)</td>
<td>As a donor-only reference strain for FRET study to couple with acceptor CBHI-Venus</td>
<td>2, 3, 8</td>
</tr>
<tr>
<td><em>T. reesei CV48</em></td>
<td>pCVt (cbh1-venus)</td>
<td>As an accepter-only reference strain for FRET study to couple with donor BiP1-GFP2; further used for tracking of secretion of the CBHI-Venus fusion protein</td>
<td>2, 3, 7, 8</td>
</tr>
<tr>
<td><em>T. reesei BV47</em></td>
<td>pBVt (bip1-venus)</td>
<td>As an accepter-only reference strain for the FRET study originally to couple with donor CBHI-GFP2 but failed to go through due to unsuccess in transformation of pCGt into <em>T. reesei</em>; later used for visualisation of the ER by CLSM</td>
<td>2, 3, 6</td>
</tr>
<tr>
<td><em>T. reesei CG</em></td>
<td>pCGt (cbh1-gfp2)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><em>T. reesei BGCV101</em></td>
<td>Coexpression of <em>bip1-gfp2</em> and <em>cbh1-venus</em></td>
<td>For FRET studies into the interaction between BiP1 and CBHI</td>
<td>2, 3, 8</td>
</tr>
<tr>
<td><em>T. reesei VG15</em></td>
<td>pVGt (venus-gfp2)</td>
<td>As a positive control for the FRET studies</td>
<td>2, 3, 8</td>
</tr>
</tbody>
</table>
2.6.1 Biolistic bombardment procedure

The uninucleate conidia of *T. reesei* Rut C-30 strain (Eveleigh and Montenecourt, 1979) were transformed by particle bombardment using the Bio-Rad model PDS-1000/He biolistic particle delivery system (Gene Gun) as described previously (Te'o *et al.*, 2002) except that the single barrel system was used in this work instead of the seven-barrel system.

2.6.1.1 Preparation of DNA coated microprojectiles

Approximately 40 mg of M-10 tungsten particles (0.7 μm, BioRad, USA) were washed in 1 mL absolute ethanol (molecular grade) three times, then washed once in 1 mL H₂O. The particles were re-suspended in 1 mL H₂O and aliquoted into 100 μL portions. The tungsten particles were kept in suspension by continuous vortexing during the following additions of DNA and reagents.

Circular DNA (500 ng) representing a particular expression vector (Section 2.5) was added to 10 μL of M-10 tungsten particles (0.7 μm, Bio-Rad, USA) and incubated for 1 min, followed by addition of 100 μL of 2.5 M CaCl₂ and a further 1 min incubation. With the tungsten particles still maintained in suspension, 40 μL of freshly prepared 0.1 M spermidine was added and mixed for 3 min. The tungsten/DNA mixture was incubated on ice and allowed to settle for at least 10 min before collection of the pellet by centrifugation. The pellet was washed in 250 μL absolute ethanol then re-suspended in 80 μL absolute ethanol.

2.6.1.2 Preparation of conidia for transformation

Freshly collected conidial suspension (70 μL) containing 5×10⁷ fungal conidia (Section 2.3.2) was plated in the middle of a PDA plate and was allowed to dry for up to 1 h before transformation by biolistic bombardment.

2.6.1.3 Biolistic bombardment

All components of the Biolistic Gene Gun were sterilized with 70% ethanol prior to use. About 10 μL of DNA-coated M-10 tungsten particles (0.7 μm, Bio-Rad, USA) containing 500 ng circular plasmid pBVt were loaded onto a macrocarrier. A rupture disk of 4485 kPa strength was used to temporarily block the helium gas from entering the barrel of the Hepta Adaptor instrument. PDA plates containing 5×10⁷ fungal conidia for bombardment were plated in the middle of the plate that was then placed at a target distance of 3 cm. The chamber vacuum was
kept at 3.72-3.85 kPa. After bombardment, PDA plates were incubated at 28 °C for 5-6 h before overlaying with 10 mL of PDA containing the appropriate amount of hygromycin B antibiotic (Calbiochem, USA; final concentration of 60 U/mL) for transformant selection, and returned to 28 °C for a further 3-5 days. Transformant colonies were picked and streaked onto PDA plates containing 60 U/mL hygromycin B and the plates were incubated at 28 °C for a second round of selection. The transformants were picked and streaked onto PDA/hygromycin B selection plates as they appeared. Transformants which survived the second round of selection were allowed to conidiate on PDA plates.

2.6.2 Protoplast transformation procedure

The protocol for the transformation of fungal protoplasts based on the work by Penttilä et al. (1987) was provided by Dr. Nina Aro (Technical Research Centre of Finland, Finland).

2.6.2.1 Preparation of protoplasts

Freshly harvested conidia were plated onto sterile cellophane discs laid onto PDA plates, and incubated for 16-20 h at 28 °C. Lysing enzymes from T. harzianum (L2265, Sigma-Aldrich, Australia) were dissolved at 5 mg/mL in MgSO₄-sodium phosphate buffer and filtered through a 0.45 µm filter to make lysing buffer. The hyphal mat, from approximately 20 plates, was washed from the cellophane discs into a plastic 150 mm Petri dish containing 40 mL lysing buffer and incubated at room temperature with occasional shaking for approximately 1.5 h or until sufficient protoplasts were produced upon microscopic examination.

Protoplasts were separated from the remaining hyphae by filtration through a sintered glass filter (porosity number 1) and rinsed with fresh MgSO₄-sodium phosphate buffer. An equal volume of 0.6 M sorbitol, 0.1 M Tris-HCl pH 7.5 buffer was added to the protoplast suspension and the mixture centrifuged at 3000 rpm for 15 min at room temperature. Following centrifugation, most of the supernatant was carefully removed and the pellet was washed three times in 1.2 M sorbitol, 10 mM Tris-HCl pH 7.5 buffer. After the final wash, the protoplasts were re-suspended in 400 µL of 1.2 M sorbitol, 10 mM Tris-HCl, 10 mM CaCl₂ pH 7.5. A sample of protoplasts was removed and a dilution series was plated onto non-selective medium (Section 2.2.3) as regeneration control.
2.6.2.2 Transformation of protoplasts
Approximately 5 µg of DNA was added to 200 µL protoplasts, followed by the slow addition of 50 µL 25% PEG 6000, 50 mM CaCl\textsubscript{2}, 10 mM Tris-HCl pH 7.5 buffer with gentle mixing and incubated on ice for 20 min. A further 2 mL of 25% PEG 6000 solution was added and mixed by gentle inversion of the tube, then incubated at room temperature for 5 min. A final 4 mL of 1.2 M sorbitol, 10 mM Tris-HCl, 10 mM CaCl\textsubscript{2} pH 7.5 solution was added before 100 µL aliquots of the transformed protoplast suspension were added to 7 mL molten overlay agar containing 60 U/mL hygromycin B, and plated immediately onto selective agar plates (Section 2.2.3). The plates were incubated at 28 °C for 7 days. Colonies were streaked and patched onto second-selection PDA plates, containing 60 U/mL hygromycin B as they appeared.

2.7 Measurement of intracellular fluorescence
Fifty mg (wet wt) of 5-day-old mycelia of the transformants and the nontransformed Rut C-30 grown in the CLS medium were sonicated in 5 mL of tricarballylate buffer (TCA buffer) pH 7.5 (Santerre Henriksen et al., 1999) for 4 × 30 seconds using a sonicator (Branson Sonifier, Smith Kline Co, Danbury, Conn., USA). The disrupted cells were then centrifuged at 4 °C to pellet the debris. A 100 µL aliquot of each lysed supernatant containing the intracellular proteins was loaded onto a clear 96 well microtiter plate (Greiner, USA) and the fluorescence was recorded on the FluoStar Galaxy plate reader (BMG Labtechnologies, Australia). Fluorescence from VenusYFP and GFP2 was detected using the following excitation/emission filter sets: 485/520-P nm (for VenusYFP) and 405/520-P nm (for GFP2). Fluorescence readings were carried out in duplicate and the supernatant from the culture of the nontransformant Rut C-30 was used to determine the level of background fluorescence in the samples.

2.8 Extraction of fungal genomic DNA
Genomic DNA was extracted from fungal mycelia using an adaptation of the technique described by Lee et al. (www.fgsc.net/fgn35/lee35.pdf). Fungal conidia were grown for three days on sterile cellophane discs placed on PDA plates. Mycelia were harvested by scraping off from the cellophane surface, frozen at -20 °C overnight, and then lyophilized by a vacuum pump system. Freeze-dried mycelia were ground to a fine powder, which was then mixed with the extraction buffer (100 mM Tris-HCl, 250 mM NaCl, and 25 nM EDTA, 0.5% (w/v) SDS). An organic extraction step using an equal volume of buffer-saturated phenol (Invitrogen, Life Technologies,
Australia) removed the bulk of cellular debris. Further organic extraction of genomic DNA involved one chloroform:phenol (1:1) step and one chloroform:isoamyl alcohol (24:1) extraction step, which were carried out using Eppendorf (Germany) 15 mL phase lock gel tubes (light) as recommended by the manufacturer. DNA concentration was determined by absorbance reading at 260 nm on an Eppendorf BioPhotometer.

2.9 Isolation of fungal total RNA
Total RNA was extracted from mycelia disrupted by a homogenizer using the Trizol Reagent (Invitrogen, Life Technologies, Australia) as per the manufacturer’s instructions. Large amount of cell debris was removed by an additional centrifugation at 10,000 rpm for 10 min at 4 °C, prior to the chloroform extraction step. Eppendorf 1.5 mL Phase Lock Gel tubes (light or heavy) were used for organic extraction steps. The RNA pellet was dissolved in DEPC-treated H₂O with a 10 min incubation at 50 °C to assist in the complete dissolution of the pellet. Total RNA quality was judged by the appearance of a 2 µL sample separated on a 1 % (w/v) agarose gel stained with ethidium bromide, in conjunction with an OD₂₆₀nm/OD₂₈₀nm ratio of greater than 1.7 for RNA samples diluted 1 in 100 in TE buffer. RNA concentration was determined by the OD₂₆₀ reading using DU 800 spectrophotometre (Beckman Coulter, USA) and stored at -80 °C until use.

2.10 Reverse transcription RT-PCR and Real time PCR
Target cDNAs were synthesised from the total RNA (Section 2.9) using the Superscript II reverse transcriptase (Invitrogen, Life Technologies, Australia) and random hexamer primers (Promega, Madison, WI) provided in the kit. The sequences of the real time PCR primers (Table 2-2) adopted in this study were designed using Oligoperfect software (Invitrogen, Life Technologies, Australia).

For quantitative real-time quantitative PCR, the mRNA levels were quantified using the Rotogene-3000 Real-Time Thermo cycler (Corbett Research, Australia). PCR mixtures containing 0.3 µM primers in 25 µL of final SYBR mastermix (Invitrogen, Life Technologies, Australia) were cycled for 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. The relative quantity was determined by the Ct value (defined as the fractional cycle number at which the fluorescence passes the fixed threshold). The normalised value representing mRNA expression in each sample was calculated as relative quantity of relevant
primers divided by the relative quantity of the housekeeping actin gene. Controls without template (no cDNA) were also performed on each PCR plate. Samples were run in duplicate.

2.11 Nucleic acid hybridisation techniques

2.11.1 Generation of DNA probes

Digoxigenin (DIG) labelled DNA probes were used for Northern blotting and Southern blotting detection procedures. The probes were generated by a standard PCR reaction using the PCR DIG labelling mix (Roche, Germany) as instructed by the manufacturer.

The 1600 bp actin gene (act1) probe was amplified from T. reesei genomic DNA using the primers actin.fwdpr and actin.revpr (Table 2-2) with a 56 °C annealing temperature. The 720 bp gfp2 probe was amplified from the pBGt plasmid DNA with the primers gfp2EcoSnabfwd.pr and gfp2AflBamrev.pr with a 60 °C annealing temperature. The 720 bp venus probe was amplified from the pCVt DNA using the primers venusEcoSnabfwd.pr and venusAflBamrv.pr (Table 2-2) with a 60 °C annealing temperature step. Following PCR and separation by electrophoresis, the DNA probes were excised from the agarose gel (Section 2.4).

2.11.2 Preparation of genomic DNA for Southern blotting

Good quality genomic DNA (4 µg) was digested overnight at 37 °C with the restriction enzyme BamHI in the appropriate buffers. The digested DNA was electrophoresed on a 1 % (w/v) agarose gel at 80 V for approximately 90 min. A picture was taken under UV illumination and the genomic DNA was transferred onto a membrane as described below (Section 2.11.4).

2.11.3 Detection of RNA transcripts by Northern blotting

2.11.3.1 Preparation of RNase–free solutions

All clean glassware was sterilised at 121 °C for 30 min in an autoclave and later baked overnight at 180 °C. The electrophoresis tank was washed thoroughly in Pyroneg and rinsed in Milli Q H₂O followed by two rinses in diethylpyrocarbonate (DEPC) treated H₂O and a rinse in 100 % molecular biology grade ethanol to aid drying. Work benches and pipettes were washed with Pyroneg, then wiped down with 70 % ethanol.
All water and most solutions used were treated with DEPC to inactivate any RNase activity. DEPC was added at 0.1 % (v/v) concentration and solutions were incubated overnight at room temperature before autoclaving at 121 °C for 30 min. Other solutions were either purchased RNase-free from the supplier or prepared from RNase-free chemicals with DEPC-treated solutions (for example, solutions containing Tris).

The MOPS buffer was produced as a 10 × stock solution using DEPC-treated H₂O and contained 200 mM MOPS, 50 mM sodium acetate and 10 mM EDTA with pH adjusted to 8.0 with NaOH. For some later experiments, 10 × MOPS buffer was purchased from Eppendorf (Germany). The RNA loading buffer contained 250 μL deionised formamide, 83 μL 37 % formaldehyde, 50 μL 10 × MOPS buffer and 10 μL 2.5 % DEPC-treated bromophenol blue. The RNA Gel loading buffer was purchased from Eppendorf (Germany). Solutions used for blotting, hybridisation and detection are described in Section 2.1 and were prepared to be RNase-free.

2.11.3.2 Preparation of RNA for Northern blotting
Total RNA (20 μg) was mixed with three volumes of RNA loading buffer and denatured at 65 °C for 10 min. Samples were electrophoresed on a 1.2 % agarose/formaldehyde gel at 70 V for 2 h as described by Sambrook et al. (2001). The gel was stained in 0.5 g/mL acridine orange in DEPC-treated H₂O for 10 min, then destained for 15 min in DEPC-treated H₂O and a picture taken under UV illumination. Transfer of RNA onto the positively-charged nylon membrane and detection of gene-specific RNAs is described in 2.11.4. The hybridisation temperature for detection of act1, gfp2, and venus specific RNAs was 56 °C. The intensity of expression was normalised against the actin transcript levels.

2.11.4 Blotting and detection procedure
Transfer of nucleic acids from agarose gels to positively-charged nylon membrane (Roche, Germany) was carried out using the BioRad vacuum blotter (BioRad, USA) with 10 × SSC buffer as recommended by the manufacturer. Nucleic acids were fixed onto the membrane by baking at 120 °C for 20 min. Hybridisation and detection of specific genes or mRNAs were carried out using the DIG Luminescent Detection Kit for Nucleic Acids (Roche, Germany) based on the
manufacturer’s instructions. Membranes were incubated in 20 mL reconstituted DIG-Easy Hyb granules (Roche, Germany) in a Hybrid oven at the relevant hybridisation temperature (ranging from 50 °C to 60 °C depending on the experiment) for 1 h (see above). The DIG-labelled DNA probe was denatured at 100 °C for 10 min then added to fresh Hybridisation buffer (20 ng/mL), pre-warmed to the hybridisation temperature. The probe was allowed to hybridise to the blot overnight. Following hybridisation, the blot was washed twice for 5 min with 2 × SSC, 0.1 % SDS at room temperature and twice for 15 min with 0.1 × SSC, 0.1 % SDS at room temperature before rinsing in washing buffer. The membrane was blocked in blocking solution for 1 h at room temperature then incubated for 30 min with the anti-Digoxigenin-AP antibody (Roche, Germany) diluted 1 in 10,000 in fresh blocking solution. Excess antibody was removed by two 15 min washes at room temperature in washing buffer before 1-2 min incubation in Alkaline Phosphatase buffer. The membrane was inserted in a hybridisation bag with approximately 500 µL ready-to-use CDP-star (Roche, Germany). The substrate was spread evenly across the membrane and the excess squeezed from the bag. The membrane was exposed to CL-Xposure film (Pierce, USA) and developed using Kodak GBX developing solutions.

2.12 Extraction of intracellular proteins of T. reesei

Intracellular protein extraction was carried out using the protocols described previously (Lombraña et al., 2004). Mycelia from liquid cultures were collected by centrifugation (10,000 rpm for 10 min at 4 °C), washed in cold 1 × PBS, then frozen under liquid nitrogen and stored at -80 °C. The frozen mycelial pellet was ground in a mortar to a fine powder under liquid nitrogen and approximately 0.5 g was added to 5 mL of chilled extraction buffer (Table 2-1) containing 1 mM PMSF and 0.08 % (w/v) protease inhibitor cocktail (Sigma-Aldrich, USA). The sample was mixed by vortexing and incubated at 4 °C for approximately 1 h. Following the incubation, the lysis supernatants were obtained by centrifugation at 10,000 rpm for 10 min at 4 °C and stored at -20 °C until used.

2.13 Western blotting

Total protein concentration of both intracellular extracts and extracellular medium was measured using a commercial protein quantification assay kit (Bio-Rad, USA) and a DU 800 spectrophotometre (Beckman Coulter, USA). Bovine serum albumin served as a reference.
standard. Assays were performed in duplicate and the samples for Western blotting were diluted to normalise to the lowest total protein concentrations.

Liquid cultures (no protease inhibitor used here) and/or cellular protein extracts were centrifuged at 10,000 rpm for 5 min at 4 °C and the supernatants collected. A denaturation treatment of protein samples was carried out by boiling in a water bath for 5 min only as stated. Otherwise, the protein samples were directly mixed with NuPAGE® LDS 4× LDS loading buffer (Invitrogen, USA) and incubated on ice for 5 min without boiling before running on a gel. The above boiled/unboiled samples were separated on a NuPAGE Novex Bis-Tris 4–12 % (w/v) gradient gel (Invitrogen, USA) at 200 V for 60 min in 1 × NuPAGE SDS running buffer (Invitrogen, USA). The gel was then transferred onto a PVDF membrane (BioRad, USA) for 2 h at 300 V constant in NuPAGE transfer buffer (Invitrogen, USA) using the XCell Blot module (Invitrogen, USA). Following transfer, the membranes were rinsed in 3 % (w/v) skim milk buffer in 1 × PBS, 0.02 % (v/v) Tween 20 (BDH Chemicals Ltd., England) then blocked in this buffer for 30 min. The membranes were probed with different primary antibodies (Table 2-4) diluted in 3 % skim milk buffer in PBS overnight. The membrane was washed three times for 5 min in 1 × PBS and probed with the corresponding secondary antibodies (Table 2-4) diluted in 3 % skim milk buffer in PBS for 1 h. Excess antibody was removed by three washes for 5 min in 1 × PBS before the membrane was incubated either with readymade Alkaline Phosphatase substrate (Promega, USA) or enhanced chemilluminescent (ECL) substrate (Pierce, USA) in the dark. For the ECL system, the chemilluminescent signals on the PVDF membranes were detected by scanning with a Kodak MM 4000 imaging facility (Kodak, USA).
Table 2-4 Antibodies used in Western blotting experiments.

<table>
<thead>
<tr>
<th>Protein to be detected</th>
<th>Antibody name</th>
<th>Monoclonal or polyclonal</th>
<th>Origin/Source</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Antibody name</th>
<th>Conjugate</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BiP1</td>
<td>Anti-BiP</td>
<td>Monoclonal</td>
<td>Yeast/Mouse</td>
<td>1: 100</td>
<td>Santa Cruz Biotechnology Inc, USA</td>
<td>Anti-mouse IgG</td>
<td>HRP</td>
<td>1:3000</td>
<td>Pierce, USA</td>
</tr>
<tr>
<td>CBHI</td>
<td>Anti-CBHI</td>
<td>Polyclonal</td>
<td>Fungus/Rabbit</td>
<td>1: 100</td>
<td>Primalco Biotech, Finland</td>
<td>Anti-rabbit IgG</td>
<td>APS</td>
<td>1:3000</td>
<td>Calbiochem, USA</td>
</tr>
<tr>
<td></td>
<td>Anti-CBHI</td>
<td>Monoclonal</td>
<td>Fungus/Mouse</td>
<td>1: 50</td>
<td>Primalco Biotech, Finland</td>
<td>Anti-mouse IgG</td>
<td>HRP</td>
<td>1:3000</td>
<td>Calbiochem, USA</td>
</tr>
<tr>
<td>VenusYFP</td>
<td>Anti-GFP</td>
<td>Polyclonal</td>
<td>Aequorea victoria jellyfish/ Rabbit</td>
<td>1: 1000</td>
<td>Sapphire Biosciences, Australia</td>
<td>Anti-rabbit IgG</td>
<td>HRP/APS</td>
<td>1:3000</td>
<td>Pierce, USA</td>
</tr>
</tbody>
</table>
2.14 CLSM studies

Three confocal microscope systems were applied for the imaging work: (i) a Leica SPM2 laser scanning confocal microscope (Leica, Germany) with full spectral capabilities; (ii) an Olympus FV 1000 with partial spectral capabilities (Olympus, Japan); (iii) an Olympus FV 300 system (Olympus, Japan). Microscopy conditions are summarised in Table 2-5. Images were manipulated using the Image J software available from http://rsb.info.nih.gov/ij/. Figures were edited and assembled using the programs, Adobe Photoshop (version 7.0) and Adobe Illustrator CS2.

2.14.1 Sample preparation for microscopic observation of living fungal cells and dye loading

Method 1: *T. reesei* Rut C-30 hyphae were grown on a standard plate with PDA medium at 28 °C for 48 h. An agar block of the size 20 mm² according to Hickey *et al.* (2004) bearing hyphae was cut out and placed, hyphal side down, in 0.9 % NaCl containing 33 μM FM® 4-64 (Invitrogen, USA) and transferred to microscopic observation after incubation for 3 h at room temperature with the stain.

Method 2: *T. reesei* Rut C-30 conidia were cultured at 28 °C for 48 h on sterilised piece of cellophane spread on a PDA plate. A small piece of cellophane (5×10 cm) was cut out and incubated at room temperature for 1 h with 40 μL of 33 μM FM® 4-64 on a coverslip, hyphal side down before imaging (modified from the method reported by Cole *et al.* (1997).

Method 3: A 1×10⁸ conidial suspension of Rut C-30 was pipetted into a 250 mL conical flask containing 50 mL CLS medium. After shaking at 250 rpm for 48 h at 28 °C, 100 μL of fresh cultures were taken out into a 1.5 mL Eppendorf tube and centrifuged at 3000 rpm for 5 min followed by three times of washing with PBS. Pellets were then re-suspended in 1000 μL of 33 μM FM® 4-64 and incubated at room temperature for 1 h. The stained samples were then washed in PBS for 5 min three times and finally resuspended in 500 μL of PBS. Samples of 100 μL from the stained cell suspensions were placed onto 16-well chambered coverslips (Molecular Probes, USA) for confocal imaging.
Method 4: A $2 \times 10^5$ conidial suspension of Rut C-30 was inoculated into the wells of a 16-well chambered coverslip each containing 100 µL of the medium composed of 1 % (w/v) lactose in MM salts (Section 2.2.1) semi-solidified with 0.1-0.7 % (w/v) agarose (ultra-pure bioreagent, low melting, Sigma USA) or 0.1-0.7 % (w/v) methyl cellulose (MC) (M0512, 4000 cp, Sigma USA). Cells were observed after incubation for 24 h at 28 °C by a standard inverted light microscope to check fungal growth. Preparation of stock MC (2 %): two grams of MC powder were added in a beaker containing 100 mL of boiling water and the solution was heated and stirred until the solution was clear. After that, the stock MC solution was kept at 4 °C until use.

2.14.2 Investigation of the optical properties of GFP2 and VenusYFP expressed in T. reesei
A Leica SPM2 laser scanning confocal microscope (Leica, Germany) was employed for collecting emission spectra of GFP2 and VenusYFP every 10 nm from 490 nm to 620 nm range using live BG29 (excited at 405 nm) and BV47 (excited at 488 nm) transformants. Photobleaching tests were carried out with the same Leica confocal microscope using 50 % power (10 mW) of 405 nm (for GFP2) or 514 nm (for VenusYFP) for 120 min at a magnification of 63× with a scan rate of 1 scan/second. A 63× objective was employed. Data were plotted using the OriginPro (ver. 7.5) software.

2.14.3 Live cell imaging
2.14.3.1 Imaging of expression of the fluorescent proteins, GFP2 and VenusYFP, in live T. reesei transformants
Fresh cultures (100 µL) of various transformants and Rut C-30 in the CLS medium (pH 6.5) were transferred into 1.5 mL Eppendorf tubes, centrifuged at 3000 rpm for 5 min. The pellets were washed in 500 µL of PBS, three times at room temperature and resuspended in 500 µL of PBS. Aliquots (100 µL) of the cell suspension representing each strain were placed onto 16-well chambered coverslips (LabTek® Nunc, USA) for confocal imaging.

2.14.3.2 Imaging of live T. reesei stained with various chemical stains
To image chemical staining of live fungi, 100 µL samples of fresh cultures of transformants and Rut C-30 in the CLS medium (pH 6.5) were collected into 1.5 mL Eppendorf tubes and
centrifuged at 3000 rpm for 5 min. The pellets were washed in 500 μL of PBS, three times at room temperature. The washed hyphae were then incubated with chemical stains at room temperature (see Table 2-5 for detailed staining conditions). Following staining, cells were washed with PBS for three times and finally resuspended in 500 μL of PBS. Aliquots (100 μL) of the stained cell suspensions were placed in 16-well chambered coverslips (LabTek® Nunc, USA) for confocal imaging. All chemical stains were obtained from Molecular Probes Inc, USA.
Table 2-5 Conditions for staining and imaging of cell organelles in the secretory pathway in living *T. reesei* hyphae using CLSM.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Target component</th>
<th>Staining conditions</th>
<th>Confocal microscope setup</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Laser power (Full intensity %)</td>
<td>Excitation laser (nm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 %</td>
<td>565-720</td>
</tr>
<tr>
<td>FM® 4-64</td>
<td>Cell membranes</td>
<td>33.3 μM, 1 h, room temperature</td>
<td></td>
<td>488/559</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIOC₆(3)</td>
<td>ER</td>
<td>5 μg/mL, 1 h, room temperature</td>
<td></td>
<td>500-540</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV47 transformant expressing BiP1-Venus</td>
<td>ER</td>
<td>Venus YFP</td>
<td>5-10 %</td>
<td>530-550</td>
</tr>
<tr>
<td>ER-Tracker™ Red</td>
<td>ER</td>
<td>1 μM, 1 h, room temperature</td>
<td></td>
<td>565-700</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BODIPY® FL C5-ceramide (Cat No. D3521; Invitrogen, USA)</td>
<td>Golgi</td>
<td>4.2 μM, 1 h, room temperature</td>
<td>10 %</td>
<td>500-560</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BODIPY® TR ceramide (Cat No. D7540; Invitrogen, USA)</td>
<td>Golgi</td>
<td>2 μg/mL, 1 h, room temperature</td>
<td>10/17 %</td>
<td>565-700</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BODIPY® FL C5-Sphingomyelin (Cat No. D3922; Invitrogen, USA)</td>
<td>Golgi</td>
<td>2.5 μg/mL, 1 h, room temperature</td>
<td>10 %</td>
<td>490-600</td>
</tr>
<tr>
<td>SYTO® 40 (Cat No. S11350; Invitrogen, USA)</td>
<td>Nuclei</td>
<td>10 μM, 1 h, room temperature</td>
<td>10 %</td>
<td>410-480</td>
</tr>
</tbody>
</table>
2.14.3.3 Imaging of expression and secretion of CBHI-Venus in the transformant CV48 over culture time

Fresh cultures (100 µL) of the transformant CV48 and the nontransformant Rut C-30 grown in the CLS medium (pH 6.5) were removed after 12, 18, 24, 48, 72, 96 and 120 h of growth and transferred into 1.5 mL Eppendorf tubes. The samples were diluted with 1.0 mL PBS to disperse the hyphae. Aliquots of 100 µL of cell suspension were then placed onto 16-well chambered coverslips (LabTek® Nunc, USA) for confocal imaging. The chambered slides were observed as rapidly as possible.

2.14.4 Indirect immunofluorescence analysis

The samples used in these analyses were semi-thin (1 µm) resin-embedded fungal cells. Semi-thin (1 µm) resin sections were cut from blocks prepared for TEM (block preparation procedures see Section 2.15) using a Reichert Ultracut FC4 ultramicrotome (Leica, Germany) and mounted onto clean glass slides. Sections were blocked in 10 % (v/v) FBS (Sigma-Aldrich, Australia) in PBS for 30 min and then incubated with a mixture of primary antibodies (see Table 2-6 for details) at room temperature for 1 h. Sections were washed in PBS, three times for 5 min and then incubated with a mixture of secondary antibodies (see Table 2-6 for details) at room temperature for 1 h. To view fixed cells in the immunofluorescence analysis or when imaging of semi-thin resin-embedded sections, stained specimens were mounted with 10 µL of ready-to-use anti-fade mountant reagent (Invitrogen, USA) and covered with a coverslip before observation.
Table 2-6 Antibodies used in immunofluorescence analysis with semi-thin resin sections with CLSM. Ex, excitation; Em, emission.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
<td><strong>Protein to be stained</strong></td>
</tr>
<tr>
<td>The transformant BV47 and host strain Rut C-30</td>
<td>BiP1</td>
</tr>
<tr>
<td></td>
<td>VenusYFP</td>
</tr>
<tr>
<td>Rut C-30</td>
<td>Vps10p</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
<td><strong>Fluorophore tag</strong></td>
</tr>
<tr>
<td>Anti-mouse IgG</td>
<td>Alexa Fluor® 546</td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>FITC</td>
</tr>
<tr>
<td>Anti-mouse IgG</td>
<td>Alexa Fluor® 546</td>
</tr>
</tbody>
</table>
2.14.5 FRET analysis

To prepare living fungal hyphae for FRET image acquisition, 100 µL samples of fresh 24 h-old cultures of transformants (Table 2-7) and the nontransformant Rut C-30 in the CLS medium (pH 6.5) were transferred into 1.5 mL Eppendorf tubes and centrifuged at 3000 rpm for 5 min. The pellets were washed in 500 µL of PBS three times at room temperature and resuspended in 500 µL of PBS. Aliquots of 100 µL of the stained cell suspensions were placed onto 16-well chambered coverslips (Molecular Probes, USA) for confocal imaging.

The FRET data collection was carried out using an Olympus FV 1000 laser scanning confocal microscope system equipped with an inverted IX81 microscope (Olympus, Japan). A 60× N.A. 1.2 Oil objective was employed. GFP2 was excited with 405 nm laser line and detected in the Donor channel from 500-530 nm. VenusYFP was excited with the 473 nm laser and detected in the Acceptor VenusYFP channel from 530-550 nm (Table 2-7). A series of raw images were acquired for FRET analysis based on sensitised emission methodology and were evaluated in FV ASW 1.7b program (Olympus, Japan). The acquisition of these images used for FRET analysis is summarised in Table 2-7.

Table 2-7 FRET acquisition for FRET correction and analysis using Olympus FV 1000 confocal laser scanning microscope and FV ASW 1.7b software.

<table>
<thead>
<tr>
<th>Image name</th>
<th>Sample</th>
<th>Microscopic Observation</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Excitation</td>
</tr>
<tr>
<td>A</td>
<td>Donor only (transformant BG29)</td>
<td>Donor (405 nm)</td>
</tr>
<tr>
<td>B</td>
<td>Donor (405 nm)</td>
<td>Acceptor (530-550 nm)</td>
</tr>
<tr>
<td>C</td>
<td>Acceptor only (transformant CV48)</td>
<td>Donor (405 nm)</td>
</tr>
<tr>
<td>D</td>
<td>Acceptor (473 nm)</td>
<td>Acceptor (530-550 nm)</td>
</tr>
<tr>
<td>E</td>
<td>Double-labelled (transformant VG15 and BGCV101)</td>
<td>Donor (405 nm)</td>
</tr>
<tr>
<td>F</td>
<td>Acceptor (473 nm)</td>
<td>Acceptor (530-550 nm)</td>
</tr>
<tr>
<td>G</td>
<td>Donor (405 nm)</td>
<td>Acceptor (530-550 nm)</td>
</tr>
</tbody>
</table>
2.15 IEM studies

Fresh fungal cell cultures (500 µL) of the recombinants and nontransformant Rut C-30 were collected into 1.5 mL Eppendorf vials and fixed in 4 % (w/v) paraformaldehyde (Proscitech, Australia) and 1 % (v/v) glutaraldehyde (Proscitech, Australia) in PBS pH 7.4, for 2 h at room temperature, followed by three washes with PBS. Samples were then dehydrated in a graded ethanol series and infiltrated in LR White resin (London Resin Co., UK). The samples were embedded in gelatin capsules and polymerized at 60 °C for 48 h. Ultrathin sections (50-70 nm) were cut using a Reichert Ultracut FC4 ultramicrotome (Leica, Germany) and mounted onto 300 mesh nickel grids.

For post-embedding labelling, ultrathin sections were mounted onto 300 mesh, Pioloform coated nickel grids (Proscitech). Post-embedding labelling was carried out as follows. Sections were incubated in 0.05 M glycine (Sigma-Aldrich, Australia) in PBS for 15 min, followed by incubation in the blocking solution 5 % (v/v) FBS (Sigma-Aldrich, Australia) with 5 % BSA (Sigma-Aldrich, Australia) in PBS for 30 min. After three washes in the incubation buffer (0.1 % BSA in PBS), sections were incubated with primary antibodies at room temperature for 1 h. Primary antibody concentrations were determined by a titration series. Sections were washed in incubation buffer (5 × 3 min) and incubated with the secondary antibody (5-nm gold goat anti-rabbit conjugate (BioCell Intl., UK; 1 in 150 in incubation buffer) and the 10-nm gold goat anti-mouse conjugate (BioCell Intl., UK; 1 in 100 in incubation buffer) for 1 h (see Table 2-8 for details). The specificity of the antibodies was checked with the transformants and the nontransformant, Rut C-30, by using gold-conjugates without primary antibodies. Sections were stained with 2 % (v/v) aqueous uranyl acetate for 10 min and Reynold’s lead citrate for 4 min. Sections were examined with a CM10 transmission electron microscope (Philips, The Netherlands) at an operation voltage of 100 kV.
Table 2-8 Antibodies used in IEM experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein to be stained</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
<th>Manufacturer</th>
<th>Gold particle size</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Name</td>
<td>Origin/Species</td>
<td>Manufacture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BiP1</td>
<td>Yeast/Mouse</td>
<td>1: 50</td>
<td>Santa Cruz Biotech Inc, USA</td>
<td>Anti-mouse IgG</td>
<td>10 nm</td>
</tr>
<tr>
<td>BV47 and host strain Rut C-30</td>
<td>VenusYFP</td>
<td>Polyclonal anti-GFP</td>
<td>Aequorea victoria jellyfish/Rabbit</td>
<td>1: 100</td>
<td>Sapphire Biosciences, Australia</td>
<td>Anti-rabbit IgG</td>
<td>5 nm</td>
</tr>
<tr>
<td></td>
<td>Vps10p</td>
<td>Monoclonal anti-Golgi</td>
<td>Yeast/Mouse</td>
<td>1: 100</td>
<td>Invitrogen, USA</td>
<td>Anti-mouse IgG</td>
<td>15 nm</td>
</tr>
<tr>
<td>CV48 and host strain Rut C-30</td>
<td>Vps10p</td>
<td>Monoclonal anti-Golgi</td>
<td>Yeast/Mouse</td>
<td>1: 100</td>
<td>Invitrogen, USA</td>
<td>Anti-mouse IgG</td>
<td>10 nm</td>
</tr>
<tr>
<td>Rut C-30</td>
<td>VenusYFP</td>
<td>Polyclonal anti-GFP</td>
<td>Aequorea victoria jellyfish/Rabbit</td>
<td>1: 100</td>
<td>Sapphire Biosciences, Australia</td>
<td>Anti-rabbit IgG</td>
<td>5 nm</td>
</tr>
<tr>
<td></td>
<td>Vps10p</td>
<td>Monoclonal anti-Golgi</td>
<td>Yeast/Mouse</td>
<td>1: 100</td>
<td>Invitrogen, USA</td>
<td>Anti-mouse IgG</td>
<td>10 nm</td>
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</table>
Chapter 3 Expression of fluorescent proteins GFP2 and VenusYFP in *Trichoderma reesei*

3.1 Introduction

*Trichoderma reesei* is one of the most powerful secretors of extracellular proteins in nature and has been extensively used in industry to produce various biocatalysts for over two decades (Nevalainen and Te'o, 2003). A systematic program aiming to improve protein production by *T. reesei* relies on comprehensive understanding of protein secretion at the cellular, subcellular and molecular levels. For example, it is essential to map out the secretory pathway in *T. reesei* by visualising compartments involved in protein secretion; it is also important to localise and track secretion of proteins of interest *in vivo*. Real-time analysis of molecular processes using FRET techniques would be helpful to disclose interactions between secreted proteins such as the main cellobiohydrolase I (CBHI) and proteins involved in the secretory process such as the folding chaperone BiP1. All these approaches would require introduction and expression of genes encoding fluorescent proteins in *T. reesei*.

In the current work, coding sequences of the fluorescent proteins GFP2 and VenusYFP were fused to the gene encoding BiP1 and/or CBHI with a view to build up FRET experimental tools for research into interactions between BiP1 and CBHI *T. reesei* at a subcellular level and to obtain a *T. reesei* transformant suitable for the tracking of the secretion of CBHI through the secretory pathway. A BiP1-Venus/GFP fusion would also be used for *in-vivo* labelling of the ER in *T. reesei* hyphae.

A number of fusion DNAs expressing CBHI-GFP2, BiP1-Venus; BiP1-GFP2, CBHI-Venus; as well as Venus-GFP2 (Fig. 2-1) were constructed and subsequently transformed into *T. reesei* separately or in pairs (e.g. BiP1-GFP2/CBHI-Venus). Work described in this Chapter focuses on expression of the fluorescent proteins GFP2 and VenusYFP in *T. reesei*. To ensure sufficient and comparative levels of expression signals, all the constructs were expressed under the control of the strong *cbh1* promoter.
3.2 Results and discussion

3.2.1 Generation and initial screening of transformants

In order to obtain enough transformants for the desired purposes, *T. reesei* was transformed with circular plasmids by biolistic bombardment and protoplasting methods as described in Section 2.6. To develop transformants expressing BiP1-GFP2, CBHI-Venus, CBHI-GFP2, BiP1-Venus and Venus-GFP2, respectively, plasmids pBGt, pCVt, pCGt, pBVt, and pVGt were transformed separately into *T. reesei*. To produce transformants coexpressing BiP1-GFP2 and CBHI-Venus, both pBGt and pCVt were introduced into *T. reesei* simultaneously. After surviving two rounds of selection for resistance to hygromycin B, potential transformants carrying the pCVt (23 candidates), pBVt (11 candidates), pBGt (19 candidates), pVGt (13 candidates), and pBGCVt (13 candidates) expression cassettes were tested based on the work flow illustrated in Fig. 3-1. Unfortunately, the possibility to use CBHI-GFP2/BiP1-Venus pair for FRET study was not to be explored further as no transformants carrying pCGt survived the screening for resistance to hygromycin B at the beginning of the screening work. Further efforts to obtain CG transformants were not undertaken since there were enough transformant candidates to continue on for FRET studies (Chapter 8).
Figure 3-1 Work flow for the screening and characterisation of T. reesei transformants.

3.2.1.1 Measurement of intra- and extracellular fluorescence

Because the DNA-constructs bip1-venus, bip1-gfp2 and venus-gfp2 have an ER-retention signal HDEL, the fluorescence of the BV (BiP1-Venus), BG (BiP1-GFP2), VG (Venus-GFP2) and BGCV (BiP1-GFP2/CBHI-Venus) transformants was expected to be found inside the hyphae. Thus, proteins extracted from the hyphae (Section 2.12) of these transformants were subjected for the reading of intracellular fluorescence originating from VenusYFP and/or GFP2 (Fig. 3-2B, C, D and E). On the other hand, the CV and BGCV transformants were expected to secrete CBHI-Venus into the growth medium. Therefore, the culture supernatants of these transformants were read for fluorescence originating from VenusYFP (Fig. 3-2A and E). Fluorescence readings were carried out in duplicate and the supernatant from the culture of a nontransformant Rut C-30 was used to determine the level of background fluorescence in the samples. This background
fluorescence varied from batch to batch. Therefore, the relative intensity of fluorescence of GFP2 and/or VenusYFP was assessed against negative control, the nontransformant Rut C-30, of which the fluorescence intensity above the background intensity.

**Figure 3-2** Fluorescence determination of GFP2 and VenusYFP in the *T. reesei* transformants by fluorometry (continued onto next page). A, VenusYFP fluorescence of the culture supernatants of the CV transformants. B, VenusYFP fluorescence of the cellular extracts of the BV transformants. C, GFP2 fluorescence of the cellular extracts of the BG transformants. D, GFP2 and VenusYFP fluorescence of the cellular extracts of the VG transformants. E, GFP2 fluorescence of the cellular extracts and VenusYFP fluorescence of the culture supernatants of the BGCV transformants. All the transformants were grown in the CLS medium (pH 6.5) for 3 days. Excitation/emission filter 485 nm/520-P nm and 405 nm/520-P nm were used for detecting VenusYFP and GFP2, respectively.
After grown for three days in the CLS medium (pH 6.5), the transformants CV48, CV29, CV63 (Fig. 3-2A); BV47, BV78, BV40 (Fig. 3-2B); BG49, BG29, BG89 (Fig. 3-2C); VG15, VG41, VG43 (Fig. 3-2D) and BGCV101, BGCV104, BGCV117 (Fig. 3-2E) displayed strong intracellular and/or extracellular fluorescence indicating strong expression compared to the nontransformant Rut C-30 and were therefore selected for further studies. Sufficient fluorescence signals are important for microscopy observation of the molecular reporters, which is particularly true for FRET study. In addition, the results showed that the selected VG (VG15, VG41 and VG43) (Fig. 3-2D) and BGCV transformants (BGCV101, BGCV104 and BGCV117) (Fig. 3-2E)
expressed both GFP2 and VenusYFP, and therefore were potentially suitable for FRET studies as dual-labelled samples. It should be noted that in the transformants VG15 and BGCV101 which were later applied as a FRET positive control and the actual FRET sample respectively (Chapter 8), the fluorescence levels of both GFP2 (FRET donor) and VenusYFP (FRET acceptor) were about at the same level when related to the negative control; this was thought to favour FRET occurrence. It was interesting to see that the fluorescence levels of GFP2 and VenusYFP were much lower in the BGCV transformants (blue bars in Fig. 3-2 E) compared to those in the other transformant strains (Fig. 3-2A-C) indicating generally lower expression of GFP2 and VenusYFP in the BGCV strains. Co-transformation with both bip1-gfp2 and cbh1-venus may result in a lower number of gene copies integrating in the genome as seen in the selected strain BGCV101 (Fig. 3-4) therefore resulting in lower expression of the fusion proteins of BiP1-GFP2 and CBH1-Venus in this transformant. Relatively lower expression levels of GFP2 and VenusYFP were found in the VG transformants compared to VG15 (Fig. 3-2D). This finding supported the possibility that a lower copy number of expression cassettes may have been responsible for the lesser expression of the fluorescent proteins GFP2 and VenusYFP since the VG15 carried four copies of the venus-gfp2 gene (I will come back to this result when I describe Southern blotting analysis in Section 3.2.2.1) which displayed strong fluorescence of GFP2 and VenusYFP (Fig. 3-2D).

3.2.1.2 PCR amplification of the expression cassettes

PCR amplification from the genomic DNAs was carried out for the three strongest fluorescing transformants of each strain. The analysis showed that two BV transformants (BV47 and BV78), two CV transformants (CV48 and CV63), two VG transformants (VG15 and VG41), two BG transformants (BG29 and BG89) and only one BGCV transformant (BGCV101) demonstrated a PCR product of the expected size using appropriate primers (Fig. 3-3A-E): BV (bip1-venus 1365 bp), CV (cbh1-venus 1365 bp), VG (Venus-GFP2 2085 bp), BG (bip1-gfp2 1365 bp) and BGCV (bip1-venus 1365 bp as well as cbh1-venus 1365 bp) (Fig. 3-3A-E). The PCR data indicated that the above-mentioned genes had been successfully integrated into the genomic DNA of the corresponding transformants.
Figure 3-3 PCR amplification from genomic DNAs extracted from the three strongest fluorescing transformants. A, BV expressing bip1-venus. B, CV expressing cbh1-venus. C, BG expressing bip1-gfp2. D, VG expressing venus-gfp2. E, BGCV coexpressing both bip1-gfp2 (E1) and cbh1-venus (E2). Plasmid DNAs PBVt, pCVt, pVGt and pBGt were used as positive controls correspondingly. Genomic DNAs were extracted from the transformants grown in the CLS medium (pH 6.5) for 3 days. The sequence information about the primers used in the PCR analysis is described in Table 2-2.

BV47, BG29, CV48, VG15 and BGCV101 were chosen for further characterisation studies based on PCR amplification of the expected sized product (Fig. 3-3) and strong intra- and/or
extracellular fluorescence (Fig. 3-2). The selected transformants were tested by PCR for homologous integration of the expression DNA into the cbh1 locus using primers internal to the respective coding regions (details in Table 2-2). None of the selected transformants were positive for homologous integration of the transforming DNA into the corresponding cbh1 locus (data not shown). This was confirmed by the Southern blotting analysis below.

3.2.2 Characterisation of the selected transformants

3.2.2.1 Southern blotting for detecting the gene copy numbers

Chromosomal DNA was extracted from the transformants BV47, BG29, CV48, VG15 and BGCV101 and digested with BamHI restriction enzyme. Southern blots were probed with the gfp2 or venus fragment DNA (Section 2.11.1) for confirmation of the presence of gfp2 and/or venus and for determining their copy number within the genome of the T. reesei transformants (Fig. 3-4).

![Southern blotting analysis of the selected transformants](image)

**Figure 3-4** Southern blotting analysis of the selected transformants BGCV101 (carrying both bip1-gfp2 and cbh1-venus), VG15 (carrying venus-gfp2), CV48 (carrying cbh1-venus), BV47 (carrying bip1-venus) and a nontransformant Rut C-30 probed with gfp2 (A) and venus (B) DNA fragments. Circular plasmid DNA pBGt and pCVt were used as positive controls for the gfp2 and venus probes, respectively.

As shown in Fig. 3-4, there was at least one copy of each of the gfp2 and venus expression cassettes integrated into the genome of the transformant BGCV101. There were at least four copies of each of the gfp2 and venus expression cassettes integrated into the genome of
transformant VG15. At least two copies of *venus* expression cassettes were detected in transformant CV48, and BV47 contained two copies of *venus*. At least two *gfp2* copies were detected in the BG29 transformant. No *gfp2* nor *venus* signal was detected from the genomic DNA of the nontransformant Rut C-30. When considering both the fluorescence and the Southern blotting results, the strong fluorescence emitting from BV47, CV48 and BG29 was likely as a result of the insertion of multiple copies of the expression cassettes into the genome as discussed in the end of the Section 3.2.1.1.

In the instance whereby one copy of the expression DNA was integrated into the fungal genome and at the *cbh1* gene locus, the expected sizes of the DNA fragments following digestion with *BamHI* and using the *gfp2* and/or *venus* probe(s) were 7800 bp for pBVt and pBGt and 6240 bp for pVGt. It was not possible to determine whether the pCVt DNA fragment had integrated at the *cbh1* locus from using *BamHI* because *BamHI* cuts internally of the *cbh1* core fragment and at the start of the *pki* promoter (just after the truncated terminator fragment, Fig. 2-1). No homologous integration of the expression DNA into the *cbh1* locus was detected in any of the following transformants as judged by the absence of bands at the expected sizes of 7800 bp (for BG29 and BV47) and 6240 bp (for VG15), respectively (Fig. 3-4).

### 3.2.2.2 Expression of *gfp2* and *venus* in *T. reesei*

**Northern blotting**

Total mRNA of the transformants BV47, BG29, CV48, VG15 and BGCV101 was extracted from cultures grown in the CLS medium (pH 6.5) (Section 2.9) for three days for the Northern blotting analysis using *gfp2* and *venus* for hybridisation probes. Both the *gfp2* and *venus* transcripts at the expected size of 2400 bp were detectable in the transformants BGCV101 and VG15 (Fig. 3-5). *Venus* transcript of the predicted size of 2400 bp was detected in BV47 and CV48. In addition, a 2400 bp *gfp2* transcript was found in the BG29 transformant. These results confirmed that the introduced DNAs were successfully expressed at the mRNA level. No *gfp2* nor *venus* transcripts were detected in the nontransformant Rut C-30.
Figure 3-5 Northern blotting analysis of total RNA of the selected transformants BGCV101, VG15, CV48, BV47, BG29, and a nontransformant Rut C-30 probed with gfp2 (A), venus (B) and actin (C). Total RNAs were extracted from the transformants grown in the CLS medium (pH 6.5) for 3 days. Ten µg of total RNA was loaded per well.

A high gene copy number is likely to be associated with the high levels of mRNA and fluorescence for example in the transformant VG15 (Fig. 3-4 and 3-5). All lines of evidence (fluorescence readings, gene copy number and the amount of VG15 specific mRNA transcript) suggested that the fusion protein was effectively expressed under the cbh1 promoter. However, high copy gene number seemed not always to result in high levels of mRNA transcripts as seen, for example, in BG29 which had at least two copies of gfp2 gene in the genome (Fig. 3-4A) whilst showing a relatively low level of gfp2 mRNA (Fig. 3-5A). This may be because some copies of the gfp2 gene were integrated into a nonactive site of the chromosomal DNA or due to mRNA instability in this specific strain.

**Western blotting**

Expression of the two fluorescent proteins GFP2 and VenusYFP in the *T. reesei* transformants BV47, BG29, CV48, VG15 and BGCV101 was next investigated by Western blotting. It is essential for the studies that the fusion protein will hold together in the cell during labelling of organelles, tracking of protein secretion and investigating FRET. In terms of tracking CBHI secretion, it was also important to ascertain that the fusion protein became secreted out of the cell.
as part of the secretion process. This aspect was studied with the transformant CV48 by detecting the CBHI-Venus fusion protein in its culture supernatant in this section to make sure that the protein, in fact, was coming out of the secretory pathway (Fig. 3-6A) and further in both intracellular extracts and the extracellular culture medium (Sections 7.2.2.3 and 7.2.3 respectively). Because of the presence of the ER targeting tag HDEL, BiP1-Venus, BiP1-GFP2, and Venus-GFP2 proteins were expected to localise inside the hyphae of the transformants BV47 (Fig. 3-6B), BG29 (Fig. 3-6C) and VG15 (Fig. 3-6C) correspondingly. Western blotting with the transformant BGCV101 coexpressing BiP1-GFP2 (expected to localise in the ER) and CBHI-Venus (expected to be secreted into the culture medium) was conducted using both intracellular and extracellular samples (Fig. 3-6E). The above protein samples were immuno-probed with both the anti-GFP antibody (monoclonal) and anti-CBHI antibody (polyclonal).

**Figure 3-6** Western blotting analysis of the selected transformants. The transformants included CV48 expressing CBHI-Venus (A), BV47 expressing BiP1-Venus (B), BG29 expressing BiP1-GFP2 (C), VG15 expressing Venus-GFP2 (D) and BGCV101 expressing BiP1-GFP2/CBHI-Venus (E). A loading of $5 \times 10^8$/50 mL spores were grown in the CLS medium (pH 6.5) for 3 days. Rout C-30 was the transformation host strain. All samples were boiled for 5 min before SDS-PAGE separation in this experiment. A 20 µg sample of total protein was loaded per well.
When probed with the anti-GFP antibody which recognises both GFP2 and VenusYFP, the nontransformant Rut C-30 proteins (intra- or extracellular) did not show any binding (top pictures in Fig. 3-6A-E). The weak bands in the Rut C-30 lane at the top of Fig. 3-6D resulted from the loading bleeding from both the protein ladder lane and the cellular lysate sample of VG15.

The protein sizes deducted from the gene sequences of BiP1, GFP2, VenusYFP, and CBHI were 78, 27, 27 and 46 kDa, respectively. Therefore, the theoretically calculated sizes of the products of BiP1-Venus, CBHI-Venus, and Venus-GFP2 were 104, 73 and 54 kDa correspondingly, when hybridized with the anti-GFP antibody. Proteins of the expected sizes were detected in the intracellular and extracellular protein samples of the selected transformants (top pictures in the Fig. 3-6A-E; Fig. 3-1) suggesting the fusion proteins were expressed successfully. The presence of multiple bands was particularly evident in the culture medium of CV48 (Fig. 3-6A) suggesting cleavage and/or degradation of the fusion protein CBHI-Venus in the culture medium plus boiling of the sample (Section 5.2).

Some unexpected bands of a lower size than expected (34 kDa and below) were also found in the samples when probed with the monoclonal GFP antibody (top pictures in Fig. 3-6A, C-E). The 34 kDa band was seen both in the samples containing intracellular and extracellular proteins (Fig. 3-6C-E). The emergence of the 34 kDa band in the cellular extracts (Fig 3-6B-E) was interpreted as a result of boiling of the samples before loading on the gel. This was confirmed by later experiments (Section 5.2 and Fig. 5-1C). It is likely that the constantly appearing 34 kDa band represents the GFP2/VenusYFP protein despite of the position of the molecular marker of 27 kDa because of the general inaccuracy of protein size assessment in Western blotting (Anderson, 2001). The higher molecular weight (34 kDa as opposed to the expected 27 kDa) could not be explained by glycosylation because the GFP2 or VenusYFP DNA sequences have no N-linked or O-linked glycosylation sites (Moin et al., 2000).

In the culture supernatant of the CV48 transformant, higher molecular weight polypeptides greater than the expected 73 kDa for the fusion protein CBHI-Venus may be due to heterogenous glycosylation of the CBHI protein. The T. reesei ALKO3713 which is a transformant of Rut C-30, has been shown to add various sized N-glycans on the endogenous CBHI therefore resulting in a product of a greater size than expected (Maras et al., 1997). Glycosylation is also known to
vary between different culture batches and conditions (Stals et al., 2004a; Stals et al., 2004b). Detailed discussion on the transformant CV48 is presented in Chapter 7.

Apart from testing the production of the fusion proteins by the anti-GFP antibody, a polyclonal CBHI antibody was also applied to check CBHI expression with the same samples probed with the anti-GFP antibody of each transformant (bottom pictures in Fig. 3-6A-D) except in the case of BGCV101 where the anti-GFP was used to probe the cellular extract (top picture in Fig. 3-6E) and the anti-CBHI was used to probe the extracellular culture medium (bottom picture in Fig. 3-6E). When using the CBHI antibody, the signals for the CBHI-Venus fusion protein and native CBHI were expected to appear at around 73 and 46 kDa, respectively. The results showed that a 46 kDa CBHI was produced inside and/or outside from Rut C-30 (left lanes in the bottom pictures in Fig. 3-6A-E). In addition, both the CBHI-Venus fusion protein and native CBHI were seen in the culture medium of CV48 (bottom picture in Fig. 3-6A) providing indirect evidence that the fusion protein was coming through the secretory pathway. The CBHI-Venus fusion protein and native CBHI were also detected in the intracellular protein extract of BGCV101 (bottom picture in Fig. 3-6E) implicating that expression of heterologous fluorescent protein as a fusion partner did not affect CBHI secretion in these two transformants.

The molecular characteristics of the selected transformants are summarised in Table 3-1 (see next page).
Table 3-1 Summary of the primary characterisation of the selected transformants used in this study.

<table>
<thead>
<tr>
<th>Final selected transformant (Fig. 2-1)</th>
<th>Vector (Fig. 3-3)</th>
<th>Expected DNA size integrated into the genome (bp)</th>
<th>Integration of cassette into the cbh1 locus (Section 3.2.2)</th>
<th>Copy number of gfp2/venus gene into the genome</th>
<th>Expected size of transcripts of gfp2/venus (bp) (Section 3.2.2.2; Fig. 3-5)</th>
<th>Expected size of the fusion protein (kDa) (Section 3.2.2.2; Fig. 3-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV47</td>
<td>bip1-venus</td>
<td>1365</td>
<td>No</td>
<td>2</td>
<td>2400</td>
<td>104</td>
</tr>
<tr>
<td>BG29</td>
<td>bip1-gfp2</td>
<td>1685</td>
<td>No</td>
<td>at least 2</td>
<td>2400</td>
<td>104</td>
</tr>
<tr>
<td>CV48</td>
<td>cbh1-venus</td>
<td>1685</td>
<td>No</td>
<td>at least 2</td>
<td>2400</td>
<td>Not detected</td>
</tr>
<tr>
<td>VG15</td>
<td>venus-gfp2</td>
<td>2085</td>
<td>No</td>
<td>4</td>
<td>2400</td>
<td>54</td>
</tr>
<tr>
<td>BGCV101</td>
<td>bip1-gfp2 cbh1-venus</td>
<td>1365 1685</td>
<td>No</td>
<td>1 1</td>
<td>2400</td>
<td>104 73</td>
</tr>
</tbody>
</table>

*a* for primer details see Fig. 3-2.

*b* genomic DNA was digested using the restriction enzyme *SnBI* (refer to Section 2.11.1).
3.2.2.3 CLSM analysis

Optical properties of GFP2 and VenusYFP expressed in T. reesei

The theoretical emission peaks of the fluorescent proteins GFP2 and VenusYFP are 509 nm (Ghukasyan et al., 2007) and 524 nm (Nagai et al., 2002) respectively. The emission overlap was checked in the T. reesei transformants expressing the gfp2 and venus genes with the system at use. In particular, emission overlap between GFP2 and VenusYFP should be avoided in the FRET study using this pair since the FRET detection method depends on the photobleaching sensitivity of the donor and acceptor. Thus, the transformants BG29 expressing BiP1-GFP2 and BV47 expressing BiP1-Venus were used as representatives for investigation of the emission properties of GFP2 and VenusYFP in T. reesei by CLSM. Figure 3-7A demonstrates the emission spectra of GFP2 and VenusYFP in the transformant BG29 and BV47, respectively, when excited at 405 nm (for GFP2) and 488 nm (for VenusYFP) laser. The most distinctive emissions of the fluorescent protein GFP2 and VenusYFP ranged from 520 to 560 nm for GFP2 and from 520 to 570 nm for VenusYFP indicating that emissions of the two fluorescent proteins greatly overlap. The emission peak of GFP2 and VenusYFP was at 524 nm and 537 nm, respectively suggesting they both exhibited right-shifted emission peaks (15 nm shift for GFP2 and 13 nm shift for VenusYFP) comparable to a pure form of GFP2 (Prasher et al., 1992) and VenusYFP protein in vitro (Prasher et al., 1992; Nagai et al., 2002). The shift is likely associated with the microenvironment where GFP2 and VenusYFP were expressed. Considering the large emission overlap between GFP2 and VenusYFP, subsequential confocal imaging mode was applied with the signal collection of 510-530 nm and 530-550 nm for GFP2 and VenusYFP separately at excitation 405 nm laser (for GFP2) and 473/514 nm (for VenusYFP) for standard fluorescence imaging of VenusYFP and GFP2 but not for xyλ scanning (details in Section 2.14.2) and for FRET imaging (details in Section 2.14.5).
Figure 3-7 Optical properties of the fluorescent proteins GFP2 and VenusYFP expressed in *T. reesei*. A, Emission spectra of GFP2 (excited at 405 nm) and VenusYFP (excited at 488 nm) in the *T. reesei* transformants BG29 and BV47, respectively. B, Photobleaching of GFP2 and VenusYFP in live BG29 and BV47 hyphae over 120 min (one scan per min) using 50% of the laser power at excitation of 405 nm (for GFP2) and 514 nm (for VenusYFP) and emission at 510-530 nm (for GFP2)/530-550 nm (for VenusYFP). Cells used for the above experiments were cultured in the CLS medium (pH 6.5) for 24 h.

In the photobleaching experiment, it took about 20 min only for GFP2 and 90 min for VenusYFP to bleach to 50% of the full fluorescence intensity (Fig. 3-7B). This preliminary photobleaching experiment showed that both GFP2 and VenusYFP tended to bleach considerably quickly with GFP2 being more photosensitive than VenusYFP. Reasonable photobleaching resistance of a FRET donor is required for FRET determination by the acceptor bleaching method (Lalonde et al., 2008). This implies that in the GFP2/VenusYFP FRET pair, it should not be recommended to measure FRET signal in live cells using the acceptor bleaching method since the donor (GFP2) is not relatively photostable. In the later FRET experiment (Section 8.2), the conventional sensitised emission method was used for FRET analysis. In order to minimise photobleaching in the subsequent confocal microscopic imaging work, a significantly reduced power intensity of the excitation laser beam was used, typically 5-10% of the full excitation laser power for both GFP2 and VenusYFP.
Imaging of the fluorescence of GFP2 and VenusYFP directly in the living *T. reesei* transformants

In this thesis, the fluorescent proteins GFP2 and VenusYFP were used as fluorescent molecular reporters to tag the ER-resident folding chaperone BiP1 and the main secreted protein CBHI in *T. reesei*. The data from Southern blotting (Section 3.2.2.1 and Fig. 3-4), Northern blotting (Section 3.2.2.2 and Fig. 3-5) and Western blotting (Section 3.2.2.2 and Fig. 3-6) showed that the constructs tagged with GFP2 or VenusYFP were successfully expressed in the selected transformants. Subsequently, fluorescence was now to be localised in the living fungal hyphae using CLSM. This was carried out with the transformants BV47, CV48, BG29. Fresh hyphae were sampled after growth in the CLS medium (pH 6.5) for 24 h, and then washed in PBS three times before imaging (for sample preparation see *Method 3* in Section 2.14.1).
<table>
<thead>
<tr>
<th></th>
<th>A 24-h-old live BV47 cell</th>
<th>B 24-h-old live CV48 cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Venus</td>
<td>DIC</td>
</tr>
<tr>
<td>A</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>C</td>
<td>24-h-old live BG29 cell</td>
<td>D 24-h-old live Rut C-30 cell</td>
</tr>
<tr>
<td></td>
<td>GFP2 Ch</td>
<td>DIC</td>
</tr>
<tr>
<td>C</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 3-8** CLSM of living hyphae of the *T. reesei* transformants grown in the CLS medium (pH 6.5) for 24 h. A, BV47; B, CV48; C, BG29; D, Rut C-30. Excitation at 405/473 nm and emission at 510-530/530-550 nm were used for viewing GFP2 and VenusYFP, respectively. DIC image was achieved with a 473 nm laser. Bar=10 µm.

The intracellular fluorescence of BiP1-Venus was of high intensity in the living hyphae of BV47 (Fig. 3-8A). BiP1-Venus fluorescence was detected dominantly in a punctate-like fashion though
some extended, yet short filaments of fluorescence were also seen. A typical ER network was not observed through the entire hyphae. The above distribution of BiP1-Venus fluorescence suggests a new localisation site where BiP1-Venus appeared as ER-derived punctate-like bodies in addition to the expected localisation on tubular membranes (Fernández-Ábalos et al., 1998; Wedlich-Söldner et al., 2002; Maruyama et al., 2006). A detailed account of the application of the BiP1-Venus fusion as an ER marker in living cells is given in Section 6.3.

In the CV48 strain, a bright fluorescence was mainly visualised distributed in a filamentous network tentatively identified as the ER (Fig. 3-8B) with plenty of discrete, dotty or elongated “bodies” assumed as ER-derived membrane structures later confirmed by IEM (described in Chapter 7). In some cases, distinctively high fluorescence was located to the septa similar to what has been seen previously for a glucoamylase-GFP fusion protein in Aspergillus niger (Khalaj et al., 2001). Gordon et al. (2000a, 2000b) pointed out the following possibilities to explain the occurrence of a secretory protein on the septa: (i) the fusion protein was trapped on the septa but freely diffusible within the intracellular matrix or (ii) the recombinant protein might be secreted by secretory vesicles joining the flow of vesicles containing wall polymers for septum formation. In this study, CBHI-Venus also appeared on the septum indicating the presence and accumulation of the fusion protein in the septum. The transformant CV48 expressing the CBHI-Venus fusion protein was employed as the FRET acceptor in pairing with the donor BiP1-GFP2 (Chapter 8). This strain was also used to track secretion of the dominant secreting protein CBHI over a time course of 120 h (Chapter 7).

The intracellular fluorescence in the transformant BG29 expressing the BiP1-GFP2 fusion protein strain appeared as small and concentrated spots throughout the hyphae (Fig. 3-8C), different to a conventional ER network. This distribution pattern was similar to what was observed in BV47 which had a similar DNA-construct integrated into the same host strain (Rut C-30) except that the fluorescent protein VenusYFP in BV47 was replaced with GFP2 in the strain BG29. Both BG29 and BV47 had two copies of the expression cassettes integrated into the genome of Rut C-30 (Fig. 3-4). Therefore, overexpression of the fusion protein may have occurred in BG29 similarly to that discovered for BV47 (Section 6.3). Further investigation of the possible reason(s) for the “unusual” punctate fluorescence pattern of BiP1-GFP2 in the transformant BG29 was not
conducted due to time restrictions. The transformant BG29 expressing the BiP1-GFP2 fusion protein was used as a FRET donor in pairing with the acceptor CBHI-Venus later in Chapter 8.

There was no obvious difference in the overall morphology between the selected transformants and the nontransformant Rut C-30 by CLSM (data not shown). The nontransformant *T. reesei* Rut C-30 did not express any intracellular fluorescence (Fig. 3-8D). The selected transformant strains constructed here and their intended uses are shown in Table 3-2.

**Table 3-2** Expression vectors used in this study and their intended applications (for detailed features of each vector, refer to Section 2.5 and Fig. 2-1).

<table>
<thead>
<tr>
<th>Vector</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBVt (<em>bip1-venus</em>)</td>
<td>Used as an ER marker for real-time visualisation of the ER morphology in live fungal cells (Section 6.3)</td>
</tr>
<tr>
<td>pCVt (<em>cbh1-venus</em>)</td>
<td>Used as a molecular reporter to allow tracking CBHI-Venus secretion (Chapter 6);</td>
</tr>
<tr>
<td></td>
<td>Used as a partner to coexpress with pBGt in <em>T. reesei</em> to create the BGCV transformant (see below);</td>
</tr>
<tr>
<td></td>
<td>Used as an acceptor for the FRET study (Chapter 7)</td>
</tr>
<tr>
<td>pBGt (<em>bip1-gfp2</em>)</td>
<td>Used as a partner to coexpress with pCVt to create the BGCV transformant (see below);</td>
</tr>
<tr>
<td></td>
<td>Used as a donor for the FRET study (Chapter 7)</td>
</tr>
<tr>
<td>pBGCVt (coexpression of <em>bip1-gfp2 and cbh1-venus</em>)</td>
<td>Used as a FRET sample for studies on interactions between Bip1 and CBHI (Chapter 7)</td>
</tr>
<tr>
<td>pVVGt (<em>venus-gfp2</em>)</td>
<td>Used as a positive control for the FRET study (Chapter 7)</td>
</tr>
</tbody>
</table>

### 3.3 Conclusions

Expression DNAs encoding the fluorescent proteins GFP2 and VenusYFP were successfully introduced into *T. reesei* as a tag for either the ER-resident folding chaperone BiP1 and/or the dominantly secreted CBHI, expressed under a strong *cbh1* promoter. The characterisation of selected transformants CV48, BV47, BG29, VG15 and BGCV101, was carried out at the both nucleic acid and protein levels. Analysis of the transformants suggested that GFP2 and VenusYFP were expressed successfully and therefore can serve as tools in further studies into the secretory pathway of *T. reesei* and to be explored in FRET studies (Chapter 8). GFP2 and
VenusYFP were sensitive to photobleaching, therefore care must be taken during the imaging work to protect the fluorophores. CLSM observations of the fluorescence of the fusion proteins such as the BiP1-Venus and the CBHI-Venus in the living hyphae of the transformants BV47 and CV48 showed that enough signal for the various planned analyses was obtained. The results also provided some information on the potential effects of high protein expression on the ER morphology discussed further in the later chapters.
Chapter 4 Optimisation of sample preparation for microscopy studies

4.1 Introduction
The secretory pathway of filamentous fungi has been shown to be conserved in many aspects in relation to the unicellular yeast *S. cerevisiae* and higher eukaryotes (Bennett and Scheller, 1993). However, there are also differences due to the hyphal mode of growth of the filamentous fungi. The general aim of this study was to map out the secretory pathway in the industrially-exploited fungus *T. reesei* and the major tasks to this project were to visualise the main cellular organelles involved in protein secretion and to follow secretion of the main cellulose, CBHI through the ‘land-marked’ secretory pathway. A potential interaction between the ER chaperone BiP1 and the main secreted cellulase CBHI was also of interest. In addition to molecular biology methods, CLSM and EM were largely used to achieve the above aims.

Before progressing into visualisation of the ER and Golgi apparatus in living *T. reesei* by CLSM, the following points were considered:

(i) a suitable culture medium for the induction of the *cbh1* promoter with a minimum fluorescence background in confocal imaging of living fungal cells would be needed;
(ii) a special sample preparation setup was required to allow visualising live fungal cells during the experiment, especially in long-time culture;
(iii) staining and imaging conditions for the general membrane dye FM® 4-64 for the live *Trichoderma* hyphae had to be established.

The work discussed in this chapter is focused on the optimisation of the experimental conditions for CLSM based on the above considerations.

4.2 Results and discussion
4.2.1 Non/least-fluorescent media for organelle observation of living *T. reesei* by CLSM
Earlier studies establishing culture conditions for the production of CBHI in *T. reesei* Rut C-30 showed abundant protein production when the fungus was cultivated on the medium containing 1 % (w/v) cellobiose, 1 % (w/v) lactose, and 1.5 % (w/v) soy hydrolysate (CLS) in minimal medium salts (MM) (pH 5.5) (Curach *et al*., 2004). Therefore, this work started with culturing *T. reesei* in the CLS medium (pH 5.5). Fluorescence of a selection of media containing different
ingredients including 2 % (w/v) agar, trace elements, 1.5 % (w/v) soy hydrolysate, 1 % (w/v) lactose, 1 % (w/v) cellobiose (Table 4-1), was tested to establish their suitability to fluorescence microscopy (Fig. 4-1). To make the media easier to stick to the glass slides, 2 % (w/v) agar was used to solidify the media.

Table 4-1 Fluorescence emitted from the culture medium ingredients under a confocal microscope.

<table>
<thead>
<tr>
<th>Group</th>
<th>Medium ingredient(s)</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>488 nm laser (10 %)</td>
</tr>
<tr>
<td>1</td>
<td>2 % agar</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>MM + 1 % 100× trace elements* + 2 % agar</td>
<td>+ +</td>
</tr>
<tr>
<td>3</td>
<td>MM + 2 % agar</td>
<td>+~~</td>
</tr>
<tr>
<td>4</td>
<td>1.5 % soy hydrolysate + 2 % agar</td>
<td>+</td>
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<tr>
<td>5</td>
<td>1 % lactose + 2 % agar</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1 % cellobiose + 2 % agar</td>
<td>+~~</td>
</tr>
<tr>
<td>7</td>
<td>MM + 1 % 100× trace elements + 2 % agar + 1 % cellobiose + 1 % lactose + 1.5 % soy hydrolysate (CLS medium)</td>
<td>+ + + + +</td>
</tr>
</tbody>
</table>

- invisible/non-significant fluorescence
+~~ just discernible fluorescence
+ very weak fluorescence
++ weak fluorescence
+++ moderate fluorescence
++++ strong fluorescence
+++++ very strong fluorescence

*refer to Section 2.2 for the recipe of 100× trace elements

The CLS medium (pH 5.5) emitted strong background fluorescence that resulted mainly from soy hydrolysate, cellobiose and trace elements or combination of all of them (Table 4-1). Lactose (1 % w/v), which induced the cbh1 promoter in Rut C-30 (Janas et al., 2002), did not give significant fluorescence. In addition, 2 % agar and MM salts were not obviously fluorescent under the observation conditions (Table 4-1). Based on the above results, a basic cbh1 inducing microscopy-compatible medium for T. reesei Rut C-30, identified as “lactose medium” in this
work, was formulated containing 1 % (w/v) lactose and MM salts for the early stage imaging work (Yu et al., 2005).

The lactose medium was then tested for protein production and the secreted proteins separated on SDS-PAGE to briefly assess the protein production on this medium. The results were compared to protein production in the CLS medium (Fig. 4-2). Gel results indicated that, after three days’ culture, proteins ranging from 60-85 kDa (lane 2 in Fig. 4-2) corresponding to possibly glycosylated forms of CBHI (Nykänen et al., 2002) and other cellulases were efficiently produced in the CLS medium (pH 5.5) (lane 2 in Fig. 4-2). In contrast, a lower amount of proteins was produced on 1 % lactose (lane 3 in Fig. 4-2). MM-only did not induce any protein production (lane 5 in Fig. 4-2). Addition of 0.5 % methyl cellulose (MC, used as medium thickener later in Section 4.2.2) did not result in a significant difference in production of secreted proteins suggesting the 0.5 % MC in 1 % (w/v) lactose medium would not affect protein production (lane 4 in Fig. 4-2).

![Figure 4-1 SDS-PAGE separation of proteins secreted by Rut C-30 grown in different culture media. Lane 1: protein marker; lane 2: the CLS inducing medium (pH 5.5); lane 3: 1 % lactose + MM; lane 4: 1 % lactose + MM + 0.5 % MC; lane 5: MM. A total of 1×10⁸ conidia per 50 mL were inoculated into 50 mL of medium in a 250 mL flask and the culture were shaken at 28 °C for three days.](image-url)

The focus of the microscopy studies discussed in this Chapter was on visualising intracellular membranous compartments stained by a membrane dye FM® 4-64, selected for the purpose. We hoped to minimise fluorescence background originating from medium ingredients while achieving reasonable induction of the hyper-secreting cbh1 promoter. Therefore, live cell imaging of FM® 4-64 stained membranes for a time course of 24 h was carried out based on the
lactose medium containing 0.5 % MC at the early stage of this work (details in Section 4.2.2), while the CLS medium was used instead to efficiently induce protein production in T. reesei throughout the whole project with pH 6.5 rather than 5.5 (investigated later in Section 5.2).

4.2.2 Sample preparation of living fungal cells for CLSM and dye loading

The published preparation methods for live cell imaging of fungal cells are either problematic in keeping microscopy observation consistent or not supportive for long-time culture/observation (reviewed in Section 1.3.2.1). Four different methods of sample preparation and staining with FM® 4-64 were therefore investigated. In Method 1, the Rut C-30 hyphae were grown on PDA medium and a 20 mm² block of the PDA medium was cut and stained with FM® 4-64 for 3 h before imaging. In Method 2, Rut C-30 conidia were cultured on a piece of cellophane spread on a PDA plate and a 5×10 cm piece of cellophane was cut out and stained with FM® 4-64 for 1 h before imaging. In Method 3, Rut C-30 grown in the liquid CLS medium was collected and washed off with PBS before stained with FM® 4-64. In Method 4, Rut C-30 was grown in the lactose medium solidified with 0.1-0.7 % (w/v) agarose or 0.1-0.7 % (w/v) MC in the wells of a 16-well chambered coverslip before imaging (details in Section 2.14.1).

Results showed that Method 1 offered the possibility to focus on the immobilised hyphae and to obtain a good staining (Fig. 4-3). However, it was hard to control the thickness of the agar block and cutting the agar blocks was also time-consuming. In addition, it took longer (here 3 h) than usual (1 h staining for FM® 4-64) to get the stain internalised by cells due to poor penetration through the agar block.
Figure 4-2 Confocal image of the hyphae of *T. reesei* Rut C-30 grown on a PDA block for 48 h. The block was cut to a 20 mm\(^2\) block which was stained with 33 \(\mu\)M FM® 4-64 for 3 h at room temperature. This figure shows investigation of sample preparation using Method 1.

**Method 2** was easy to use but the cellophane emitted strong fluorescence background making it impossible to obtain an image (data not shown).

**Method 3** resulted in good staining and excellent images (Fig. 4-4A) therefore offering an option for fast imaging of living fungal cells. The fluorescence background from the medium could be removed by washing, and this method was later used to image the live fungal hyphae cultured in the CLS medium which supported strong induction of the *cbh1* promoter when required (Fig. 4-1). However, this method was compromised due to time-consuming and tedious washing steps. Another disadvantage of this method is that hyphal aggregation due to centrifugation does not allow demonstration of the natural distribution and growth status of the growing hyphae in the authentic cultivation environment. The aggregation can be partially rectified by diluting the samples with PBS solution to disperse the hyphae (Fig. 4-4B).
Figure 4-3 Confocal images of the hyphae of *T. reesei* Rut C-30 grown in the CLS medium (pH 5.5) for 48 h. A, Rut C-30 stained with FM® 4-64. B, DIC image of the edge of an aggregated hyphal clump. Cells were harvested and washed for three times with 1× PBS before staining with 33 µM FM® 4-64 for 1 h at room temperature. This figure shows investigation of sample preparation using Method 3.

In the **Method 4**, a semi-solid medium containing 0.1-0.5 % methyl cellulose (MC) which had no obvious inhibitory effect on growth of fungi (Fig. 4-5 F-H) compared to the control medium was used (Fig. 4-5A). In experiments with different MS concentrations, 0.7 % MC (Fig. 4-5I) and 0.1-0.7 % agarose showed inhibition against the growth of fungi (Fig. 4-5 B-E). MC is a hydrophilic chemical compound derived from cellulose and dissolves in hot water, forming a clear viscous solution or gel. It is sold under a variety of trade names and is used as a thickener and emulsifier in various food and cosmetic products. Like cellulose, it is not toxic. Aqueous methylcellulose solutions have been used to slow cell motility for closer inspection by microscopy (Metcalf, 1977; Kahn *et al.*, 2004; Fiedler *et al.*, 2008). Changing the amount of methylcellulose in solution allows one to adjust the solution’s viscosity (Brokaw, 1966). Our results showed that 0.5 % MC in the *T. reesei* culture medium provided reasonable viscosity, efficiently immobilising the movement of fungal conidia and hyphae therefore having a beneficial effect on imaging of living fungal cells. This method is easy to use and facilitates keeping the cultures free of contamination over longer experiments. Importantly, MC is not fluorescent therefore allowing *in situ* staining of cells in the semi-solid medium by premixing the medium with the particular dye without disturbing growing cells (Yu *et al.*, 2005). To add to its benefits, MC seemed to have no significant effect on protein production of Rut C-30 in a lactose medium (lanes 3 and 4 in Fig. 4-2). In contrast, low concentrations of agarose, even as low as 0.1 %, seemed to negatively affect
fungal growth (Fig. 4-5 B-E). This probably was because of the special jelly-like nature of agarose, hard to penetrate by fungal hyphae.

**Figure 4-4** Effect of the medium solidifier MC and agarose in lactose medium on hyphal growth. The lactose medium was composed of 1 % lactose in MM salts (pH 5.5). Initial spore inoculation: 2×10⁶/mL. Culture conditions: 16-well chambered coverslip with 100 μL of culture medium in each well, incubated at 28 °C for 48 h (no shaking). Scale bar represents 20 μm. A, Lactose medium without agarose or MC; B, Lactose medium with 0.1 % agarose; C, Lactose medium with 0.3 % agarose; D, Lactose medium with 0.5 % agarose; E, Lactose medium with 0.7 % agarose; F, Lactose medium with 0.1 % MC; G, Lactose medium with 0.3 % MC; H, Lactose medium with 0.5 % MC; I, Lactose medium with 0.7 % MC. This figure shows investigation of sample preparation using **Method 4**.

The basic *cbh1* inducing microscopy-compatible medium, identified as “lactose medium” containing 1 % (w/v) lactose and MM salts was used for imaging of the hyphae stained with FM4-64 described in the following section.

**4.2.3 Characterisation of the membrane-selective stain FM® 4-64 in living *T. reesei***

The membrane-selective stain FM® 4-64 was the first fluorescent stain to be tested in this work as part of methods development. The theoretical emission data is extracted based on the use of methanol as the solvent for this amphiphilic styryl dye ([www.invitrogen.com](http://www.invitrogen.com)) and its emission
spectrum varies between different cell types (Betz and Bewick, 1992) because of its aromatic nuclei (Fig. 1-10D). Therefore, the emission of the FM® 4-64 dye had to be established in the environment relevant for the current research. The culture of *T. reesei* Rut C-30, grown in the lactose medium for 24 h, containing the dye, showed emission in the range 560-720 nm, with the maximum at 750 nm. This indicated the emission spectrum of this dye in *T. reesei* spectrally shifted by 104 nm towards the longer wavelength compared to the emission data provided by the manufacturer (Fig. 4-6) (Yu et al., 2005). Such a large shift may have been due to the effect of the cellular environment on the dye molecules.

Figure 4-5 Comparison of emission spectra of FM® 4-64 in methanol and *T. reesei* for excitation at 488 nm. Left curve: FM® 4-64 in methanol (adapted from the information on www.invitrogen.com); Right curve: FM® 4-64 in *T. reesei* Rut C-30 grown in the lactose medium for 24 h. Δ shift = 104 nm.

We also explored the time course of staining of the fungal hyphae with FM® 4-64 by CLSM over 135 min using the semi-solid medium setup (Fig. 4-7). Examination of the early dye uptake revealed that plasma membrane staining was immediate and staining became more pronounced after 20 min in the dye-containing medium. Subsequently, light staining of the hyphal cytoplasm was seen increasing with time. The internalisation of the dye approached saturation after 60 min. At 135 min, numerous organelles were visible and oversaturated.
In the fungal or other eukaryotic cells, successive staining of the cell plasma membrane followed by the membranes of other intracellular structures is time-dependent (Bolte et al., 2004). It seemed that after 60 min, most of the internal membranes in T. reesei were visibly stained (Fig. 4-7) (Yu et al., 2005), therefore, 60 min was applied as a standard staining time using the concentration of 33.3 µM FM® 4-64 as suggested by the manufacturer.

**4.3 Conclusions**

In this Chapter, protocols for confocal fluorescence microscopy studies were developed to minimise fluorescence background originating from the medium ingredients to facilitate long-time live cell imaging and to work out the staining conditions for FM® 4-64 to allow an overview of the membraneous organelles in preparation for further microscopy studies.

A commonly-used CLS medium (pH 5.5) which efficiently induces the expression of the cbh1 promoter, under which the various genes and gene fusions discussed in Chapter 3 were expressed, was initially chosen as the medium to grow T. reesei for CLSM. It was found that the CLS medium emitted strong background fluorescence (Table 4-1 and Fig. 4-1) resulting mainly from soy hydrolysate, cellobiose and trace elements or their combination. Therefore, a medium containing 1 % (w/v) lactose and MM salts was developed to avoid the background fluorescence.
Lactose medium was further supplemented with 0.1 % MC to make a semi-solid medium to be applied in staining and imaging of the living hyphae of *T. reesei* over time using FM® 4-64 by CLSM. Time course of staining the *T. reesei* Rut C-30 with the membrane-selective stain showed that FM® 4-64 was taken up by most of membranous compartments after 60 min incubation at room temperature. Special labelling of the ER and Golgi apparatus is discussed in the next chapter (Sections 6.3 and 6.4 respectively). The spectral characteristics of FM® 4-64 are very different to that from e.g. VenusYFP which was tagged to the ER-resident folding chaperone BiP1 and the high-secreting protein CBHI in this work (Section 6.3 and Chapter 7). Therefore, the FM® 4-64 was later used in *T. reesei* in conjunction with the genetically targeted VenusYFP (Chapters 6 and 7, Fig. 6-7A and Fig. 7-5C).

It should be noted that since strong induction of the *cbhI* promoter was required for the expression of the various recombinant proteins produced in this work, it was decided to go back to using the CLS medium and subsequent washing of the samples (*Method 3*) for imaging of live fungal hyphae (Section 6.3 and Chapters 7 and 8). Optimisation of the culture conditions and sample preparation for Western blotting are discussed in the next chapter.
Chapter 5 Optimisation of the culture medium-the effect of initial medium pH on the expression of the CBHI-Venus fusion protein

5.1 Introduction
The cultivation pH is an important parameter in the production of enzymes by T. reesei (Denison, 2000; Xiong et al., 2004). It has been reported that the production of cellulases was favoured by a low pH 4 or 4.5 (Bailey et al., 1993a; Yang, 2004). For CBHI production, it is generally accepted that the optimal production pH is 5-6 and a rather low pH is essential for good production of cellulases with the Rut C-30 strain (Bailey et al., 1993a). In our laboratory, pH 5.5 has been applied for years as an initial pH of a standard culture medium for induction of cbh1 promoter. On the other hand, fluorescence of GFP and its variants has shown to be sensitive to low pH (Patterson et al., 1997; Kneen et al., 1998; Nagai et al., 2002) and low pH induces the production of proteases that are known to affect yields of heterologous proteins (Archer and Peberdy, 1997; van Den Wymelenberg et al., 1997; Gouka et al., 1997a). Previous research results suggest that GFP-related fusion proteins can be used to track protein secretion as long as the pH is held above 6.0 (Gordon et al., 2000b). VenusYFP is a heterologous protein produced in Trichoderma and may therefore be subjected to degradation by protease(s) which are active especially around pH 4-5 (Gordon et al., 2000b).

5.2 Results and discussion
The effect of the initial medium pH (5.5-8.0) on the yield of the fusion protein CBHI-Venus in T. reesei, was studied with the CV48 transformant (refer to Section 2.5 and Fig. 2-1 for the construct design) by reading the medium fluorescence intensity and Western blotting of proteins in 72 h-old culture supernatants of the CV48 transformant. In the extracellular medium, the fluorescence intensity increased with a higher initial medium pH and the highest fluorescent peak was produced by the culture with the starting pH of 6.5 (Fig. 5-1A). When the pH was greater than 6.5, the medium fluorescence intensities dropped down. The expression of the CBHI-Venus fusion protein examined by Western blotting coincided with the spectrometry measurement indicating that the extracellular production of CBHI-Venus was maximal in the cultivation with the starting pH of 6.5 compared to lower pHs (5.5 and 6.0) and higher pHs (7.0, 7.5, and 8.0) (Fig. 5-1A and B).
Figure 5-1 Effect of initial culture medium pH on production of the CBHI-Venus fusion protein and sample preparation for Western blotting. A, Relative fluorescence intensity of the 72 h-old culture medium with different initial pHs. Initial spore inoculation of 2×10⁶/mL. Cultivation conditions: 28 °C, 250 rpm. The fluorescence of the supernatants was measured in a 96-well plate using a Victor plate reader with the excitation and emission filters of 485 nm and 520-P nm separately. Duplicate wells for the same sample were run. B, The effect of the initial pH on the fusion CBHI-Venus production in the 72 h-old culture supernatants as determined by Western blotting. The antibody used was the polyclonal anti-GFP antibody and the protein samples were boiled for 5 min in NuPAGE® 4X LDS Sample Buffer. before loading the gel (20 µg of total protein each lane). C, Western blotting analysis of the 120 h-old boiled (for 5 min water bath at 100 °C in NuPAGE® 4X LDS Sample Buffer) and unboiled culture supernatants (5 min on ice in NuPAGE® 4X LDS Sample Buffer); 10 µg of total protein each lane.
Interestingly, there was no visible band detected from the medium at pH 5.5 (Fig. 5-1B), which is generally considered as the optimal pH for production of CBHI (Mandels and Reese, 1957). The pH below 5.0 may cause irreversible loss of fluorescence (Kneen et al., 1998). Our finding of the absence of CBHI-Venus in the CLS medium with initial pH 5.5 supported this view suggesting low acidic conditions are likely responsible for VenusYFP fluorescence disappearance as previously observed for GFP (Webb et al., 2001). Although expression of the fusion protein remained high between pH of 7-8, the protein was not strongly fluorescent. The reason for this could be because of the total amount of fluorescent proteins (including the fusion and cleaved forms) was still relatively low compared to that of fluorescent proteins produced under other pHs. In Fig. 5-1B, the boiled supernatant samples with initial pH 6.0-8.0 demonstrated two major bands having sizes of 73 and 27 kDa representing the CBHI-Venus fusion protein and VenusYFP alone, respectively. A few bands between 73 and 27 kDa were also seen on the blot of proteins from cultures with the initial pH 6.5 and 7.0. These intermediate bands could be the result of degradation by endogenous proteases. One would also assume that these bands are cleaved off from the fusion protein due to boiling of the sample. Therefore, boiled and un-boiled culture supernatants collected from CV48 grown at pH 6.5 for 120 h were analysed by Western blotting (Fig. 5-1C). The results confirmed the above assumption. For this reason, all samples processed for further Western blotting experiments were incubated with the NuPAGE® 4X LDS Sample Buffer onto ice for 5 min without boiling before loading to gel to keep the fusion protein intact as much as possible. In order to maximise the yields of the CBHI-Venus fusion in the CLS medium, pH 6.5 was chosen for the initial culture medium pH in further work.

5.3 Conclusions
The recombinant fusion protein CBHI-Venus was successfully expressed in T. reesei and secreted into the culture medium. The fusion protein was detected in the culture medium as long as the initial medium pH was held above 6.0. Initial pH 6.5 resulted in the highest production of VenusYFP into the culture supernatant of CV48 and was therefore considered optimal for high-level production and used to cultivate all transformants.

Denaturation by boiling of the protein samples containing the CBHI-Venus fusion protein caused cleavage at the fusion site as indicated by Western blotting. Proteolytic degradation may also have occurred in addition to boiling of the samples therefore resulting in the appearance of
multiple bands of lower molecular sizes. For this reason, cold-denaturation of the protein samples on ice was adopted for further Western blotting analysis with protein samples from BV47 (Section 6.3) and CV48 (Chapter 7).

Note that initial visualisation of the membrananeous structures in the nontransformant Rut C-30, described in the following chapter (Section 6.2) was carried out with cultures grown on the CLS medium pH 5.5.
Chapter 6 Visualisation of the major organelles in secretory pathway of the filamentous fungus *Trichoderma reesei*

6.1. Introduction
With the optimised methods for sample preparation (Chapter 4), commercially available chemical stains specific for different membrane-bound cellular organelles and the fusion protein BiP1-Venus constructed in the work (Chapter 3), visualisation of the major organelles involved in the secretory pathway was carried out at both the CLSM and EM levels.

6.2 Staining of membranes with FM® 4-64
6.2.1 Introduction
FM® 4-64 is membrane selective and stains many organelle membranes. This stain has also several advantages for live cell staining such as photo-stability and low toxicity (Read and Hickey, 2001) making it excellent for overall visualisation of the membraneous network in *T. reesei*. In Section 4.2.3, the emission spectrum of FM® 4-64 was measured and the staining conditions were set up. Work described in this section is a continuation from these preliminary experiments.

6.2.2 Results and discussion
Staining of 24 h-old Rut C-30 (grown on CLS medium pH 5.5) with FM® 4-64 was visualised throughout the hyphae with intense labelling of the vesicle membranes (arrow in Fig. 6-1A), cell membrane (arrow head in Fig. 6-1A) and a filamentous network (narrow arrow in Fig. 6-1A) assumed to be the ER membranes. It is believed that FM® 4-64 inserts into the outer leaflet of the surface of the membrane and hence becomes intensely fluorescent (Fischer-Parton *et al.*, 2000).
Figure 6-1 Labelling of the membrane-based organelles in Rut C-30 with the stain FM® 4-64 alone (A) and in conjunction with the ER marker DIOC₆(3) (B) and the Golgi marker BODIPY® FL C-5 ceramide (C). The arrow, arrowhead and narrow arrow indicate stained membranes of assumed vesicles, cell membrane and ER membranes, respectively.

Double labelling of the membranes was carried out to distinguish different membraneous organelles in the secretory pathway (e.g. ER and Golgi). In the double labelling experiments with FM® 4-64 and the ER-dye DIOC₆(3) (Fig. 6-1B) or the Golgi dye BODIPY® FL C-5 ceramide (Fig. 6-1C), obvious colocalisation of the FM® 4-64-stained membranes with the ER and Golgi
was obtained as reported previously (Yu et al., 2005). However, the reticular staining pattern of BODIPY® FL C-5 ceramide seemed more like the ER than the Golgi bodies which are supposed to appear as dispersed throughout the cytoplasm. Therefore, the colocalisation between FM® 4-64-stained membranes and Golgi is still not certain. ER and Golgi labelling are discussed in detail in Sections 6.3 and 6.4.

One of the current debates regarding FM® 4-64 staining in live cells is organelle specificity (Bolte et al., 2004). The ER or Golgi membranes have not been previously shown to be stained with FM® 4-64 (Fischer-Parton et al., 2000; Bolte et al., 2004) with two exceptions, a study on Fucus cells (Belanger and Quatrano, 2000) and on a BY-2 cell line (Bolte et al., 2004). Our findings that FM® 4-64 colocalised with the ER marker DIOC6(3) provided another positive example to suggest FM® 4-64 can stain the conventional ER reticulum network. Colocalisation between FM® 4-64 and the Golgi marker BODIPY® FL C-5 ceramide was also seen but it was difficult to conclude that FM® 4-64 stained the Golgi due to the ER-like staining pattern of BODIPY® FL C-5 ceramide.

6.2.3 Conclusions
FM® 4-64 stained major secretory organelles including the ER, vesicles and cell membrane in living T. reesei. It can be used as an experimental tool for general visualising of membrane-based components in living T. reesei hyphae. It is also useful for multicolour imaging experiments as explored later in Sections 6.2, 6.3, 6.4 as well as in Chapter 7.

6.3 Visualisation of the ER
6.3.1 Introduction
In eukaryotic cells, some of the most important protein processing steps occur in the lumen of the ER. These include removal of the secretion signal, folding, primary glycosylation and proteolytic processing (van Vliet et al., 2003). Proteins are then packaged into vesicles that bud off from the ER and deliver their cargo to the Golgi apparatus where they may be modified further (Rothman, 1994) (described in Section 1.2.1). The ER consists of a network of membranous tubules that extend throughout the cell (Terasaki et al., 1984). Morphology studies into this important secretory compartment will add valuable information on its behaviour in protein secretion and overexpression of both membrane-bound (BiP1) and secreted proteins (CBHI).
As noted in Section 1.3.2.2, commonly-used fluorescent markers targeting the ER such as dicarbocyanine fluorophore DIOC$_6$(3) (Terasaki et al., 1984; Terasaki and Reese, 1992) and ER-Tracker™ series (http://www.invitrogen.com) are not capable of specifically differentiating the ER from other membrane-organelles (Terasaki and Reese, 1992; Cole et al., 2000). As an alternative to these dyes, antibodies against ER-specific antigens such as the glycoprotein endoplasmin or chaperone BiP (or GRP 78), can be applied to assist visualisation of the ER, in combination with IEM (Koch et al., 1987; Soltys et al., 1996); however this application is limited to fixed cells. The anti-BiP antibodies have been applied to label the ER in the unicellular yeast Saccharomyces cerevisiae. The third option for organelle labelling is provided by genetically encoded fluorescent proteins such as GFP and its variants that can be used as fluorescent tags attached to an organelle-specific protein. Although the wild-type GFP gene has not been functioning properly in many organisms including filamentous fungi (Fernández-Ábalos et al., 1998), the modified GFP genes have been successfully expressed in A.nidulans, Ustilago maydis, A. oryzae (Fernández-Ábalos et al., 1998; Wedlich-Söldner et al., 2002; Maruyama et al., 2006) and in T. reesei (Yu et al., this work).

Considering the specificity problems associated with the use of chemical dyes for ER labelling, it was decided to explore visualisation of the ER architecture in the fungal hyphae using the T. reesei transformant BV47 expressing the BiP1-Venus protein (Chapter 3). Using this tool, the ER structural modifications were visualised in T. reesei during protein overexpression.

6.3.2 Results and discussion
6.3.2.1 Determination of BiP1-Venus expression by RNA analysis
The expression of bip1 and bip1-venus mRNA was examined by quantitative real time PCR (Fig. 6-2). The host strain Rut C-30 did not express bip1-venus mRNA which was highly expressed in the BV47 transformant. Both the transformant and nontransformant T. reesei strains expressed native bip1 mRNA and the expression was greater in the transformant BV47. The reason for the greater bip1 mRNA level in BV47 than in Rut C-30 was because the total expression of bip1 in BV47 was attributed to expression of the endogenous bip1 gene and bip1-venus fusion. This increase in bip1 mRNA expression was not unexpected as the transformant BV47 had two copies of the venus expression DNA (Fig. 3-4B) indicating overexpression of BiP1-Venus in the transformant.
Figure 6-2 Quantitative real time PCR analysis for relative intensity of bip1 and bip1-venus mRNA transcript levels in the transformant BV47 and the nontransformant Rut C-30 with respect to the housekeeping gene actin. Cells were grown in the CLS medium (pH 6.5) for 24 h at 28 °C.

6.3.2.2 Expression of the BiP1-Venus protein in the transformant BV47 as determined by Western blotting

BiP1-Venus expression was detected both intracellularly and extracellularly by Western blotting using the monoclonal anti-BiP and polyclonal anti-GFP antibodies. Although the bands on the blot were overexposed/overloaded when hybridised with the anti-BiP antibody (Fig. 6-3A), it was still possible to see two bands, one at around 104 kDa corresponding to the expected size of the BiP1-Venus fusion protein, and another at around 78 kDa corresponding to the expected size of the BiP protein in the intra- and extracellular samples of BV47 (lanes 1 and 2 in Fig. 6-3A). In the cell extract of Rut C-30, only the ~78 kDa BiP proteins was detected (lane 3 in Fig. 6-3A). No BiP protein was found in the culture medium of Rut C-30 (lane 4 in Fig. 6-3A). The anti-GFP antibody recognised a 104 kDa signal that matches the size of the fusion BiP1-Venus in both the intracellular extract and culture supernatant of BV47 (lanes 1 and 2 in Fig. 6-3B) but not in Rut C-30 (lanes 3 and 4 in Fig. 6-3B). In addition, the polyclonal anti-GFP antibody recognised lower molecular weight proteins in the size range of 10-60 kDa which possibly was due to non-specific binding of this antibody (Fig. 6-3B). These results indicated that the BiP1-Venus fusion protein was successfully introduced and expressed in T. reesei and both the endogenous BiP and the BiP1-Venus fusion proteins were secreted into the culture medium in BV47.
It was interesting to find that the native ER-resident chaperone BiP1 and the BiP1-Venus fusion protein with an ER retention sequence HDEL were exported from the ER and secreted to the culture medium (lanes 2 in Fig. 6-3A and B). It is generally accepted that under normal physiological conditions, the luminal proteins in the ER such as BiP1 and Calreticulin, are efficiently retained in the ER with the retention sequences HDEL/KDEL (Pelham, 1990). However, there is also considerable amount of evidence that tagging with ER retention motifs is not always sufficient to obtain efficient retention (Pimpl and Denecke, 2000). A possible explanation for the export of BiP1 from the ER and secretion outside the fungal hyphae could be overproduction of the BiP1 protein in the *T. reesei* transformant due to presence of multiple copies of the *bip1*-venus expression DNA; the resulting BiP1 molecules may have escaped the routine recycling pathway between the ER and the Golgi, normally faced by the native BiP1 protein. This possibility is supported by previous studies where overexpression of fusion proteins has resulted in the secretion of the resident proteins (such as BiP) in yeast (Dean and Pelham, 1990; Belden and Barlowe, 2001), plant cells (Crofts *et al.*, 1999; Galili, 2004; Petruccelli *et al.*, 2006) and mammalian cells (Wiest *et al.*, 1997; Okazaki *et al.*, 2000; Ravindran *et al.*, 2008).
In addition, intrinsic hyphal cell wall lysis could also result in leakage of BiP1 molecules into the culture medium (Pimpl et al., 2006). However this is unlikely in our case where the hyphae examined were only 24 h-old.

6.3.2.3 Visualisation of the ER structure in the transformant BV47 expressing the BiP1-Venus fusion protein

Live cell imaging

For visualisation of the ER, the fluorescence emitted by the BiP1-Venus was first observed in the live BV47 transformant by CLSM. Live cell imaging of the BV47 transformant showed a high level of fluorescence indicative of strong expression of the VenusYFP inside the fungal hyphae grown in the CLS medium for 24 h (Fig. 6-4A). The BiP1-Venus fluorescence was seen intracellularly in a punctate pattern and as short filamentous structures in addition to a small amount of the expected distribution of tubular membranes as what has been reported in filamentous fungi by fusing GFP to different ER-resident protein sequences (Fernández-Ábalos et al., 1998; Wedlich-Söldner et al., 2002; Maruyama et al., 2006). No typical extensively interconnected ER network was observed in the BV47 hyphae (Fig. 6-4A). No VenusYFP fluorescence was detected in the nontransformant Rut C-30 (Fig. 6-4B). Similar spherical fluorescence inclusions representing the ER structures have also been found in the yeast Pichia pastoris (Rossanese et al., 1999) and Saccharomyces cerevisiae where the term “BiP bodies” (Nishikawa et al., 1994) was used to refer to the BiP-enriched ER sub-compartments.
**Figure 6-4** Visualisation of the ER in the living transformant BV47 and nontransformant Rut C-30 by CLSM. Live cell imaging of BV47 (A) and Rut C-30 (B) grown in the CLS medium (pH 6.5) for 24 h. Staining of living 24 h-old BV47 (C) and Rut C-30 (D) with the ER marker ER-Tracker™ Red. Excitation at 473/559 nm and Emission at 520-
550/560-720 nm were used for viewing VenusYFP and ER-Tracker™ Red, respectively. DIC image was achieved by a 473 nm laser. Scale bars represent 5 µm.

Subsequently, dual-labelling with the ER marker ER-Tracker™ Red was performed in the living transformant BV47 and the nontransformant Rut C-30 in order to examine the ER structures in more detail. The staining of the ER using the ER-Tracker™ Red in both the transformant BV47 and nontransformant Rut C-30 displayed an interconnected network sprinkled with some punctate fluorescence throughout the hyphae (Fig. 6-4C and D). When superimposed with the staining with the ER-Tracker™ Red, the fluorescence of the BiP1-Venus fusion protein showed no obvious colocalisation in the BV47 (Fig. 6-4C). The above results suggested that the morphology of the ER in BV47 appear as punctate-like structures different from the typical reticulum network (discussed in the next section).

**IF and IEM analysis**

The morphology of the ER in BV47 and Rut C-30 was further investigated by immunolabelling using both the anti-GFP antibody and anti-BiP antibody at the CLSM (Fig. 6-5) and EM levels (Fig. 6-6) to correlate and confirm the findings from live cell imaging.

Analysis of the morphology of the immunofluorescence staining of semi-thin resin-embedded sections using anti-BiP antibody demonstrated staining in a filament-like pattern and some punctate staining throughout the hyphae in the nontransformant strain Rut C-30 (Fig. 6-5B). There was no GFP antibody staining visible in the nontransformant Rut C-30 (Fig. 6-5B). In contrast, the transformant BV47 showed more abundant punctate labelling with very few filament-like structures using the anti-BiP antibody (Fig. 6-5A). Double-staining of BV47 sections with the anti-BiP and anti-GFP antibodies showed a high level of colocalisation of the two antibodies (Fig. 6-5A) suggesting BiP1-Venus was in a fusion form. These results confirmed that the apparent change in the ER morphology with the BiP1-Venus fusion protein escape from the conventional ER network in the transformant BV47 compared to that in the nontransformant Rut C-30. The “aggregated” BiP in the “BiP bodies” caused by overexpression may have been further externalised/secreted from the hyphae into the culture medium as indicated by Western blotting (Fig. 6-3A) thereby releasing the ER stress caused by protein overexpression.
Immunofluorescence staining of the semi-thin resin-embedded sections of BV47 (A) and Rut C-30 (B) with the anti-BiP and anti-GFP antibodies. All cells were grown in the CLS medium (pH 6.5) for 24 h. The secondary antibodies for the GFP antibody and BiP antibody were Alexa Fluor® 488 and Alexa Fluor® 546, respectively. Scale bars represent 10 µm.

Figure 6-5 Immunofluorescence staining of the semi-thin resin-embedded sections of BV47 (A) and Rut C-30 (B) with the anti-BiP and anti-GFP antibodies. All cells were grown in the CLS medium (pH 6.5) for 24 h. The secondary antibodies for the GFP antibody and BiP antibody were Alexa Fluor® 488 and Alexa Fluor® 546, respectively. Scale bars represent 10 µm.

To improve the resolution of the transformed ER structures, analysis of the modified ER structures was extended by localising the fusion protein using TEM and IEM. Ultrastructural investigation of sections of the nontransformant Rut C-30 revealed abundant reticular ER membranes and often these membranes appeared as stacks exhibiting numerous smooth and parallel profiles throughout the hyphae (Fig. 6-6D1). In contrast, sections of the recombinant strain BV47 demonstrated a relative lack of membrane stacks with predominantly single or disrupted membrane patterns in the ER (Fig. 6-6A). Other cellular structures and components including mitochondria (M), cell wall (CW) and vacuoles (V) were also visualised in the hyphae (Fig. 6-6A-E).
Figure 6-6 IEM of the transformant BV47 and nontransformant Rut C-30. A, B and C, BV47; D1, D2 and E, Rut C-3. A, D1 and D2, anti-BiP antibody staining; B and E, anti-GFP antibody staining. C, double staining with the anti-BiP and anti-GFP. The secondary antibodies for GFP antibody and BiP antibody were conjugated with gold particles sized 5 nm and 10 nm, respectively. Cells were grown in the CLS medium (pH 6.5) for 24 h. CW: cell wall, ER:

When probed with the anti-BiP antibody, the sections demonstrated rich gold staining on the membrane stacks in Rut C-30 (Fig. 6-6D1) while much less gold labelling was visible on the ER membranes in the BV47 transformant (arrowhead in Fig. 6-6A). Corresponding well to the fluorescence pattern seen in the BV47 with CLSM (Fig. 6-4A and 6-5A), gold labelling of the BiP antibody was also localised to punctate-like “bodies” in BV47 (arrow in Fig. 6-6A). In most cases, these structures were surrounded with electron-lucent membranes approximately 90-160 nm in diameter (Fig. 6-6A and C). Similar “bodies” stained with the BiP antibody were also seen in Rut C-30 but at a lower level (arrow in Fig. 6-6D2). In addition, the BiP antibody labelling was found sparsely in the cytoplasm of BV47 and also in Rut C-30 but with more abundant amount (stars in Fig. 6-6A, C, D1 and D2) suggesting translocation of the overexpressed/misfolded proteins, e.g. BiP1-Venus in BV47 from the ER to the cytosol, maybe by activation of ERAD (ER associated protein degradation) (Pimpl et al., 2006). There was no evidence of localisation of BiP1 or BiP1-Venus in the cell wall as seen with the secreted endogenous protein celllobiohydrolase I (CBHI) and heterologous calf chymosin in T. reesei (Nykänen, 2002) indicating their complete externalisation from the hyphae.

In comparison to the staining pattern revealed by the BiP antibody, immunogold labelling with the GFP antibody showed strong localisation in heavily labelled punctate structures (Fig. 6-6B). This punctate staining pattern was further confirmed by using the anti-GFP primary antibody in BV47 (Fig. 6-6B). Double labelling with the anti-BiP and anti-GFP primary antibodies showed BiP1 and VenusYFP were mainly colocalised in punctate, membrane-bound structures in the transformant BV47 overexpressing the fusion protein (Fig. 6-6C) confirming again that the BiP1-Venus was in a fusion form. No significant gold labelling was detected when the nontransformant strain Rut C-30 cells were treated with the anti-GFP primary antibody (Fig. 6-6E).

High level of expression due to the use of strong inducible promoters and high gene copy numbers may lead to overloading of the secretory pathway (Sagt et al., 1998) and saturation of the protein production and processing capacity in the ER (Hsu and Betenbaugh, 1997). Rut C-30
is a high cellulase-secreting mutant originating from the *T. reesei* wild type QM6a (Eveleigh and Montenecourt, 1979) and has a high content of ER, which has been proposed to relate to its high cellulase secretion capacity (Ghosh *et al.*, 1982). In Rut C-30, the high expression levels of cellulases may exceed the protein processing capacity thus inflicting ER stress, resulting in the increase of expression of molecular chaperone proteins such as BiP1 by the activation of unfolded protein response (UPR) (Vattemi *et al.*, 2004; Duriez *et al.*, 2008). This activation is coupled to a redistribution of BiP to the cytosol (Rao *et al.*, 2002). Thus, we could assume that overexpression of the BiP1-Venus protein triggered the activation of UPR through BiP relocation to the cytosol in *T. reesei*, therefore reinforcing the fact that BiP plays an active role in the quality control within the secretory pathway. Although the majority of the ER in Rut C-30 appeared non-disturbed and consisted of tubular membranes, some ER dilation was visible as punctate-like bodies (arrow in Fig. 6-6D2). These bodies resembled Russel body-like dilated ER structures in yeast, noted by Umebayashi *et al.* (1998), formed as a result of accumulation of misfolded protein aggregates.

On top of the ER stress caused by hypersecretion of cellulases in the host strain Rut C-30, overproduction of the recombinant BiP1-Venus fusion protein in the transformant BV47 carrying two copies of the gene fusion expressed under the strong cbh1 promoter (Paloheimo *et al.*, 1993; Nevalainen *et al.*, 2005) may further contribute to ER stress and result in an overflow of the protein folding machinery. BiP relocation to the cytosol and the considerable morphological changes in the ER characterised as BiP “bodies” could be a natural response to the burden loaded to the ER. This type of modification of the ER may assist in forming an ER sub-domain to which overproduced and potentially misfolded proteins can be deposited to wait for further processing.

**6.3.2.4 Intracellular localisation of BiP1-Venus in the *T. reesei* transformant BV47**

The investigation into subcellular localisation of the BiP1-Venus fusion protein in BV47 was conducted in the same manner to visualisation of the ER structures in the same strain, discussed above. This included live cell imaging and immuno-labelling studies by confocal microscopy and TEM (see below).
Live cell imaging of the transformant BV47 by co-staining with the membrane stain FM® 4-64 and the Golgi marker BODIPY® TR ceramide

The occurrence of a number of punctate BiP “bodies” observed in BV47 prompted us to characterise the subcellular localisation of the BiP1-Venus fusion protein to clarify whether the fusion protein localised in the vesicles which also appeared as spherical structures or in the putative punctate-like Golgi. Some round structures, assued as vesicles, could be seen in the DIC image of the live BV47 (Fig. 6-4A). They could be any type of vesicles, i.e. transport vesicles, secretory vesicles, etc. as light microscopy is not able to resolve these structures. In the merged image of BiP1-Venus fluorescence and DIC, it was clear that the fluorescence was not associated with these vesicles (Fig. 6-4A). In order to confirm the fusion protein did not localise in the vesicles, a membrane marker FM® 4-64 was used to stain live BV47 to investigate the colocalisation (Fig. 6-7A). The result showed that there was no or very little presence of the VenusYFP fluorescence inside the vesicle whose membranes were stained with FM® 4-64 indicating that the recombinant protein was not likely to have localised inside the vesicles in BV47 (Fig. 6-7A). Compared to the typical size of secretory vesicles (50-80 nm in diametre) (Aridor and Balch, 1996), the “BiP bodies” (punctate structures) described in this work (90-160 nm in diametre) were considerable larger, suggesting these structures are likely to represent an ER-derived sub-compartment rather than customary secretory vesicles. This also implied that BiP secretion as determined by immunoblotting may have been dependent on the conventional vesicular transport machinery.
Figure 6-7 Colocalisation of the BiP1-Venus fusion protein with the membrane stain FM® 4-64 (A) and the Golgi marker BODIPY® TR ceramide (B) in the living transformant BV47. Cells were grown in the CLS medium (pH 6.5) for 24 h. A, Live BV47 stained with the Golgi stain BODIPY® TR ceramide. Scale bars represent 5 µm.
Colocalisation of BiP1-Venus fluorescence with the Golgi stain BODIPY® TR ceramide was also visualised in the living hyphae of BV47. The staining pattern of the Golgi with BODIPY® TR ceramide appeared as punctates in the hyphae of BV47 which resembled the fluorescence pattern of BiP1-Venus (Fig. 6-7B). However, no obvious colocalisation was visualised between the staining of BODIPY® TR ceramide and BiP1-Venus fluorescence indicated the escaped BiP1-Venus was not likely to have localised in the Golgi.

**Immunolabelling**

Subsequent immunofluorescence staining of the semi-thin sections of hyphae showed no obvious colocalisation between the staining of the anti-GFP and the anti-Golgi bodies (Fig. 6-8A), therefore being consistent with the results by costaining of the live BV47 with the Golgi marker BODIPY® TR ceramide where colocalisation was not apparent either. These data strongly suggested that BiP1-Venus hardly appeared in the Golgi (Fig. 6-7B). The evidence provided by IEM study further confirmed the “BiP bodies” (thick arrows in Fig. 6-8B) localised away from the Golgi apparatus (thin arrows in Fig. 6-8B) and the gold staining pattern of the GFP-antibody was obviously different from that of the Golgi-antibody in both BV47 (Fig. 6-8B) and Rut C-30 (thin arrow in Fig. 6-8C).

**Figure 6-8** Immunolabelling of the BiP1-Venus fusion protein using the anti-Vps10p and anti-GFP antibodies by CLSM and IEM (see next page). A, Immunofluorescence staining of semi-thin resin-embedded sections (1 μm thick) of BV47. The secondary antibodies against the anti-GFP and anti-Vps10p were tagged with FITC and Alexa Fluor® 546 and imaged at excitation of 488/543 nm and at emission of 500-560/560-660 nm, respectively. DIC images were achieved by a 488 nm laser. Scale bars represent 20 μm. Double staining of the ultra-thin sections of BV47 (B) and Rut C-30 (C) with the anti-Golgi (represented by 20-nm-gold particles) and anti-GFP (represented by 10-nm-gold particles). CW: cell wall, ER: endoplasmic reticulum, M: mitochondria. Thick arrows represent ER-derived punctate-like “BiP bodies” labelled by the anti-GFP antibody and thin arrows represent the Vps10p antibody labelling.
The size of the single Golgi profile in *T. reesei* was about 10-60 nm (described in Section 6.4.2), therefore it is not surprising that the Golgi labelling using 20 nm gold particles appeared as discrete grains of gold. HDEL-containing proteins such as BiP1 recycle between the ER and the Golgi (Dean and Pelham, 1990). Our data that the BiP1-Venus fusion protein was not found in the Golgi suggests that, at this sampling time point of 24 h, the amount of the fusion protein in the Golgi was beyond the detection limit or the fusion protein may have already left the Golgi.

### 6.3.3 Conclusions

The BiP1-Venus fusion protein construct was initially designed for FRET study and was further used as an ER probe in this work to allow visualisation of the ER architecture in *T. reesei*. However, in contrast to expectations, images from CLSM and IEM showed that, the ER structure in the transformant BV47 appeared unusual with an abundance of punctate structures and fewer tubular membranes demonstrating modified spatial organisation of the ER, different to other filamentous fungi studied so far. On the other hand, the nontransformant Rut C-30 demonstrated
that the morphology of the ER was characterised by a typical network of parallel tubular membranes and some punctate-like bodies through the hyphae corresponding to what has been reported previously. The ER structural modifications in BV47 appeared to have been caused by overproduction of the BiP1-Venus fusion protein and these findings may contribute and help better understand the biology of the secretion of heterologous proteins in T. reesei. For example, overexpression of heterologous proteins can cause ER stress resulting in saturation of ER retention capability and here BiP1 had escaped from the ER lumen forming so-called “BiP bodies” to provide a sub-ER domain to process overexpressed (recombinant) protein. BiP was also directed for secretion possibly to alleviate ER stress. In addition, overexpression seems to have affected recycling of BiP from the Golgi to the ER since no fusion protein was found localised to the Golgi. The finding that the BiP1-Venus fusion protein was absent in the vesicles suggested the relocalisation of BiP could be independent of traditional vesicular transport along the secretory pathway.

The use of a transformant with one copy of the bip1-venus DNA integrated in the genome may have circumvented the problem of overexpression and its effects on the ER. Also, expression of the fusion construct under a native bip1 promoter instead of the strong cbh1 promoter may have eased the burden at least to some extent. However, since the ER-resident molecular chaperone proteins are generally expressed at low levels in all cells (Gething and Sambrook, 1992), this may have compromised acquiring the amount of signal required for efficient imaging. Even though it might be of interest to carry out further studies into the natural ER organisation by expressing a single copy of the BiP1-Venus fusion protein at the endogenous locus under the native bip promoter, this arrangement may invite additional problems. Therefore, even though when not an optimal solution, expression of the bip1-venus DNA in T. reesei clearly was efficient in lighting up the ER and opened some interesting vistas into the modifications to the ER upon protein overproduction.

6.4 Visualisation of the Golgi apparatus

6.4.1 Introduction

From the ER, secretory proteins proceed to the Golgi for further post-transcriptional modifications before they are sent to their intended destinations (Breakspear et al., 2007). Despite the conservation in the secretory machinery, physical organisation of the Golgi apparatus differs
enormously across kingdoms (daSilva et al., 2004). Although the classical dictyosome organisation of the Golgi compartment is not commonly found in filamentous fungi (Markam, 1994), typical Golgi-associated functions are present in the fungal cell and the term Golgi-like structures, Golgi equivalents, or dictyosomes, is normally used (Conesa et al., 2001). So far, very limited data are available about the morphological architecture of the Golgi apparatus in filamentous fungi. As described in Section 1.3.2, the morphology of the Golgi in filamentous fungi varies between strains and different formations have been reported in T. reesei based on different experimental setups (Ghosh et al., 1990; Nykänen, 2002; Kuratsu et al., 2007). Therefore, there is a need for further visualising of the Golgi apparatus in T. reesei in order to explore protein processing and secretion in this industrially-exploited fungus.

6.4.2 Results and discussion
In earlier work (Section 6.2), a fluorescent Golgi marker BODIPY® FL C-5 ceramide was used to co-stain T. reesei Rut C-30 with FM® 4-64 but it was found that staining was not specific to the Golgi structure as it appeared as an interconnected network throughout the hyphae which was assumed to be ER membranes (Fig. 6-1C). Thus, two other commonly-used chemical fluorescent Golgi probes, BODIPY® FL C-5 sphingomyelin and BODIPY® TR ceramide were applied here to fluorescently stain the Golgi apparatus in the living T. reesei Rut C-30 strain for visualising the Golgi morphology in T. reesei. After incubation with BODIPY® FL C-5 sphingomyelin (2.5 μg/mL) at 28 °C for 1 h, the staining pattern of the Golgi apparatus was characterised by distinct punctate bodies seen throughout the cytoplasm (Fig. 6-9A). When stained with BODIPY® TR ceramide under the same conditions, some elongated structures (arrow in Fig. 6-9B) were observed in addition to fluorescent spherical dots similar to that seen by BODIPY® FL C-5 sphingomyelin (arrowhead in Fig. 6-9B). Further immunofluorescence staining of semi-thin sections embedded in resin was conducted using a Golgi-specific antibody, the anti-Vps10p antibody (Fig. 6-9C). The confocal images indicated a similar punctate staining pattern through the hyphae as that was seen in the chemical staining experiments (Fig. 6-9A).

Figure 6-9 Staining of the Golgi apparatus in living T. reesei Rut C-30 grown in the CLS medium (pH 6.5) (see next page). A, BODIPY® FL C-5 Sphingomyelin. B, BODIPY® TR ceramide. C, Indirect immunofluorescence staining of 24 h-old resin-embedded sections probed with the anti-Golgi antibodies (the secondary antibody was conjugated with Alexa Fluor® 546 nm fluorophore). D, IEM of 24 h-old Rut C-30 probed with the anti-Golgi antibodies conjugated with 10 nm gold particles. E, Negative control for IEM experiment (no anti-Vps10p antibody was
applied). Arrow and arrowhead point to punctate and elongated staining structures, respectively. Abbreviations: ER-endoplasmic reticulum, M-mitochondria, CW-cell wall.
Closer examination of the Golgi apparatus was carried out at the EM level using the same anti-Vps10p antibody for comparison with the immunofluorescence studies. IEM results revealed that the Golgi apparatus was organised in spherical bodies with no apparent surrounding membrane (see gold particle clusters in Fig. 6-9D). The size of the single profile of Golgi varied between 10-60 nm in diameter. However, it was not possible to estimate the size of the total Golgi body due to the lack of serial sectioning data. There was no obvious subcellular distribution difference of these Golgi bodies between the distal tip and the sub-apical portion in the hyphae. The spherical gold-labelled bodies detected by IEM are in line with the fluorescent punctate bodies stained with BODIPY® FL C-5 sphingomyelin and BODIPY® TR ceramide in the living Rut C-30 (Fig. 6-9A and B respectively). Further, a similar distribution of the punctate-like Golgi bodies was seen in resin-embedded sections stained with the Vps10p antibody as studied by indirect immunofluorescence analysis using CLSM (Fig. 6-9C). These results showed that the T. reesei Golgi was punctate in shape and randomly distributed throughout the hyphal cytoplasm without polarised localisation pattern of the Golgi concentrated at the tip region previously reported in the hyphae of Candida albicans (Rida et al., 2006).

These punctate-like Golgi bodies have also been found in other fungi such as P. tinctorius (Cole et al., 2000), A. oryzae (Akao et al., 2006; Kuratsu et al., 2007), A. nidulans (Breakspear et al., 2007), Laetisaria arval (Hoch and Howard, 1980), Sclerotium rolfsii (Roberson and Fuller, 1986) and Allomyces macrogynus (Sewall et al., 1989). It is noted that these data concerning the punctate-like Golgi structures in various fungi were mainly based on ultrastructural observations without specific probing (Hoch and Howard, 1980; Roberson and Fuller, 1986; Sewall et al., 1989; Cole et al., 2000) with a few exceptions where GFP was fused to Golgi-resident proteins i.e. CopA (Breakspear et al., 2007), SNAREs (Kuratsu et al., 2007) and 1,2-alpha-mannosidase (FmanIBp) (Akao et al., 2006). Among these studies, Cole et al., combined BODIPY® FL C-5 sphingomyelin staining of P. tinctorius and ultrastructural investigation at the EM level to visualise the Golgi organisation (Cole et al., 2000). In T. reesei, published studies on the Golgi morphology have also been carried out mainly at the ultrastructural EM level and have shown various formations considered as the Golgi body. For example, a semicircle Golgi-equivalent has been described in the Rut C-30 hyphae (Nykänen, 2002). In addition, electron-dense vesicles have been detected in T. reesei Rut C-30 that were supposed to fulfil the processing role of the Golgi complex (Kurzatkowski et al., 1993). However, Ghosh et al. (1990) claimed that there
were no characteristic Golgi structures in either the wild type QM6a or the high protein secreting Rut C-30 strains (Ghosh et al., 1990; Kurzatkowski et al., 1993). This claim was based on the notion that conventional stacked Golgi organelles were not detected in *T. reesei* (Gould et al., 1992) but were seen in *S. cerevisiae* (Preuss et al., 1991; Rambourg et al., 1993) and *Pichia pastoris* (Gould et al., 1992). The morphology of the Golgi apparatus in *T. reesei* appears not strain-specific since different organisation patterns have been seen in the same strain Rut C-30 as discussed above and further no staining pattern difference was observed between the host strain Rut C-30 (Fig. 6-9D) and the transformant CV48 expressing CBHI-Venus in this project (Fig. 7-5F). It is likely that sample preparation, staining or observation methods may affect the appearance of the Golgi. Our IEM findings showing the punctate-like Golgi structures using a Golgi-specific antibody will contribute to the existing information on the Golgi morphology of filamentous fungi.

Synthesised proteins destined for secretion are trafficked along the secretory pathway from the ER to Golgi for further transcriptional modifications via COP-coated vesicle fusion machinery (discussed in Section 1.2.4), therefore the function of the Golgi depends on membrane trafficking to and from the ER (Ward et al., 2001). Thus, our IEM findings that no obvious surrounding membrane was seen for the Golgi structures using the antibody raised against the yeast Vps10p (a late-Golgi trans-membrane protein) is unusual since functions associated with Golgi are present in the fungal cell (Conesa et al., 2001). The apparent lack of Golgi membranes was possibly a result of the method used for sample preparation i.e. fixation or embedding. The ultrastructure-friendly freeze-substitution technique was not used in our sample preparation. In the studies where this technique has been applied, the Golgi appeared as electron-dense structures with possible, yet hazy, poorly defined membrane envelopes (Hoch and Howard, 1980; Roberson and Fuller, 1986; Sewall et al., 1989; Cole et al., 2000; Nykänen et al., 2002). In our case, the fine membrane structures were unlike to have been preserved because of the fixation method (4 % 4 % paraformaldehyde and 1 % glutaraldehyde, details in Section 2.15) applied for IEM. The protocol did not include higher concentration of aldehydes and osmium tetroxide for post fixation which favour membrane preservation (Hayat, 2000).

The punctate-like staining pattern of Golgi by the chemical dyes (Fig. 6-9A and B) agrees well with that was seen by immunostaining (Fig. 6-9C and D). The commercially available fluorescent
Golgi markers BODIPY® FL C-5 sphingomyelin and BODIPY® ceramide can be used for labelling of the Golgi apparatus in living *T. reesei* and the former is better than the latter in terms of specificity as BODIPY® TR ceramide may also stain ER membranes (Cole et al., 2000) (arrow in Fig. 6-9B). However, one would still be able to differentiate the Golgi apparatus from other organelles by its punctate staining pattern suggesting the BODIPY® TR ceramide is also useful for the localisation of the Golgi. This is particularly true for co-staining with green fluorescent probes e.g. GFP because the BODIPY® TR ceramide emits red fluorescence whereas BODIPY® FL C-5 sphingomyelin emits green fluorescence therefore causing an overlapping problem in the dual staining work with green fluorophores. This optical advantage of the BODIPY® TR ceramide was also used in the current study for the colocalisation studies with VenusYFP in both the transformants BV47 (Fig. 6-7B) and CV48 (Fig. 7-5B).

Antibodies against the Golgi-specific proteins such as Vps10p (Nothwehr et al., 2000), YPT1 (Segev et al., 1988), SEC7 (Franzusoff et al., 1991; Rossanese et al., 1999), KEX2 (Redding et al., 1991), SEC14 (Cleves et al., 1991), RHO1 (McCaffrey et al., 1991) and OCH1-HA (Rossanese et al., 1999; Callewaert et al., 2001) have been used to label the Golgi in fungi by immunofluorescence microscopy, but not by EM yet as described in this study. High affinity specificity of antigen-antibody binding allows immunolabelling to be more reliable than chemical staining. This is especially true when antibodies are applied in conjunction with EM studies which provide subcellular ultrastructures of high resolution. However, immunolabelling can only be applied to the fixed specimens but not to living cells. This limitation can be overcome by the strategy of fusion of the genetic material encoding fluorescent proteins such as GFP to the genes encoding Golgi-resident proteins i.e. SNAREs (Kuratsu et al., 2007), CopA (Breakspear et al., 2007) and 1,2-α-mannosidase (Akao et al., 2006). In terms of labelling specificity, expressing the GFP-tagged Golgi-specific constructs is as good as immunostaining. However, this genetic method, compared to the conventional immunolabelling method, is more complicated and the correct expression can be affected by many factors such as the expression promoter, RNA stability and gene copy number.

**6.4.3 Conclusions**

The morphology of the Golgi apparatus in filamentous fungi differs from that in plants and mammalian cells where it appears as classical flat stacks. Our work with light microscopy and
EM has indicated that the Golgi apparatuses in *T. reesei* appeared as punctate bodies. The Golgi membrane invisibility could be associated with the chemical fixation method used in this study which failed to preserve the delicate Golgi membranes. The information obtained on the Golgi morphology has raised some issues on visualisation of the structure of the Golgi body in filamentous fungi such as labelling specificity of different probes and reliability of visualisation methods. To visualise the Golgi in the live fungi, the chemical stain BODIPY® FL C-5 sphingomyelin was more specific than BODIPY® TR ceramide. Immunolabelling using Golgi-specific antibodies, especially when combined with EM, was found to be more reliable in terms of staining specificity compared to chemical labelling, but limited to the fixed samples. The physical existence and visualisation of this important organelle in the secretory pathway provides an additional tool for studies addressing protein secretion through the secretory pathway. This is the first study to attempt labelling the Golgi in filamentous fungi by immunostaining.
Chapter 7 Tracking secretion of the cellobiohydrolase I (CBHI)-Venus fusion protein through the hyphae of *Trichoderma reesei*

7.1 Introduction

Cellobiohydrolase I (CBHI; Cel7A,) is the major secreted cellulase in *T. reesei* (described in Section 1.1.3). It accounts for about 60\% of the total amount of the secreted proteins by *T. reesei* therefore serving as an excellent model for studying efficient protein secretion (Kubicek *et al.*, 1993a; Pakula *et al.*, 2000; Pakula *et al.*, 2003). The subcellular location and trafficking of CBHI has been studied previously by indirect immunofluorescence microscopy and IEM (Nykänen *et al.*, 1997; Nykänen, 2002) as well as by immunocytochemistry and enzyme cytochemistry (Chapman *et al.*, 1983). Pakula *et al.* (2000) investigated the kinetics of CBHI production *in vivo* with 2D analysis of the metabolically labelled CBHI protein allowing monitoring of protein maturation in the secretory pathway (Pakula *et al.*, 2000). Maturation, processing and secretion of CBHI have also been studied under the conditions where secretion process was interfered by treating *T. reesei* with chemical drugs, i.e. dithiothreitol (DTT) to inhibit the intrachain disulphide bond formation during the folding of proteins (Pakula *et al.*, 2000), brefeldin A (BFA) to inhibit protein transport and the Ca\(^{2+}\)-ionophore A23187 to inhibit protein synthesis (Pakula *et al.*, 2003). In general, the above studies have provided considerable information on protein secretion from morphological, biochemical and molecular biological points of view. However, “visible” data on protein production and localisation *in vivo* has remained limited.

The application of fluorescent proteins such as green fluorescent protein (GFP) as molecular tags has enabled the visualisation of various proteins in their natural environment (Broekhuijsen *et al.*, 1993; Archer *et al.*, 1994; Gouka *et al.*, 1997b; Gordon *et al.*, 2000b; Schwartz and Patterson, 2003). In these studies, GFP has been proven to be an excellent fusion partner for proteins of interest as it rarely affects the function and localisation of the fusion protein (Reits and Neefjes, 2001). In addition, GFP does not pose a large metabolic burden on the cells in terms of growth rate (Cha *et al.*, 2000) and is generally believed to be non-toxic to living cells (Alexander *et al.*, 1997) although cytotoxicity of GFP has been reported in the NIH/3T3, BHK-21, Huh-7, and HepG2 cell lines inducing apoptosis (Liu *et al.*, 1999). Venus fluorescent protein VenusYFP, developed from GFP, matures more efficiently and faster than GFP (Nagai *et al.*, 2002).
Although the secretion of CBHI in *T. reesei* has been studied using different techniques, the dynamic process of protein secretion *in vivo*, however, has not been fully characterised. In the current chapter, the transformant strain CV48 expressing a CBHI-Venus fusion protein was used to track progress of CBHI through the secretory pathway *in vivo* over a time course of 120 h using confocal fluorescence microscopy and other methods. Samples of cells and/or culture supernatants collected at 12, 18, 24, 48, 72, 96 and 120 h were measured for expression of the CBHI-Venus fusion protein by mRNA transcript analysis (Section 7.2.2.1), direct imaging of the fluorescence emitted by VenusYFP (Section 7.2.2.2) and Western blotting (Section 7.2.2.3). After the detection of VenusYFP fluorescence at the 24 h time point and establishment of an apparent association with cellular organelles at the time points from 24 and 48 h, the subcellular localisation of CBHI-Venus was subsequently carried out by IEM with hyphae cultured for 24 and 48 h (Section 7.2.2.4). Further colocalisation studies with some organelle-specific fluorescent dyes using CLSM were carried out with 24 h-old live cells (Section 7.2.2.5), which showed sufficient fluorescence signals of VenusYFP as well as active association with the major secretion organelles. Following the above intracellular investigations into expression of CBHI-Venus in the hyphae, the dynamics of secretion of the fusion protein into the culture medium was monitored over a time course of 120 h (Section 7.2.3).

### 7.2 Results and discussion

#### 7.2.1 Expression vector pCVt and the *T. reesei* transformant CV48

An expression plasmid pCVt coding for the CBHI-Venus fusion protein, initially created for the FRET study to address potential interaction between the ER-resident chaperone BiP1 and CBHI (Tables 2-3 and 3-2), was applied here for tracking secretion of the main cellulbiohydrolase I (CBHI) in *T. reesei* hyphae. As described in Section 2.5, the pCVt vector contained the *venus* gene fused to the C-terminus of the gene coding for the CBHI core under the *cbh1* promoter followed by the *cbh1* signal sequence (ss) (Fig. 2-1). This expression plasmid was introduced into *T. reesei* Rut C-30 and a transformant strain CV48 expressing strong VenusYFP fluorescence was obtained (refer to Fig. 3-2A). The transformant CV48 contained at least two copies of the *cbh1-venus* fusion gene ectopically integrated into the genomic DNA (Fig. 3-4B).

Native *T. reesei* contains one copy of the *cbh1* gene (Ilmén *et al.*, 1997) and the best strategy to track CBHI secretion would be with a transformant containing one copy of the fusion construct.
integrated into the endogenous *cbh1* locus. However, even if such strains were available, they have suffered from instability and occasional ‘disappearance’ of the fluorescent signal from the hyphae (unpublished observations). Therefore, the CV48 expressing strong fluorescence was considered suitable for the tracking of CBHI secretion.

### 7.2.2 Intracellular aspects of synthesis of the CBHI-Venus fusion protein in *T. reesei*

#### 7.2.2.1 Expression of the CBHI-Venus fusion protein as determined by transcript levels over time

The relative expression levels of total *cbh1* and *cbh1-venus* transcripts in the transformant CV48 were examined by quantitative real time PCR over a time period of 120 h (Fig. 7-1). As the total expression of *cbh1* in CV48 was attributed to expression of both the endogenous *cbh1* gene and the *cbh1-venus* fusion DNA, the relative intensities of the *cbh1* mRNA transcripts of the endogenous *cbh1* gene in the transformant strain CV48 were roughly accounted by subtracting the relative expression value of *cbh1-venus* value from that of the total *cbh1* (Fig. 7-2). As a result, the *cbh1-venus* mRNA levels in CV48 were found greater than that of the endogenous *cbh1* transcripts at the early culture stages from 12 to 48 h (1.9-6.4 fold) but decreased after 72 h (0.23-0.48 fold).

As shown in Fig. 7-1, the *cbh1-venus* message was detectable at a low level at 12 h and increased slightly by 24 h of cultivation. The level of the *cbh1-venus* transcript appeared to peak at 48 h and decrease clearly after 72 h of cultivation. In contrast to the expression pattern of the fusion *cbh1-venus* transcripts over time, the amount of the native *cbh1* transcripts were lower at early culture stages from 12 to 24 h but increased considerably by 48 h and peaked at 96 h. At 120 h, the expression level of the native *cbh1* transcript decreased but still remained at a considerably high level.
Relative expression of cbh1-venus and cbh1 mRNAs in the total RNA of the transformant CV48 measured by real-time RT-PCR. Values were normalised to the housekeeping actin mRNA and average values of duplicate real-time PCR runs are shown. The “native cbh1 mRNA” represents the calculated results by subtracting the relative expression value of cbh1-venus value from that of the total cbh1.

Because CV48 had at least two copies of the cbh1-venus expression DNA (Fig. 3-4B) while the endogenous cbh1 is present as a single copy, it was expected to see higher amount of the cbh1-venus mRNA than the endogenous cbh1 transcripts as was the case. This finding also implied that the mRNA transcripts of the cbh1-venus fusion gene would have similar stability to that of the native message from the cbh1 gene in CV48. However, the expression of the cbh1-venus transcripts seemed to have been suppressed at the later cultivation stages after 72 h possibly due to transcriptional repression through the UPR triggered by overexpression of the CBHI-Venus fusion protein and subsequent ER stress. This phenomenon, also known as RESS (repression under secretion stress) (Nevalainen et al., 2005; Guillemette et al., 2007; Gasser et al., 2008), decreases the biosynthetic burden of the secretory pathway by downregulating expression of genes encoding secreted proteins (reviewed by (Schröder and Kaufman, 2005).

7.2.2.2 Visualisation of intracellular fluorescence of CBHI-Venus in the living fungal hyphae by CLSM

The development of fluorescence inside the hyphae by expression of CBHI-Venus in the transformant CV48 grown in the CLS medium (pH 6.5) was monitored over time by CLSM (Fig.
The fluorescence of VenusYFP was faint but discernible with diffuse distribution throughout the hyphae at 12 h of growth (Fig. 7-2A1) and became much brighter at the later stages. At 18 and 24 h, the fluorescence appeared as a filamentous network assumed to represent the ER membranes indicating apparent association of the fusion protein with the ER, which plays an important role in protein folding, modification and quality control. Meanwhile, sparsely-distributed fluorescent spots and bright fluorescence at the septa were also visible (Fig. 7-2B1 and C1). After 48 h, the CBHI-Venus started localising into discrete rounded spots resulting in the emergence of numerous fluorescent spots of high intensity (Fig. 7-2D1, E1, F1 and G1) suggesting the fusion protein was packed into secretory vesicles for transport. These fluorescent vesicles seemed to fuse to vacuoles and formation of vacuoles was especially obvious at a late stage of 120 h (Fig. 7-2G1). The formation of vacuoles could be a natural response to heterologous protein production in T. reesei as previously reported in yeast (Holkeri and Makarow, 1998). Although there is evidence that macromolecules may be directed to the vacuole via receptor-mediated endocytosis in yeast (Weisman and Wickner, 1988; Dulic and Riezman, 1989), the manner in which the recombinantly expressed CBHI-Venus was targeted from the vesicles to the vacuoles remains unclear. Fluorescence became visible in the cell wall by 72 h (Fig. 7-2E1) and became more intense at the late growth stages of 96 and 120 h (Fig. 7-2F1 and G1) indicating the fusion protein was trapped in the cell wall. A possible explanation for the cell wall retention is that the cell wall polymers rigidify in the old hyphae which makes the cell wall less penetrable.

**Figure 7-2** CLSM of CBHI-Venus expression in the CV48 transformant over a time course of 120 h (see next page). Cells were grown in the CLS medium (pH 6.5). The nontransformant Rut C-30 was imaged as comparison. Images were taken in VenusYFP channel (1) with ex/em of 473 nm/530-550 nm and DIC channel (2) also with a 473 nm laser and merged (3). Bar=5 μm in all images.
Generally, distribution of fluorescence between apical and non-apical cells was found to be very similar suggesting the *cbh1* promoter is active in all cells through the hyphae and the *cbh1* signal sequence does not target the protein to the hyphal apex only but also to the cell wall of the sub-
apical cells. This equal distribution of a recombinant protein has been observed previously for the heterologous protein barley cysteine Endopeptidase B produced in the *T. reesei* (Nykänen *et al.*, 1997). The lack of spatial bias of the fluorescence provides additional support to the view proposed by Nykänen *et al.* (2002) that protein secretion does not occur solely from the hyphal tips.

### 7.2.2.3 Intracellular expression of the CBHI-Venus fusion protein analysed by Western blotting

Progression of the CBHI-Venus fusion protein inside the hyphae was analysed by Western blotting with cell extracts from CV48 grown in the CLS medium over a time course of 120 h, using a polyclonal anti-GFP antibody and a monoclonal anti-CBHI antibody (details in Section 2.13 and Table 2-4). In the earlier work (Section 5.2 and Fig. 5-1C), it was discovered that denaturation of the protein samples by boiling caused cleavage at the fusion site of the CBHI-Venus protein produced by CV48. Therefore, both intracellular and extracellular protein samples processed for Western blotting experiments here were incubated with the NuPAGE® 4X LDS Sample Buffer on ice for 5 min without boiling before loading into the gel to keep the fusion protein intact (Section 5.2).

When probed with the anti-GFP antibody, a single band corresponding to the expected size of 73 kDa for CBHI-Venus was visible in the culture supernatants at all time points (Fig. 7-3, top picture), suggesting no cleavage of the fusion protein CBHI-Venus occurred inside the hyphae through the whole time course and the fusion protein remained stable. At 12 h, the CBHI-Venus level was detectable in the hyphae although considerably low. This result was well consistent with the live CLSM findings where weak fluorescence was discernible also at 12 h (Fig. 6-2A1). VenusYFP’s maturation is 1.5 min as measured *in vitro*, which is more efficient and much faster than that of other GFP variants (about 0.5-2 h) (Frieda *et al.*, 2003). Fast maturation of VenusYFP and efficient expression under the *cbh1* promoter allowed immediate detection of the fusion protein which is important for the tracking of protein expression. In our study, the appearance of the *cbh1-venus* transcripts (Fig. 7-1), the emergence of intracellular fluorescence of CBHI-Venus as determined by CLSM (Fig. 7-2A1) and the detection of intracellular CBHI-Venus by Western blotting using GFP antibody (top picture in Fig. 7-3) were all evident at the time point of 12 h. Intracellular expression of CBHI-Venus appeared to increase dramatically at
18 h, then grew gradually and stayed at a considerably high level until 48 h. Protein production declined steadily thereafter and was of faint intensity at 120 h. The declined expression level of CBHI-Venus at the late culture stages from 72 to 120 h may be a result of the decline in the amount of *cbh1-venus* transcripts (Fig. 7-2).

**Figure 7-3** Western analysis of CBHI-Venus extracted from mycelia of the CV48 transformant over a 120 h culture period. Cells were grown in the CLS medium (pH 6.5). Cellular extract samples were incubated with the NuPAGE® 4X LDS Sample Buffer on ice for 5 min and loaded straight into the gel without boiling to keep the fusion protein intact. The blots were probed for VenusYFP using the anti-GFP antibody (top) and anti-CBHI antibody (bottom); 15 µg of total protein was loaded in each lane. The expected size of CBHI-Venus is 73 kDa and that of native CBHI is 46 kDa, as indicated by the arrows.

When using the monoclonal anti-CBHI antibody (bottom picture in Fig. 7-3), fusion protein bands at size of 73 kDa were detected in the cell extracts with similar pattern to that seen with the anti-GFP antibody over the time course (top picture in Fig. 7-3). It was also noted that the bands representing endogenous CBHI at the expected size of 46 kDa were hardly detectable until 72 h and onwards when the signals were tailing off. At 96 h, the endogenous CBHI was at the
maximal level and in line with the transcript synthesis kinetics showing the highest native cbh1 mRNA amount also at 96 h (Fig. 7-1). It was expected that the yield of the fusion protein CBHI-Venus in CV48 was greater than that of endogenous CBHI since this strain contains at least two copies of the cbh1-venus gene and only a single copy of the native cbh1.

7.2.2.4 Subcellular localisation of the CBHI-Venus in CV48 by IEM
A more detailed subcellular localisation of the CBHI-Venus fusion protein in the CV48 transformant was carried out by IEM. In Section 7.2.2.2 and Fig. 7-2, it was shown that, after 48 h, no apparent association of CBHI-Venus with the ER membranes was visualised in living CV48 hyphae. Therefore, only cultures grown in the CLS medium (pH 6.5) for 24 and 48 h were selected for further EM study using the anti-GFP antibody (Fig. 7-4). Due to failure in immunostaining detection using the monoclonal anti-CBHI antibody, the anti-CBHI antibody was disregarded in this work and staining with the anti-GFP antibody was considered reliable to reflect localisation of the CBHI-Venus as it appeared intact on immunoblots both intracellularly and extracellularly (Fig. 7-3 and 7-6).

Characteristic of the ultrastructure of both the recombinant CV48 and the transformation host Rut C-30 was the occurrence of a large amount of normal ER cisternae throughout the hyphae (Fig. 7-4A-E), similar to that seen in T. reesei QM6a (Eveleigh and Montenecourt, 1979), Rut C-30 and recombinant T. reesei expressing barley cysteine endopeptidase B and CBHI (Nykänen et al., 1997). Interestingly, in CV48, the ER membranes seemed extended to some distorted membrane structures detected with a gold label for CBHI-Venus in 24 (arrows in Fig. 7-4A and C) and 48 h-old samples (arrow in Fig. 7-4D) and this distortion became more prominent at 48 h. The morphology of these ER-derived structures varied from elongated spheres (arrows in Fig. 7-4A and D) to fused irregular bodies (arrow in Fig. 7-4C). It was noted that in Rut C-30, no such aberrant ER membranes were seen (Fig. 7-4E).
**Figure 7-4** IEM micrographs showing subcellular localisation of the CBHI-Venus fusion protein in the hyphae of the transformant CV48. The CV48 transformant grown in the CLS medium (pH 6.5) for 24 h (A and C) and 48 h (B and D) was stained with the anti-GFP antibody. A 24 h-old nontransformant strain Rut C-30 treated with the anti-GFP antibody was set up as a control (E). The CV48 transformant grown in the CLS medium (pH 6.5) for 24 h was stained with the anti-Vps10p antibody to demonstrate the morphology of the Golgi in CV48 (F). The secondary antibodies were conjugated with 10 nm diameter gold particles. Arrowheads, arrows, stars and double arrowhead point to gold staining of CBHI-Venus in the normal ER membranes, distorted ER sub-domain, the putative Golgi bodies and vesicle, respectively. CW: cell wall, ER: endoplasmic reticulum, M: mitochondria, N: nuclei.
Protein overexpression can lead to structural changes in the ER (Mullins, 2004). For example, ER dilation in pancreatic beta-cells was caused by overexpression of an islet-specific glucose-6-phosphatase catalytic subunit-related protein (Shameli et al., 2007). Thus, we assumed that the changes of the ER architecture and formation of the ER sub-domains filled with the CBHI-Venus fusion protein in CV48 were likely to be a result of overexpression of the recombinant fusion protein CBHI-Venus. Similar phenomenon was also seen earlier in the transformant BV47 where punctate “BiP bodies” were associated with the overexpression of the BiP1-Venus fusion protein (Section 6.3, Fig. 6-4A, 6-5A, 6-6A-D).

The transformant CV48 contained at least two copies of *venus* gene and therefore the bulk synthesis of recombinant protein may have overloaded the ER mechanism causing ER distortion. As a result, the overproduced CBHI-Venus may have aggregated in the ER-derived structures to await further processing, i.e. being directed to the Golgi for further modification or relocated to vacuoles for degradation. In addition to localisation to the above ER-derived domains, CBHI-Venus was also visualised in the classical tubular ER membranous cisternae at 24 (arrowheads in Fig. 7-4A and C) and 48 h (arrowheads in Fig. 7-4B and D). These data suggested that the ER was actively involved in protein processing and secretion at early the culture of 24 and 48 h.

Gold label was also seen in the vesicles at the 24 and 48 h-old hyphae (double arrowhead in Fig. 7-4B). No label for CBHI-Venus in the cell wall was detected at these time points indicating smooth protein externalisation with no obvious cell wall retention. This result agrees well with the findings by Western blotting of the secretion of CBHI-Venus into the extracellular culture medium where the fusion protein was of high intensity at 24 and 48 h (Fig. 7-6). No or only sporadic labelling was observed in the sections of the nontransformant Rut C-30 treated with the GFP antibodies (Fig. 7-4C).

The anti-Vps10p antibody was used to label the Golgi apparatus in CV48 and the data demonstrated that Golgi appeared as punctate structures with no obvious surrounding membrane in CV48 (stars in Fig. 7-4D) as seen earlier in the nontransformant Rut C-30 (Fig. 6-9D). Similar punctate CBHI-Venus label without an obvious membraneous envelope of a smaller size compared to secretory vesicles was occasionally seen in the cytoplasm in CV48 at 24 h but appeared in higher numbers at 48 h (stars in Fig. 7-4A and B) suggesting localisation of CBHI-
Venus in the Golgi apparatus. Nykänen (2002) has reported that the heterologous barley EPB protein was detected in and close to an electron dense Golgi apparatus that appeared as semicircles in *T. reesei*. However, the author claimed that it was difficult to confirm that the Golgi apparatus was involved due to the lack of data about the morphology of the fungal Golgi compartment. Our EM data on the Golgi morphology stained with a Golgi-specific antibody made against a Golgi trans-membrane protein Vps10p, together with the findings from CLSM where the CBHI-Venus fusion protein was shown to be partly colocalised with the Golgi marker BODIPY® TR ceramide (Section 7.2.2.5 and Fig. 7-5B), provide strong evidence that the Golgi plays a role in the protein secretory pathway in *T. reesei* as pointed out earlier in Section 1.2.5.

7.2.2.5 Colocalisation of the CBHI-Venus fusion protein inside the live CV48 by co-staining with organelle-specific dyes

The data from live cell imaging of the CBHI-Venus fluorescence by CLSM and IEM study using the anti-GFP antibody showed apparent association of the CBHI-Venus fusion protein with cellular organelles at the 24 h-old hyphae. In addition, the fluorescence at this time point was bright and therefore sufficient for visualising colocalisation with organelle-specific markers by CLSM. Thus, only 24 h-old live cells were applied in the colocalisation study. For this purpose, three fluorochromes, ER-Tracker™ Red, BODIPY® TR ceramide and FM® 4-64 were employed as organelle markers for the ER, the Golgi apparatus and the endo-membranes respectively in dual-labelling of the living hyphae of CV48 grown for 24 h to investigate subcellular localisation of the CBHI-Venus fusion protein (Fig. 7-5).
Figure 7-5 Colocalisation of the CBHI-Venus fusion protein with some organelle markers in the living transformant CV48 by CLSM. The live CV48 was stained with ER stain ER-Tracker™ Red (A), Golgi stain BODIPY® FL C-5 (B), and membrane stain FM® 4-64 (C). Arrows represent colocalisation (yellow). Arrowhead indicates the vesicle. Scale bar= 5 nm in all images.

In the living hyphae of CV48, the ER-Tracker™ Red dye stained a reticulate network throughout the hyphae with occasional small fluorescent punctates present close to tubular membranes (Fig. 7-5A). The staining by the ER-Tracker™ Red dye was similar to distribution of CBHI-Venus fluorescence resulting in some overlapping of the two fluorescent probes and therefore
suggesting localisation of CBHI-Venus in the ER (arrow in Fig. 7-5A). When stained with BODIPY® TR ceramide, some sparse colocalisation of CBHI-Venus fluorescence and BODIPY staining was seen (arrows in Fig. 7-5B) coinciding with the IEM results where a punctate-like label pattern was seen using the anti-GFP antibody (stars in Fig. 7-4A, B and D). The reason for the occasional colocalisation of CBHI-Venus and BODIPY® TR ceramide may have been due to the BODIPY® TR ceramide non-specifically staining other structures as well (discussed in Section 6.4). Following labelling with FM® 4-64, CBHI-Venus was found inside vesicles (arrowheads in Fig. 7-5C) consistent with the IEM result where gold label for CBHI-Venus was found in round-shaped structures (double arrowhead in Fig. 7-4B). In addition, colocalisation was visualised in reticulate structures between CBHI-Venus fluorescence and FM 4-64; these structures are considered as the ER networks (arrows in Fig. 7-5C) therefore providing further evidence, in addition to ER staining data by IEM as well as colocalisation with ER-Tracker™ Red by CLSM that the recombinant protein had progressed to the ER for secretion.

Direct visualisation of a reporter protein such as GFP in fungal cells is a helpful technique for the analysis of protein localisation. This is particularly true when the reporter protein is co-stained with some organelle-specific stains. For example, a colocalisation work was carried out in the living hyphae of *Neurospora crassa* transformants NMR3 and NMR6, expressing GFP-labelled chitin synthase CHS-3 and CHS-6, respectively (Riquelme *et al.*, 2007). In this study, live fungi were dual-stained with specific vital fluorescent dyes to determine whether CHS-GFP colocalised with the ER, Golgi body equivalents, vacuoles and endocytic compartments using brefeldin A (BFA) BODIPY® 558/568 conjugate as the Golgi and ER marker, diacetate derivative of carboxydifluorofluorescein (CDFFDA) as the vacuolar marker and FM® 4-64 as the general endocytic organelle marker. Surprisingly, there was no colocalisation of the CHS-GFP-labelled and BODIPY®-stained membranes and the authors suggested that the CHS-GFP was not transported by a conventional ER-Golgi pathway. In contrast, our findings from dual-staining of CV48 with organelle dyes, in conjunction with the data from confocal imaging of the fluorescence of VenusYFP in the live CV48 as well as the IEM results, suggested that secretion of the fusion protein CBHI-Venus was progressing through the conventional secretory pathway via ER to Golgi and packed into secretory vesicles (refer to Section 1.2.1).
7.2.3 Secretion of the CBHI-Venus fusion protein into culture supernatants

Following the investigations into the intracellular expression and localisation of the CBHI fusion protein in CV48, secretion of CBHI-Venus into the culture medium over a time course of 120 h was tracked by Western blotting using a polyclonal anti-GFP antibody and a monoclonal anti-CBHI antibody. Like inside the hyphae, the extracellular fusion protein was relatively stable since the fusion bands at the size of 73 kDa with no lower molecular weight bands resulting from proteolytic cleavage were observed on the blots (Fig. 7-6). The presence of the intact CBHI-Venus of the expected size of 73 kDa without any cleavage in both intracellular lysates (Fig. 7-3) and extracellular supernatants from CV48 denatured by directly incubating with the NuPAGE® 4X LDS Sample Buffer on ice instead of boiling suggests that no boiling of the protein samples was important to keep the fusion protein in the intact state. The cleavage at the fusion site resulting from denaturing by boiling could explain why a GFP-alone band (27 kDa) was detected in addition to a band of the GLA::GFP fusion protein by Western blotting in a previously reported work by Gordon et al. (2000a).

When probed with the anti-GFP antibody, there was a 6 h time difference between the first appearance of the intracellular (top picture in Fig. 7-3) and extracellular CBHI-Venus (top picture in Fig. 7-6). This 6 h time lag was considered long, considering that CBHI production has been reported to take only eleven minutes from translation to secretion and only four minutes for the synthesis (Pakula et al., 2000). It was noted that such an efficient synthesis and secretion was concluded based on the developed methodology by in vivo metabolic labelling of T. reesei Rut C-30 with [³⁵S] methionine or [¹⁴C] mannose and subsequent two-dimensional gel electrophoresis (Pakula et al., 2000). Furthermore, the cultivation conditions of the above studies were also distinct from ours. For example, aliquots from T. reesei Rut C-30 cultivations were withdrawn from the bioreactor to a shake flask for metabolic labelling in a carbon-limited chemostat with lactose as a carbon source and amino acid precursor labelled with [³⁵S] methionine or [¹⁴C] mannose but excluding organic nitrogen sources and free methionine (Pakula et al., 2000). Therefore, the results obtained using different methods and conditions cannot be compared directly. In addition, expression of the fusion proteins is generally slower than that of non-fused proteins (Curach, 2005, unpublished data). In the current work, it was assumed that the delay in secretion on the CLS medium may have been due to a low efficiency of secretion of the fusion protein at the early time point of 12 h. In addition, the secreted CBHI-Venus fusion protein
seemed to have accumulated over time in the medium resulting in strong signals up to 96 h (top picture in Fig. 7-6). At 120 h, the extracellular production of the fusion protein appeared at a low level suggesting that cell-wall retention of CBHI-Venus (Fig. 7-2F1 and G1) may have contributed to this phenomenon in addition to exhaustion of nutrients at the late stages of growth.

**Figure 7-6** Western analysis of the CBHI-Venus fusion protein secreted into the culture medium of the CV48 transformant over a 120 h culture period. Cells were grown in the CLS medium (pH 6.5). Culture supernatants were incubated with the NuPAGE® 4X LDS Sample Buffer on ice for 5 min and loaded straight into the gel without boiling to keep the fusion protein intact. The blots were probed for VenusYFP using the anti-GFP antibody (top) and anti-CBHI antibody (bottom); 15 µg of total protein was loaded in each lane. The expected size of CBHI-Venus is 73 kDa and that of native CBHI is 46 kDa, as indicated by the arrows.

When probed with the monoclonal anti-CBHI antibody, bands with the size of 73 kDa representing the fusion protein were strong after 24 h (bottom picture Fig. 7-6), which is in a good agreement with what was seen with probing with the anti-GFP antibody on the immunoblot.
(top picture in Fig. 7-6). Surprisingly, the anti-CBHI antibody did not recognise extracellular endogenous CBHI throughout the whole culture of 120 h as no band at 46 kDa was visible (bottom picture Fig. 7-6). This was unexpected since with the same monoclonal anti-CBHI antibody, endogenous CBHI of the size of 46 kDa was detected in the cellular extract of CV48 after 72 h, although at low levels (bottom picture in Fig. 7-3) suggesting that the native CBHI was produced and secreted at least at late culture stages. In the earlier primary characterisation of the transformant CV48 (Section 3.2.2.2), when using the polyclonal anti-CBHI antibody, two bands representing the CBHI-Venus fusion protein and endogenous CBHI were visualised in a culture medium from 72 h-old CV48 but the 46-kDa-sized band of endogenous CBHI was considerably faint compared to the fusion protein band of 73 kDa (bottom picture in Fig. 3-6A). It remains to be clarified whether the non-detection of the extracellular endogenous CBHI was a consequence of a lower binding affinity of the monoclonal CBHI antibody or its instability in pH-lifted culture environment or both.

7.3 Conclusions
Secretion of the CBHI-Venus fusion protein in the transformant CV48 was tracked by in vivo visualisation of the fluorescence from CBHI-Venus over a time course of 120 h using CLSM. CBHI-Venus expression was also monitored over the 120 h culture time at the mRNA level by real time PCR and at the protein level by Western blotting. CV48 cells collected at early time points of 24 and 48 h were selected for further IEM localisation analysis and finally only 24 h-old living CV48 cells, which displayed sufficient fluorescence signals and demonstrated active association with secretory compartments, were used for subcellular colocalisation studies by CLSM. The cbh1-venus transcripts were detected at 12 h and intracellular fluorescence was detected also at the same time suggesting efficient and fast maturation of the fusion protein. However, extracellular CBHI-Venus was not detectable at 12 h indicating a 6 h time lag between intracellular and extracellular presence of the CBHI-Venus possibly due to a low efficiency of secretion of the fusion protein at the early stage of 12 h. The CBHI-Venus fusion protein appeared first in the ER at 12 h and its association with the ER became more apparent at 24 and 48 h. In addition to localisation to the conventional ER cisternae, the CBHI-Venus fusion protein was seen in some distorted ER membrane structures assumed to be ER-derived sub-domains. The modification of the ER organisation and formation of ER sub-domains in the CV48 transformant with at least two copies of venus gene were similar to what was seen in the BV47 transformant.
overexpressing BiP1-Venus therefore providing additional evidence to the notion that overexpression of recombinant proteins can lead to modification to the ER morphology in filamentous fungi. The association of CBHI-Venus with the Golgi apparatus was occasionally visualised at both 24 and 48 h suggesting that the Golgi forms an integral part of the secretory compartments in filamentous fungi. At the later culture stages, production of vacuoles in the hyphae and protein retention in the cell wall were seen resulting in reduction of the extracellular amount of the CBHI-Venus. The appearance of the full sized fusion protein on the immunoblots also suggested that the recombinant CBHI-Venus was processed successfully along the secretory pathway in CV48.
Chapter 8 Visualisation of protein interactions between BiP1 and CBHI in
*T. reesei*

8.1 Introduction

The ER-resident chaperone BiP plays an important role in protein quality control and secretion. BiP interacts with newly synthesised translocating polypeptides (Haas, 1991) and misfolded proteins (Sorgjerd et al., 2006). The interactions between BiP and the secreted proteins are not only crucial for translocation, but they also prevent prolonged exposure of hydrophobic regions and thus avoid uncontrolled aggregation of folding intermediates in the ER lumen (Pimpl and Denecke, 2002). In addition, BiP interacts with misfolded proteins to prevent them from entering the secretory pathway by retrograde translocation of the proteins to the cytosol and ER-associated protein degradation (ERAD) (Sorgjerd et al., 2006; Gasser et al., 2008). Visible evidence for protein-protein interactions has been mainly produced by microscopy studies. The high resolution capability of EM and the use of immunogold labelling allow visualisation of the co-localisation of the BiP chaperone and interacting proteins in organelles (Sagt et al., 1998; Xie et al., 2006; Nawa et al., 2007). Interactions of BiP and the fungal protein cutinase have been previously investigated in *S. cerevisiae* showing that the introduction of two exposed hydrophobic patches in cutinase resulted in a higher affinity for BiP therefore causing the retention of the mutant cutinase in the ER (Sagt et al., 1998). However, IEM technique can only provide correlative information regarding the relative position of proteins in the fixed cell (Day et al., 2001). Another important microscopy technique for looking into protein interactions is FRET which permits visualisation of dynamic interactions between specific protein partners within the context of a living cell (Day et al., 2001). In the work described in this chapter, FRET microscopy was attempted to visualise the interaction between BiP1 and the highly-secreted protein CBHI in *T. reesei* transformants.

8.2 Results and discussion

FRET technique is widely used to measure localised protein interaction partners (Lalonde et al., 2008). In order to study the interaction between BiP1 and CBHI in *T. reesei in vivo* by FRET microscopy, *T. reesei* Rut C-30 was transformed with the gfp2 gene and/or venus gene to obtain the FRET reporters as follows:

(i) donor: transformant BG29 expressing *bip1-gfp2*;

(ii) acceptor: transformant CV48 expressing *cbh1-venus*;
(iii) positive control: transformant VG15 expressing venus-gfp2 with a short 15 bp linker between gfp2 and venus;

(iv) FRET sample: transformant BGCV101 co-expressing bip1-gfp2 and cbh1-venus.

DNA constructs were expressed under the strong cbh1 promoter to ensure sufficient expression for the FRET study.

To examine whether the dominantly secreted protein CBHI had any interactions with the protein folding chaperone BiP1, sensitised FRET efficiencies (Sorkin et al., 2000) between GFP2 and VenusYFP were measured on a pixel-by-pixel basis (Section 2.14.5) and a series of raw images were acquired (Table 2-7). The FRET efficiency and the distance between the donor and acceptor were evaluated in Olympus FV ASW 1.7b program. The FRET efficiency the transfer efficiency (E) is a direct measure of the fraction of photon energy absorbed by the donor that is transferred to an acceptor (Berney and Danuser, 2003). It depends on the donor-to-acceptor separation distance R with an inverse sixth power of the distance (R) between the two fluorophores (E=1/(1+(R/R_0)^6) where R_0 is the Förster distance of the of donor-acceptor pair at which the FRET efficiency is 50 %) (Lakowicz, 1999). It is generally difficult to pinpoint an exact FRET efficiency value to look for, because this value is dependent upon the probe uptake, distance between the donor and acceptor molecules, autofluorescence and other factors such as background noise (Timothy and Holmes, 2006). In practice, if an efficiency reading is above 0.25 in the supposed FRET region, this value is considered to be attributed to a positive FRET occurrence (Timothy and Holmes, 2006). As seen in Fig. 7-1A, the FRET image of the transformant VG15 revealed a detectable energy transfer (with an average FRET efficiency of 0.16 between GFP2 and VenusYFP suggesting that these two proteins interacted but at a low efficiency level (less than 0.25). Negative or very low levels of FRET signals were determined in the transformant BGCV101 with the average FRET efficiency close to zero (E=0.043). The average distances between the donor and acceptor in VG15 and BGCV101 were 8.44 nm and 9.50 nm respectively.
Figure 8-1 FRET analysis by sensitised emission in the living hyphae of *T. reesei* transformants. The fungal cells were cultured in the CLS medium (pH 6.5) for 24 h. *T. reesei* transformant VG15 contained a Venus-GFP2 tandem construct with a short 5 aa linker region between therefore serving as a positive FRET control in this experiment. The transformant BGCV101 coexpressed BiP1-GFP2 and CBHI-Venus. Image acquisition conditions for the three channels were: ex 405 nm, em 510-530 nm (donor/GFP2 channel); ex 473 nm, em 530-550 nm (acceptor/VenusYFP channel); ex 405 nm, em 530-550 nm (FRET channel). The output FRET efficiency (A) and the average distance (B) between the donor and acceptor were displayed in a pseudo colour mode. The unit of the distance colour bar is nm. The FRET analysis was carried out using FV ASW 1.7b software (Olympus, Japan). The raw data to produce the
results shown in B and C were collected from 20 cells of each transformant strain. Figure C illustrates the average FRET efficiency and distance between the donor and acceptor in the transformant VG15 and BGCV101.

The FRET phenomenon is based on the dipole-dipole interaction of a suitable donor-acceptor pair. The basic conditions for a good efficiency of the energy transfer have been discussed in Section 1.3.1.3. In general, the important requirements include maximal spectral overlap between the emission of the donor and the excitation of the acceptor molecule, the distance between the centres of the dipoles should be in a range of 1-10 nm (in general < 7nm) and the relative orientation of the chromophores should be favourable (Day et al., 2001).

The positive control stain VG15 expressed Venus-GFP2 with a 5 aa linker between the donor GFP2 and the acceptor VenusYFP. The energy transfer in the VG15 was observed with an average FRET efficiency of 0.16 indicating the positive control was functional although at a low level (the FRET efficiency was less than 0.25). In studies by Zimmerman et al. (2002), a similar donor-acceptor pair with GFP2-YFP was applied for FRET measurement indicating 87 % overlap between GFP2 (donor) emission and acceptor (YFP) excitation (Zimmermann et al., 2002). This 87 % overlap is considered high compared to the spectral overlap of 66 % for the commonly-used CFP-YFP FRET pair (Zimmermann et al., 2002). Considering the estimated spectral overlap, the Venus-GFP2 pair used in the current study should also be as high or close to 87 %; the FRET efficiency of 0.16 observed with the positive control VG15 was relatively low. The amount of transferred energy depends on the distance between the donor and acceptor (typically over the range 1–10 nm) (Chen et al., 2006) and is considerably insensitive to distance changes outside the 2–8 nm range (Roy et al., 2008). Thus, we assumed that the low FRET efficiency in VG15 could have been due to the factor that the conformational distance between GFP2 and VenusYFP was too long (D=8.44 nm) after protein maturation and folding.

The failure to detect FRET activity in the transformant BGCV101 (the FRET sample) does not necessarily translate to the absence of interaction between BiP1-GFP2 and CBHI-Venus. There are several potential reasons why a FRET signal was not detected. First of all, as discussed above, FRET works efficiently in the range of 1–10 nm (Jares-Erijman and Jovin, 2003), therefore, an increase in distance between the fluorophore pair can lead to the decrease in FRET efficiency (Collins et al., 2009). Using the image software it was found that the average distance between
GFP2 and VenusYFP in the transformant strain BGCV101 was 9.50 nm. This is close to the 10 nm upper limit of the ‘allowed’ distance and thus may or may not contribute to the end result (Fig. 8-1C).

According to (Periasamy, 2003), FRET is most efficient with a greater concentration of the acceptor molecule compared to the donor concentration. However, if (Berney and Danuser, 2003) are to believed, the donor and acceptor concentrations should be of the same order of magnitude. These views have been based on work with different FRET pairs, molecular constructs and cell types. On the outset of the FRET work, when plasmid and strain construction were started (Chapter 3), it was considered important to obtain similar and high expression levels of both the donor (GFP2) and the acceptor (VenusYFP) in the T. reesei transformants. To this end, bip1-gfp2, cbh1-venus and venus-gfp2 were expressed under the strong cbh1 promoter. The double transformant and BGCV101 also seemed to contain about the same number of the gfp2 and venus expression cassettes integrated into the genome (Fig. 3-4), therefore suggesting the acceptor-to-donor ratio was 1:1. It was also noticed earlier (Section 3.2.1.1 and Fig. 3-2E) that the fluorescence levels of both GFP2 (FRET donor) and VenusYFP (FRET acceptor) were close to each other in the BGCV101 strain providing further support to the notion that the acceptor and donor were expressed at about the same level.

The approach for aiming at the same expression level appeared not to be optimal since no significant energy transfer was detected when the FRET study was carried out with BGCV101. Therefore, having the acceptor expressed at a higher level than the donor (Periasamy, 2003) may have been a more successful approach. According to Laib and Seeger (2004), the acceptor-to-donor ratio of 1:10 produced high FRET efficiency using bisintercalation dyes TOTO-1-iodide (donor) and TOTO-3-iodide (acceptor) as a FRET pair in vitro established by a fluorometric titration of the concentrations of the donor and acceptor (Laib and Seeger, 2004). Therefore it is evident that the concentrations of the donor and acceptor molecules would have to be determined in detail and different ratios experimented to improve FRET in T. reesei. This would require obtaining transformants with a variety of gene copy numbers. Attention should also be paid not to overexpress the the FRET pair too much since overexpression modifies the relative concentrations of the interacting FRET partners compared to the in vivo state (Lalonde et al., 2008).
On the technical side, FRET measurements are limited by the accuracy of quantifying fluorescence intensity, which is more prone to artifacts at weaker energy transfer signals. Therefore, it is essential to define the intensity limits of the microscopy system to be used, below which fluorescence quantification becomes too inaccurate for FRET determination (Day et al., 2001). In these cases, fluorescence lifetime imaging (FLIM) of the donor fluorophore may offer significant improvement in sensitivity for determining the physical interactions between molecules in living cells (Murakoshi et al., 2008).

In summary, the GFP2/VenusYFP pair is a good FRET pair option in terms of the spectral overlap and therefore should provide a reasonably high FRET efficiency as long as the emission overlap between the donor and acceptor can be separated by spectral imaging and linear unmixing. Attention must be paid to the linker length between the donor GFP2 and acceptor VenusYFP to ensure the donor-acceptor distance is short enough (< 7 nm) when designing the positive FRET control for the GFP2/VenusYFP pair. In addition, it will be necessary to take fluorophore orientation into consideration when interpreting FRET data.

Although no positive FRET signals were visualised in BGCV101, an interaction between BiP1 and CBHI may be occurring as evidenced by colocalisation of the fusion protein CBHI-Venus in the ER in the transformant CV48, shown by CLSM and IEM (Fig. 7-2, 7-4 and 7-5 in Chapter 7). The detection of ER-derived sub-domains filled with CBHI-Venus (Section 7.2.2.4 and Fig. 7-4A) strongly suggested that these distorted structures could be “BiP bodies” where BiP was interacting with overexpressed CBHI-Venus to alleviate the ER stress as seen earlier in the transformant BV47 (Section 6.3.2.4; Fig. 6-6A and C).

8.3 Conclusion
FRET microscopy has been widely used in measuring protein-protein interaction (Grefen et al., 2007). Although weak, the FRET signals were detected in the positive control strain VG15 suggesting the FRET pair Venus-GFP2 worked. Failure to observe FRET signals in the transformant BGCV101 could be a result of multiple factors as discussed above. Based on the available FRET tools, it was difficult to address the interaction between BiP1 and dominantly-secreted protein CBHI in T. reesei in more detail. At least two strategies will be worth trying in the future to improve the FRET efficiency: (i) shortening the linker between donor and acceptor.
in the positive control; (ii) increasing the expression ratio of the acceptor molecule by using stronger promoter for expressing of acceptor than for expressing donor or screening of higher copy gene for acceptor than for donor.
Chapter 9 Summary and concluding discussion

There is a need for a better understanding of the secretory pathway in the industrially-exploited filamentous fungus *Trichoderma reesei* to eventually lead to improvement of (heterologous) protein yields in this protein expression system. A wide range of methodologies, such as genetic, biochemical and molecular approaches, have been used to investigate protein processing, secretion and production as described in Section 1.3. However, there are far less studies into visualisation of the secretory pathway and its main organelles to support protein secretion and localised interaction studies.

This thesis started with the tasks of mapping the secretory landscape of the *T. reesei* hyphae using fluorescence tools and developing a FRET system using the GFP2/VenusYFP pair in *T. reesei* in order to visualise the interaction between an ER-resident protein folding chaperone BiP1 and the main secreted cellobiohydrolase I (CBHI). Due to difficulties in implementation of the FRET assay (Section 1.4 and Chapter 8), the recombinant *T. reesei* strains constructed, BV47 expressing BiP1-Venus (Chapter 3) and CV48 expressing the CBHI-Venus fusion protein (Chapter 3) were then used to in vivo labelling of the ER (Section 6.3) and tracking secretion of the CBHI in the *T. reesei* hyphae at both the CLSM and EM levels (Chapter 7).

9.1 Development of a series of *T. reesei* transformants

Fluorescent proteins provide an excellent tool, for example, for visualising protein interactions with FRET microscopy, labelling specific cell compartments and tracking secretion of proteins of interest using CLSM. In this work, various *T. reesei* transformants expressing the fluorescent proteins GFP2 and VenusYFP were initially generated for a FRET study targeting interactions between the protein folding chaperone BiP1 and the highly-secreted CBHI protein.

Plasmids with potential expression combinations, BiP1-GFP2 (donor)/CBHI-Venus (acceptor) and CBHI-GFP2 (donor)/BiP1-Venus (acceptor), were developed and transformed into the host strain Rut C-30 in order to maximise success in obtaining FRET signals. Venus-GFP2 with a short 5-aa linker between VenusYFP and GFP2 was developed to serve as a positive FRET control. Since no transformants expressing CBHI-GFP2 were obtained, the FRET combination of CBHI-GFP2/BiP1-Venus was discarded and the rest of the work was focused on the combination
of BiP1-GFP2 and CBHI-Venus. A series of transformants was obtained: “BG” series expressing BiP1-GFP2, “CV” series expressing CBHI-Venus, “BV” series expressing BiP1-Venus, “VG” series expressing Venus-GFP2, and “BGCV” series coexpressing BiP1-GFP2 and CBHI-Venus. The strong cbh1 promoter was used to drive efficient expression of the above fusion proteins in *T. reesei* to obtain sufficient signals for FRET studies and maximise yield. Using the same promoter for all constructs was expected to aid in obtaining comparative levels of the donor (GFP2) and acceptor (VenusYFP) to facilitate FRET. This approach was based on the notion that donor and acceptor concentration should be of the same order of magnitude (Berney and Danuser, 2003). The transformant candidates were selected according to the criteria of high expression of the fusion protein (determined by fluorescence reading and Western blotting) and efficient transcription of the fusion genes at the mRNA level (determined by Northern blotting). Southern blotting was applied to determine the gene copy number in the transformants selected by the given criteria. Five transformants named BG29, BV47, CV48, VG15 and BGCV101 were selected for further study. Molecular characterisation of these transformants showed that all carried multiple copies of the gfp2/venus gene(s) except BGCV101 which probably had one copy of gfp2 and cbh1 (Fig. 3-4), and the DNA had integrated in the genome in a random fashion.

The transformants BG29, CV48, VG15 and BGCV101 were applied as the FRET donor, acceptor, positive FRET control and as the actual FRET sample respectively. The emission spectra of GFP2 and VenusYFP were measured in the transformants BG29 and BV47, respectively (Fig. 3-7A) and the emission signal collection ranging 510-530/530-550 nm was applied for standard and FRET imaging of GFP2 and VenusYFP separately with CLSM at excitation with 405 nm laser (for GFP2) and 473/514 nm (for VenusYFP). The half time of photobleaching of GFP2 and VenusYFP was about 20 min and 90 min respectively when using 50 % of full laser intensity suggesting that both fluorescent proteins were sensitive to photobleaching and GFP2 was bleached considerably quicker than VenusYFP. Since GFP2 (donor) was not resistant to photobleaching, acceptor bleaching method should be avoided in detecting FRET signals (Lalonde *et al.*, 2008). Thus, the conventional sensitised emission method was used for FRET analysis. In addition, due to photobleaching sensitivity, reduced power intensity of the excitation laser beam, typically 5-10 % of the full excitation laser power for both GFP2 and VenusYFP was applied for standard confocal imaging work to protect the fluorophores.
The transformant BV47 carrying two copies of the *bip1-venus* gene could not be used for FRET study due to unavailability of a coupling CG transformant (expressing CBHI-GFP2); instead, this strain was used for visualisation of the ER in *T. reesei* (Chaper 6).

In addition to serving as an acceptor in the FRET work with the coupling donor transformant BG29 expressing BiP1-GFP2, the transformant CV48 expressing the CBHI-Venus fusion protein was applied to track secretion of CBHI throughout the hyphae of *T. reesei* (Chapter 7).

**9.2 Investigation into different culture media**

The CLS medium (Section 4.2.1) is one of the routine choices for protein production in *Trichoderma reesei* in this laboratory (Curach, 2005). The two features of the medium that were deemed less optimal for the studies here were emission of a strong fluorescence background resulting from soy hydrolysate, cellobiose and trace elements (Table 4-1 and Fig. 4-1) that interfered with the CLSM studies and low pH (pH 5.5) which contributed to the degradation of the fluorescent fusion proteins (Chapter 5, Fig. 5-1A and B). Even tough a microscopy-compatible “lactose medium” was developed for direct observation of the fungal cultures under a fluorescence microscope, the studies reverted back to using CLS for its ability to strongly induce the *cbhI* promoter (Fig. 2-1) under which all genes of interest were expressed. A thorough washing of fungal hyphae after cultivation in the CLS medium solved the background fluorescence problem.

**9.3 Sample preparation techniques for live cell imaging by CLSM**

Successful imaging of live fungal cells by confocal fluorescence microscopy requires hyphae staying in the same plane of focus, minimal fluorescence background as well as easy staining and manipulation of live cells during imaging. Agar block and cellophane methods tried first had inherent disadvantages in live cell imaging (Chapter 4). In order to establish fast and easy protocols for live cell imaging, two techniques, quickly observing washed live cells on a microscope slide, and observing the live cells grown in a semi-solid medium containing 0.5 % (w/v) methyl cellulose (MC) over a longer time period (*Methods 3* and *4* in Chapter 3) were investigated successfully. Addition of non-fluorescent MC into a cultivation medium to immobilise live fungi for a closer, long-time inspection is a new technique developed in the present study.
9.4 Denaturation of protein samples by boiling caused cleavage of the fusion protein

Prior to separation on SDS-PAGE, proteins are usually denatured by boiling for 5 min at 100 °C in a loading buffer, i.e. a commercial loading buffer containing SDS and a reducing agent such as DTT or 2-mercaptoethanol to achieve complete denaturation. When the samples were treated in this way, it was noted that boiled culture supernatants from the CV48 transformant blotted onto a PVDF membrane and stained with an anti-GFP antibody resulted in a number of different sized bands ranging from 27-73 kDa (Fig. 5-1B). In contrast, Western blotting of unboiled samples showed only a single band at 73 kDa corresponding to the size of the CBHI-Venus fusion implicating that denaturation by boiling caused the cleavage of the fusion protein. For this reason, unless stated otherwise, all samples processed for further Western blotting experiments in this study were treated by incubating them in a NuPAGE® 4X LDS Sample Buffer on ice for 5 min without boiling, before loading onto gels to keep the fusion structure intact.

9.5 An overview of the membraneous compartments in T. reesei Rut C-30 using the membrane-selective stain FM® 4-64

The membrane selective stain FM® 4-64 was used to stain 24 h-old Rut C-30 hyphae in order to obtain an overview of the membrane network involved in the secretory pathway in T. reesei. Dual labelling of Rut C-30 with the FM® 4-64 and the ER-dye DIOC₆(3) or the Golgi dye BODIPY® FL C-5 ceramide was further carried out for colocalisation studies. The results (Fig. 6-1) showed that FM® 4-64 was able to label the main secretory organelles including the membranes of the ER and transport vesicles as well as plasma membranes. Therefore, the FM® 4-64 can be used as an experimental tool for visualising membrane-based components in living T. reesei. However, it was not certain whether the FM® 4-64 stained the Golgi as the BODIPY® FL C-5 ceramide seemed not specific to the Golgi in the colocalisation work using the BODIPY® FL C-5 ceramide and FM® 4-64.

9.6 Visualisation of changes in the ER during expression of a BiP1-Venus fusion protein in T. reesei

BiP1 is an ER-resident chaperone and the ER retention signal of BiP, HDEL, provides an excellent tag to be attached to fluorescent proteins to visualise the ER compartment. In the current study, morphological studies of the ER in Trichoderma reesei were performed using a DNA construct expressing BiP1-Venus which had the ER retention signal HDEL tagged to the C-terminus of the BiP1-Venus fusion protein (Fig. 2-1).
The BiP1-Venus fusion protein was successfully expressed in *T. reesei* transformant BV47 based on the evidence from the following experiments: (i) PCR analysis of the *bip1*-venus fusion gene from gDNA of the transformant BV47 produced a product of 1365 bp which agreed with the expected size of the fusion gene, indicating successful integration of *bip1*-venus into the genome (Fig. 3-3A); (ii) The expected 104 kDa sized band representing the BiP1-Venus fusion protein was detected in Western blotting experiments when hybridised with the anti-BiP and anti-GFP antibodies (Fig. 6-3) suggesting the protein was in an intact form; (iii) Real time PCR using *bip1*-venus primers demonstrated that significant specific signals were detected in the transformant BV47 while no signals were found in the nontransformant Rut C-30 under the same conditions (Fig. 6-2); (iv) Obvious colocalisation of BiP1 and VenusYFP in punctate “bodies” was observed with immunofluorescence CLSM (Fig. 6-5A) and IEM (Fig. 6-6A and C). In addition, the pattern of VenusYFP fluorescence in the live BV47 hyphae (Fig. 6-4A) corresponded to immunofluorescence and immuno-gold labelling results (Fig. 6-5A, Fig. 6-6A and C).

Fluorescence images (Fig. 6-4B and 6-5B) and electron micrographs (Fig. 6-6D1 and E) of the ER in the host strain *T. reesei* Rut C-30 were characterised by a typical interconnected network of parallel tubular membranes and some punctate-like bodies adjacent to the tubular membranes through the hyphae. The punctate “BiP bodies” were more abundant in the transformant BV47 than in the nontransformant Rut C-30 as seen with EM (Fig. 6-5A, D1 and D2). Studies into the ER structure and dynamics under secretion stress contribute to the understanding protein secretion in *T. reesei*. The modification of the ER morphology in BV47 is summarised in Chapter 6.

Further subcellular localisation of the BiP1-Venus was investigated by dual staining with other organelle markers including the vesicle marker FM® 4-64 (Fig. 6-7A) and the Golgi marker BODIPY® TR ceramide (Fig. 6-7B) and anti-Vps10p antibody (Fig. 6-8A and B). These results demonstrated that no or very little colocalisation of the BiP1-Venus fusion protein with the above conventional secretory compartments suggesting a new sub-ER domain called “BiP Bodies” was formed which contained a large amount of BiP protein possibly to attenuate the ER stress by providing a temporary depository for overproduced proteins waiting further processing. The finding that the BiP1-Venus fusion protein was absent in the transport vesicles suggested the relocation of BiP could be independent of traditional vesicular transport along the secretory
pathway. In addition, overexpression seemed to have affected recycling of BiP from the Golgi to the ER since no fusion protein was found localising to the Golgi. In the EM micrographs, the label for the BiP1-Venus fusion protein was found sparsely in the cytoplasm of BV47 and nontransformant Rut C-30 (stars in Fig. 5-6A-D) indicating translocation of BiP from the ER to the cytosol possibly for degradation by the proteasome by ERAD (Pimpl et al., 2006). We could therefore assume that overexpression of the BiP1-Venus protein contributes to activation of the protein quality control mechanisms UPR and ERAD.

Although the BiP1-VenusYFP appeared to function as an ER marker in T. reesei as previously observed in A. oryzae (Maruyama et al., 2006), A. nidulans (Fernández-Ábalos et al., 1998) and U. maydis (Wedlich-Söldner et al., 2002), some BiP1-Venus seemed to have escaped from the ER into the culture medium under the conditions used in this study (Fig. 6-3) indicating the retention sequence HDEL/KDEL was not completely retaining the fusion BiP1-Venus in the ER luminal membranes and that overexpression resulted in BiP1-Venus molecules escaping the routine recycling pathway between the ER and the Golgi, normally faced by the native BiP1 protein. The saturation of the standard ER retention machinery is in turn likely to lead to the changes in the ER morphology.

Even though BiP overexpression may not have been the perfect way to mark the ER as it reflected an ER organisation modified by overexpression rather than the natural ER architecture, it has provided new information on ER dynamics under protein overexpression as discussed in Section 6.3.2.

9.7 Morphology studies of the Golgi apparatus in T. reesei
To date, the knowledge on morphology and biochemistry of the Golgi complex in filamentous fungi is very limited. In the present study, the Golgi apparatus in T. reesei was initially investigated by fluorescence staining and CLSM (Section 6.3). Following staining with the Golgi stain BODIPY® FL C-5 sphingomyelin (Fig. 6-9A) and BODIPY® TR ceramide (Fig. 6-9B), the Golgi was mainly visualised as bright punctate bodies throughout the hyphae. Staining with BODIPY® TR ceramide demonstrated some non-specific elongated staining characteristic for ER filaments (arrow in Fig. 6-9B). The punctate fluorescence staining pattern in Rut C-30 and the transformant CV48 expressing a secretory fusion protein CBHI-Venus was further confirmed by
IEM using a Golgi-specific antibody (Fig. 6-9D and 6-8, respectively). Visualisation of the Golgi in our study did not point out any particular intracellular site, but the fluorescence was distributed through the hyphae unlike to what has been seen with other Golgi markers such as GFP-tagged SNARES which appeared to have located at apical regions in A. oryzae (Kuratsu et al., 2007). Punctate shaped Golgi bodies have also been found in other filamentous fungi (Hoch and Howard, 1980; Roberson and Fuller, 1986; Sewall et al., 1989; Cole et al., 2000; Akao et al., 2006; Breakspear et al., 2007; Kuratsu et al., 2007). In T. reesei, no typical Golgi structures were observed in either the wild type QM6a and the high protein secreting Rut C-30 strain (Ghosh et al., 1990) but Nykänen (2002) claimed that the Golgi-equivalent showed as semicircles in Rut C-30. The conflicting information from these studies could be a result of the use of different sample treatments or Golgi markers.

In the EM micrographs, the size of single Golgi profile appeared at variable sizes ranging from 10-60 nm with no obvious surrounding membranes and very often residing in the vicinity of ER (Fig. 6-9D). These data are not in accordance with the previous report where electron-dense endomembraneous structure was elucidated to act as the Golgi body in wild-type yeast cells (Preuss et al., 1992). The observations showing no obvious membrane surrounding Golgi could have been because of the fixation method used in this study which may have failed to preserve the delicate Golgi membrane (discussed in Section 6.4).

9.8 Transcription of fusion genes and the endogenous cbh1 gene
Gene transcription was studied in the T. reesei Rut C-30 host and the transformants BG29, BV47, CV48, BGCV101 and VG15. The features of the gene expression cassettes are described in detail in Fig. 2-1. Production of mRNA transcripts in the above transformants was elucidated by Northern blotting using either gfp2 or venus probes. The gfp2 and/or venus transcripts were detected at the expected size of 2400 bp confirming that the expression cassettes were successfully expressed at the mRNA level. The hybridisation signals for either gfp2 or venus were specific to the transformants since no signal for the gfp2 nor venus transcript was detected in the host T. reesei Rut C-30 (Fig. 3-5). Further, quantitative real time PCR was carried out to compare the transcript levels of the fusion cbh1-venus gene and native cbh1 gene over a culture course of 120 h in the CV48 transformant (Section 7.2.2.1). The relative mRNA transcript intensity of the native cbh1 gene was determined by subtracting the relative mRNA intensity of
the cbh1-venus fusion gene from the total cbh1 mRNA (Fig. 7-1). It was noted that the cbh1-venus mRNA levels in CV48 were generally greater (1.9-6.4 fold) than those of the endogenous cbh1 transcripts at the early culture stages from 12 to 48 h. This increase could be due to the fact that the transformant CV48 had at least two copies of the cbh1-venus expression DNA (Fig. 3-4B) while the endogenous cbh1 is present as a single copy. It also implies that the mRNA transcripts of the cbh1-venus fusion gene would have similar stability to that of the native message from cbh1 gene in CV48. It has been reported that mRNA stability is an important factor for the low or undetectable protein levels of heterologous genes produced in filamentous fungi (Gouka et al., 1996). Our results showed that mRNA transcripts from the cbh1-venus fusion gene were greater than that of native CBHI at the early stages from 12 to 48 h suggesting that mRNA stability was not an issue in producing the CBHI-Venus fusion protein. However, at the late culture stages after 72 h, the cbh1-venus mRNA levels became lower compared to those of the endogenous cbh1 transcripts and displayed earlier decay. This result was possibly due to transcriptional repression through the UPR triggered by overexpression of the CBHI-Venus fusion protein and subsequent ER stress as discussed in Section 7.2.2.1.

9.9 Tracking CBHI-Venus in the secretory pathway

The aim of this part of the study was to use the transformant CV48 expressing the fluorescent fusion protein CBHI-Venus to visualise the expression and secretion of the main cellobiohydrolase I (CBHI) in T. reesei by confocal fluorescence microscopy. Extensive characterisation of the transformant CV48 was carried out using molecular biological, biochemical and electron microscopic methods (Chapter 7). Tagging CBHI with the genetically encoded VenusYFP offers a simple way to track protein secretion in fungal hyphae and the fast maturation time makes VenusYFP an especially good reporter to detect immediate expression of proteins. The success in expressing the fluorescent marker CBHI-Venus in T. reesei will open up new possibilities to more thoroughly study biological processes involved in protein production and secretion in this organism.

VenusYFP fluorescence was detected in the cytoplasm as early as 12 h at a low level of fluorescence intensity and displaying a relatively diffused distribution (Fig. 7-2A). The fusion protein appeared to have localised to the typical reticular ER filaments at the early culture stages of 18, 24 and 48 h (Fig. 7-2A-C; arrowheads in Fig. 7-4A and B). Immediately after interaction
with the ER, the fusion protein appeared in the Golgi (stars in Fig. 7-4A and B), likely to undergo further modification. From 24 h on, the CBHI-Venus protein concentrated into secretory vesicles (Fig. 7-2E-G, double arrowhead in Fig. 7-4B and Fig. 7-5C). After 72 h, the fluorescence started to appear in the cell wall (Fig. 7-2E-G) and by 120 h, some of the protein tended to concentrate into vacuoles for degradation (Fig. 7-2G). Over the culture time, the septa were stained with VenusYFP fluorescence indicating protein transport between compartments through the pores in the septa (Fig. 7-2B-D).

Expression of the fusion protein was also characterised by real time PCR in a time course of 120 h (Fig. 7-1). The cbh1-venus mRNA was just detectable at a low level at 12 h and increased slightly by 24 h in the culture grown on the CLS medium (pH 6.5). The level of the cbh1-venus transcript dramatically increased to a peak at 48 h followed by a great decrease after 72 h and continuation of a low level of transcription until 120 h. The nature of mRNA expression over time corresponded well with the amount of gold labelling in the early stage samples studied by EM where gold labelling of the fusion protein was more prominent at 48 h than that at 24 h (Fig. 7-4A and B), since VenusYFP matures rapidly and efficiently (Nagai et al., 2002). The change in the expression pattern of the cbh1-venus mRNA also agrees well with the intracellular expression of the CBHI-Venus as determined by CLSM (Fig. 7-2) and Western blotting (Fig. 7-3).

Intracellular expression of the fusion protein CBHI-Venus over time was further analysed by Western blotting method using both the anti-GFP and anti-CBHI antibodies (Fig. 7-3). Inside the hyphae of CV48, a single band corresponding to the expected size for an intact CBHI-Venus, 73 kDa, was visible in the culture supernatants sampled at 12 h onwards. This result was consistent with the findings by live CLSM where weak fluorescence was discernible also at 12 h (Fig. 7-2A) and the results by real time PCR where the mRNA transcript of cbh1-venus was detectable at 12 h (Fig. 7-1). No obvious time difference at the first sampling time point of 12 h between the appearance of cbh1-venus transcripts and the presence of intracellular fluorescence of CBHI-Venus. CLSM and Western blotting in this work confirmed that VenusYFP matured quickly as also noted by (Nagai et al., 2002; Frieda et al., 2003), therefore allowing for immediate detection of the translated fusion protein. After 18 h, the intracellular fusion protein CBHI-Venus appeared to have increased dramatically and reached the highest level at 48 h. After that, secretion declined until 120 h. The declined expression level of CBHI-Venus at the later culture stages from 72 to
120 h may be a result of declined expression of *cbh1-venus* transcripts (Fig. 7-1) due to exhaustion of nutrients in the flask-shake cultures.

The extracellular fusion protein was 73 kDa in size corresponding to the CBHI-Venus fusion protein (Fig. 7-6). It was noted that there was a 6 h time difference between the first occurrence of the intracellular and extracellular CBHI-Venus when probed with the anti-GFP antibody. This 6 h time lag was considered long since production of the native CBHI took only eleven minutes from translation to secretion and only four minutes for the synthesis in Rut C-30 where the endogenous CBHI was labelled with [³⁵S] methionine or [¹⁴C] mannose (Pakula *et al.*, 2000). As discussed in Section 7.2.3, the results obtained using different labelling methods, different protein of interest (such as CBHI-Venus fusion protein in our case and the intact endogenous CBHI in the study by Pakula *et al.*) and different culture conditions are not directly comparative. It is postulated that the delay in secretion may have been due to a low efficiency of secretion of the fusion protein at the early time point of 12 h.

It is generally accepted that a classical secretory pathway is present in *T. reesei*, which starts from the ER and follows onto the Golgi bodies and then progresses to vesicles (Ghosh *et al.*, 1982; 1984; 1990). Our data has confirmed that *T. reesei* has the intracellular facilities of a classical secretion pathway from the ER to Golgi and further via vesicles to the cell wall.

**9.10 Subcellular protein localisation of the secreted CBHI-Venus**

Intracellular localisation of the fusion protein CBHI-Venus was initially carried out in the transformant CV48 by visualising the VenusYFP fluorescence with CLSM. Subsequently, subcellular localisation of the CBHI-Venus fusion protein in the *T. reesei* hyphae was further analysed by dual labelling of live CV48 with some fluorescent organelle markers, determined by CLSM. Finally, immunoprobe localisation of the CBHI-Venus fusion protein in the hyphae of CV48 was conducted by immunofluorescence with resin-embedded semi-thin sections and post-embedding IEM.

In the current study, combining the live cell imaging and immunofluorescence labelling of semi-thin sections with confocal fluorescence microscopy as well as immunogold probing of ultrathin sections by TEM can be considered as a type of correlative microscopy. In our microscopy work,
different parts of the sample instead of the same regions/parts of a sample were used. Combined application of light and EM allowed correlating and integrating the data from visualisation of colocalisation of the fluorescence of CBHI-Venus with organelle-specific fluorophores in living cells, and immunolocalisation of the CBHI-Venus by immunofluorescence and immunogold labelling using fixed cells.

9.10.1 Protein localisation in the ER
Observation of the fluorescence of the CBHI-Venus in the living CV48 hyphae showed that fluorescence distribution was not clearly distinguished at the early stage of 12 h (Fig. 7-2A). At 18 and 24 h, a brighter fluorescence appeared in a reticulate network assumed to be ER cisternae indicating active association of the fusion protein with the ER (Fig. 7-2B and C). Colocalisation of the CBHI-Venus fluorescence and the chemical ER marker ER-Tracker\textsuperscript{TM} Red confirmed that the secreted CBHI-Venus was indeed present in the ER (Fig. 7-5A). In the IEM micrographs of cultures at 24 and 48 h, the CBHI-Venus label was detected on tubular membranous cisternae typical for the ER (arrow heads in Fig. 7-4A and B). However, association of the CBHI-Venus with the ER was less prominent at later time points (72h, 96 and 120 h) indicating that the fusion protein CBHI-Venus had left the ER and became packed into secretory vesicles (Fig. 7-2E-G). Interestingly, in CV48, the ER membranes seemed extended to some distorted membrane structures with gold label for CBHI-Venus at 24 h (arrows in Fig. 7-4A and C) and 48 h when the distortion became more prominent (arrow in Fig. 7-4D). In contrast, no such aberrant ER membranes were seen in the host stain Rut C-30 (Fig. 6-5E). The finding of the ER morphology modification and formation of an ER-derived sub-domain filled with the recombinant protein CBHI-Venus was similar to what was seen in the transformant BV47 where punctate “BiP bodies” were associated with overexpression of the BiP1-Venus fusion protein (Fig. 6-4A, 6-5A and 6-6A-C), therefore supporting the suggestion that protein overexpression of CBHI-Venus can also lead to structural changes in the ER (Mullins, 2004). The ER dilation has been reported to be caused by overexpression of an islet-specific glucose-6-phosphatase catalytic subunit-related protein in pancreatic Beta-cells (Shameli \textit{et al.}, 2007). The transformant CV48 contained at least two copies of the \textit{venus} gene, therefore, the bulk synthesis of recombinant protein may have overloaded ER mechanism causing ER distortion. As a result, the overproduced CBHI-Venus aggregated in these ER-derived structures to await further processing i.e. transport to Golgi for further modification or vacuoles for degradation.
9.10.2 Protein localisation to the Golgi

Colocalisation of the CBHI-Venus fluorescence with the staining of the Golgi marker BODIPY® TR ceramide was visualised by CLSM and the results showed that CBHI-Venus was occasionally localised to the Golgi (Fig. 7-5B). Using an anti-GFP antibody, CBHI-Venus was found in the cytoplasm as punctate-like structures in the samples of 24 and 48 h (stars in Fig. 7-4A and B) which were assumed as Golgi bodies since their shape and size agreed well with those of the Golgi apparatus stained with anti-Vps10p antibody (Fig. 7-4F). The reason for membrane invisibility was likely because the fixation method used in the IEM work failed to preserve the Golgi membrane structure as discussed in Chapter 7. In general, localisation of the CBHI-Venus fusion protein to the Golgi suggested that the Golgi apparatus did play a role in protein secretion in T. reesei.

9.10.3 Protein localisation to the secretory vesicles

Bright round-shaped spots visualised in the T. reesei hyphae after 24 h growth in the CLS medium (pH 6.5), were interpreted to represent vesicle-like structures containing CBHI-Venus protein (Fig. 7-2C-G). Dual staining of the 24 h-old living hyphae of CV48 with the vesicle membrane marker FM® 4-64 supported that the CBHI-Venus fusion protein had entered into the vesicles (Fig. 7-5C). In the IEM experiment, the recombinant CBHI-Venus was seen in vesicles more often at 48 h than at 24 h (double arrowhead in Fig. 7-4B). From 72 to 96 h, the concentration of CBHI-Venus into the vesicles was more discernible by viewing the CBHI-Venus fluorescence under a confocal microscope (Fig. 7-2E-G). These data suggested efficient transport of the CBHI-Venus fusion protein from the ER to Golgi along the secretory pathway. In terms of distribution of the vesicles filled with the CBHI-Venus fusion protein, there was no distinctive difference between the apical and sub-apical cells indicating that protein secretion activities occurred simultaneously in different compartments and secretion also happened in each compartment without long-distance transport through the septum, in addition to apical release.

9.10.4 Protein localisation to the vacuoles

In the transformant CV48 expressing the CBHI-Venus fusion protein, studied under a confocal microscope, the fluorescent vesicles seemed to have fused to vacuoles at the late growth stages, i.e. 120 h and formation of vacuoles occurred in both the apical and sub-apical segments of the hyphae (Fig. 7-2G). Vacuoles may be involved in protein secretion in filamentous fungi (Kubicek et al., 1993b) and it is also likely that functionally different sub-populations of vacuoles exist in
fungi to perform a multitude of tasks, although experimental evidence for this is not yet available (Weber, 2002). For example, a constitutive acid phosphatase was secreted via the fusion of vacuoles with the plasma membrane in *Botrytis cinerea* (Weber and Pitt, 1997). Secretion of lignin peroxidases from *P. chrysosporium* has also been proposed to occur via vacuoles (Kuan and Tien, 1989). In our case, the roles of vacuoles in *T. reesei* would be assumed to be related to the degradation of proteins (Holkeri *et al*., 1998) since the vacuole formation occurred at the late growth stages and their presence was somehow associated with a decrease in the extracellular yield of the CBHI-Venus fusion protein as measured by Western blotting (Fig. 7-6).

### 9.10.5 Protein localisation to the septa

It is currently unknown how much the septa contribute to protein secretion in filamentous fungi (Shoji *et al*., 2008). Secreted proteins fused with EGFP often localise to the septa (Gordon *et al*., 2000b; Masai *et al*., 2006), suggesting the occurrence of protein secretion directed to the septa in addition to the default route through hyphal tips. Consistent with this, plasma membrane SNAREs have also been found at the septa (Kuratsu *et al*., 2007). In the recombinant strain CV48, the septa were often labelled with the VenusYFP in both young and old hyphae (Fig. 7-2B-D) suggesting that protein trafficking occurred actively via septal pores over time in *T. reesei*.

### 9.10.6 Protein localisation to the cell wall

The CBHI-Venus fusion protein in the CV48 strain seemed to have localised throughout the cell wall at the later culture stages i.e. at 72, 96 and 120 h (Fig. 7-2E-G). Localisation to the cell wall at earlier stages was hardly seen (Fig. 7-2A-C). In a *C. albicans* study, a secreted glycoprotein was localised throughout the cell wall (Poulain *et al*., 1989). Moreover, a low-molecular weight alkaline xylanase in *T. reesei* and an invertase in *N. crassa* have been shown to localise and accumulate in the cell wall (Kurzatkowski *et al*., 1993). Although the porous nature of the nascent cell wall at the hyphal apex facilitates protein secretion (Wessels, 1993), efficient secretion of enzymes in *N. crassa* strains with deficient cell wall synthesis suggested that the cell wall acted as a barrier for protein secretion (Pietro *et al*., 1989). It has been pointed out previously that many cell wall-attached proteins can be covalently linked to 1, 3–β-glucans in the yeast cell envelope (Kapteyn *et al*., 1999; Smits *et al*., 1999), therefore it is likely that CBHI-Venus may also have reacted with the 1, 3–β-glucans in the cell wall by covalent bonding.
Localisation of the CBHI-Venus in the cell wall indicating entrapment could therefore provide a further explanation for the decrease of the amount of this protein in the extracellular medium after 96 h growth in the CLS medium (pH 6.5) (Fig. 7-6).

9.11 Comments on the FRET pair of GFP2/VenusYFP

GFP2 and VenusYFP are two improved mutant forms of GFP with a faster maturation rate and higher emission intensity compared to the previous GFP variants. GFP2 has been used as a donor to couple with YFP in FRET studies and VenusYFP has also contributed to FRET work as an acceptor together with the donor CFP (reviewed in Section 1.3.1.1). However, there were no reports of the use of GFP2/VenusYFP as a FRET pair when this work was started.

Some problems were noted when using the GFP2/VenusYFP as a FRET pair, for example, the emission spectra of the GFP2 donor and VenusYFP acceptor demonstrated significant overlap (Fig. 3-7A), making it difficult to separate emissions of the donor and the acceptor in our FRET study. This problem can be rectified by using spectral imaging and subsequent spectral un-mixing (Zimmermann et al., 2002). However, the pre-existing expertise in spectral un-mixing was not available for the current study. Thus, narrow emission collection ranging from 510-530 nm for GFP2 was adopted in our case to minimise emission overlap between the donor and acceptor. As a result, the power level of the 405 nm laser had to be elevated to reach a proper donor signal level therefore resulting in a possible bleaching effect on the donor. This donor photobleaching may have been responsible for the weak energy transfer measured in the positive control stain VG15 (Chapter 8). While this work was in progress, Verrier et al. reported their success with FRET using the same GFP2/VenusYFP pair in Vero cells with an average FRET efficiency of 0.22 % (Verrier et al., 2008). It is worth mentioning that the authors did not measure FRET with the GFP2/VenusYFP pair by microscopy but by spectrofluorimetry while in the same paper FRET microscopy was conducted with another commonly-used CFP/YFP pair. It is unclear whether the attempt not to use microscopy was because of difficulty in imaging due to the emission overlap.

Systematic studies have clearly demonstrated the importance of minimising the donor-acceptor distance for FRET efficiency (Evers et al., 2006). However, in most cases, linkers of varying length and composition need to be tested in a trial-and-error manner in order to determine an
optimal linker sequence (Ni et al., 2006). Good FRET responses have been reported with a donor-acceptor linker of 4 aa (Cai et al., 2007a), 13 aa (van der Krogt et al., 2008), 14 aa (Zhang et al., 2005), 16 aa (Bayle et al., 2008) and 17 aa (Shcherbo et al., 2009). The length of the linker separating the GFP2 donor and VenusYFP acceptor in our positive control construct was 5 aa which should be short enough to enable close proximity of the donor and acceptor. However, our data showed that the FRET efficiency in the positive control strain VG15 was relatively low (0.16) with the calculated distance of 8.44 nm suggesting the distance between the donor and acceptor depends not only on the quantity of the amino acid but also their composition since there is a considerable variation in the sizes of amino acids (i.e. the length and bulkiness of the side chain residues) (Biro, 2006).

FRET depends not only on the spectral overlap and donor-acceptor distance but also strongly on fluorescent dipole orientation (Patterson et al., 2000). An improved dipole orientation can be obtained by substituting the VenusYFP with circular permutation mutants of VenusYFP in the linker-containing constructs (van der Krogt et al., 2008). It is unclear whether the dipole alignment of the donor and acceptor was optimal for FRET in the reporter used in the current work and more work needs to be done to eventually boost the FRET signal in the GFP2/VenusYFP FRET pair.

A considerable advantage of GFP2 and VenusYFP for fluorescence microscopy is brightness and fast maturation. An option to reduce the problem of emission overlap between the donor may be to take advantage of other GFP mutants, e.g red-shifted variants such as mCherry with the maximal emission at 610 nm (Larina et al., 2009) and DsRed with the maximal emission at 558 nm (Elangovan et al., 2003). For example, VenusYFP could be tried as an acceptor to pair with DsRed and GFP2 as a donor to couple with mCherry. These FRET pairs are considered feasible in terms of overlap of donor emission and acceptor excitation based on the published data (Kwok et al., 2008; Vidi et al., 2008; Albertazzi et al., 2009).

9.12 Concluding remarks and future perspectives

The work conducted for this thesis featured visualisation of the secretory pathway in an industrially important fungus Trichoderma reesei. A series of expression constructs featuring genetic tags encoding fluorescent proteins were produced to study protein secretion and
interaction. Together with chemical organelle-specific dyes, a number of secretion-related compartments including the ER, Golgi, secretory vesicles, vacuoles and cell wall were visualised and shown to be involved in protein secretion in *T. reesei*. The VenusYFP tag was specifically used to track secretion of the main cellobiohydrolase I (CBHI) in the living fungal hyphae. In order to achieve “visible” data on the secretory pathway in *T. reesei* using the above tools, a large amount of microscopy work at both the CLSM and EM levels was carried out. Correlative microscopy was applied with an attempt to subcellularly localise Bip1-Venus in the BV47 transformant and CBHI-Venus in the CV48 transformant in live cells.

As with every thesis, there are several areas where further work could be carried out as outlined below.

(i) Subcellular localisation of the CBHI-Venus fusion protein in the CV48 strain showed that the CBHI-Venus was actively associated in the ER. However, FRET studies of the transformant BGCV101 displayed considerably low energy transfer between the donor (BiP1-GFP2) and the acceptor (CBHI-Venus). Thus, it would be helpful to clarify the interaction between BiP1 and CBHI by colocalisation studies at the IEM level by dual labelling of the same transformant BGCV101 with the anti-BiP antibody and anti-CBHI antibody.

(ii) Dual labelling work using the anti-BiP antibody and anti-CBHI antibody can be conducted by IEM with the transformant CV48 as proposed above for the BGCV101 strain to particularly address the role of native BiP1 in protein secretion in *T. reesei*.

(iii) A better FRET system with the donor GFP2 and the acceptor VenusYFP expressed at a more optimal ratio e.g. 1:10 may improve the FRET efficiency between BiP1-GFP2 and CBHI-Venus. The ratio could be manipulated by choosing promoters with different expression ability and/or screening transformants with a range of gene copies. A shorter linker between the donor (GFP2) and acceptor (VenusYFP) in the positive FRET control may produce a better control. In addition, application of FLIM technology or spectral imaging and unmixing of the GFP2/VenusYFP pair may increase opportunities to obtain stronger FRET signals.
(iv) A transformant expressing a single copy of bip1-venus gene from the native bip locus under the native bip promoter could help visualise the natural architecture of the ER in T. reesei. Comparison between this new BiP1-Venus construct and the available BV47 strain overexpressing BiP1-Venus will provide more information on the relationship between protein overexpression and the ER morphology, and further, with the ER stress.

(v) More visual information about the secretion blocks could be obtained by obstructing the secretory pathway by treating the existing transformant CV48 expressing the CBHI-Venus fusion protein with some commonly-used chemicals such as dithiothreitol (DTT) and brefeldin A (BFA).

The present study will contribute to the current understanding of the secretory pathway and protein secretion in filamentous fungi especially under the situation of overexpression of recombinant proteins in the industrially-exploited filamentous fungus T. reesei. Consequently, it will help develop better production strains, to work out strategies for removing secretion blocks and eventually, to increase recombinant protein production in filamentous fungi.
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Appendix (Publications)
Confocal microscopy of FM 4-64 tagged membranes in the living fungus *Trichoderma reesei*

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Visualization of the protein secretory pathway in the high secreting *Trichoderma reesei* (*T. reesei*) hyphae using confocal microscopy will lead to better understanding of the cellular mechanisms involved in protein secretion and contribute to the identification of bottlenecks in the secretion of foreign proteins from fungi. An introduction into visualization approaches involved the application of the fluorescent dye FM 4-64 for staining of membrane-based structures in *T. reesei* hyphae. Confocal microscopy studies were carried out with 24-hour old cultures of the *T. reesei* strain Rut-C30. Staining of the fungal hyphae and characteristics of FM 4-64 including spectral properties, time course of the labeling and double labeling with other specific organelle dyes will be discussed.

*OCIS codes*: 180.1790, 110.0180, 170.0180, 180.0180.

*Trichoderma reesei* (*T. reesei*) is a biotechnologically important organism with an excellent capability of secreting high levels of hydrolytic enzymes, e.g., cellulases into the external medium[1,2], therefore it is well known as an efficient cell factory for protein production. In spite of gram per litre levels of secreted enzymes, products from heterologously-expressed genes have remained much less abundant[2]. Research so far has shown that these proteins are lost somewhere in the secretory pathway, therefore giving an indication of intracellular secretion bottlenecks[2]. Advanced fluorescence microscopy[3,4] techniques now allow novel approaches to address the secretion bottlenecks by the visualization of the secretory pathway and proteins traveling through it. This requires fluorescent tagging of both the organelles and cell structures in the secretory pathway as well as the secreted protein(s) of interest so they can be followed along the secretory pathway using fluorescent microscopy. Since the cell structures and organelles in the secretory pathway (e.g., endoplasmic reticulum (ER), Golgi, and secretory vesicles) are membrane-based, a fluorescent probe is required to visualize the relevant components. After this initial mapping, some of the cell structures such as ER and Golgi can be studied in more detail using organelle-specific fluorophores such as an ER stain DiOC6(3) and a Golgi stain BODIPY FL C5-Ceramide.

FM 4-64, one of amphiphilic styril dyes (see http://www.probes.com), is being increasingly used for the identification of firing neurons[5], studying vesicle trafficking and organelle organization, investigating endocytosis and exocytosis, and as fluorescent tracers of cell morphology and fluid flow. It is commonly believed that FM 4-64 enters the cell primarily by endocytosis[4,6]. After internalization, FM 4-64 is distributed to different organelle membranes, probably primarily via the vesicle trafficking network whereby components of the secretory pathway can become labeled[4,7-9]. Importantly, FM 4-64 has been successfully applied in combination, for example, with the molecular fluorophore green fluorescent protein (GFP)[10]. FM 4-64 is membrane-selective and stains many organelle membranes, therefore, it can be used as a vital marker to monitor organelle organization and dynamics in general[11,12]. FM 4-64 has been used extensively in the studies of mammalian[13] and plant cells[10,14-19], and there is a reasonable amount of data on its staining characteristics in a range of fungal species including *Aspergillus nidulans*, *Borriyis cinerea*, *Magnaporthe grisea*, *Neurospora crassa*, *Phycomyces blakesleeanus*, *Puccinia graminis*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Trichoderma viride[16]*, none of which is an industrially-exploited cell factory. To date, there are no reports on the use of FM 4-64 in living *T. reesei*.

In this work, we focus on the staining characteristics of FM 4-64 in the living *T. reesei* hyphae using confocal laser scanning microscopy. Our aim is to obtain an overview of the membrane network involved in protein secretion in *T. reesei* as a prelude to localization of proteins of interest in the secretory pathway and identification of secretion blocks for heterologous gene products. We will also report on double-labeling using organelle-specific dyes in combination with FM 4-64.

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<th>Table 1. Confocal Microscopy Conditions</th>
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<td><strong>Laser Power/Full Intensity (%)</strong></td>
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<td>Carbocyanine DiOC6(3)</td>
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<td>BODIPY FL C5-Ceramide</td>
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<td>SYTO 40</td>
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The dyes FM 4-64 (for molecular structure, see Fig. 1), carbocyanine DiOC₆(3), BODIPY FL C5 Ceramide, and SYTO 40 were obtained from Molecular Probes Inc. (U.S.A.). All other chemicals were supplied by Sigma (U.S.A.).

*T. reesei* Rut-C30 strain was grown in a medium which contained (w/v) 1.5% KH₂PO₄, 0.5% (NH₄)₂SO₄, and 1% lactose on an orbital shaker at 250 rpm at 28 °C. The inoculum used was 10⁶ conidia/50 ml of growth medium in a 250-ml conical flask. Cultures were harvested by centrifugation after 24 h and the pellet was collected.

The above 24-h old cultures were directly stained with 33 μM FM 4-64 dissolved in 0.9 % NaCl in an incubator at 28 °C for 60 min for the emission spectra scans (zyl experiment). For double-staining, FM 4-64 was combined with DiOC₆(3) or BODIPY FL C5 Ceramide, or SYTO 40. For staining, we used 33 μM FM 4-64 together with 5 μg/ml carbocyanine DiOC₆(3) or 4.2 μM BODIPY FL C5 Ceramide, or 10 μM SYTO 40, incubated for 1 h at 28 °C. All dye solutions were prepared in 0.9% NaCl.

For imaging, the above stained cells were transferred into wells of a 8-well chambered coverslip (Nalge Nunc International Corp., Naperville, IL) and resuspended in a dye-free culture medium containing 1.5% KH₂PO₄, 0.5% (NH₄)₂SO₄, 1% lactose, and 0.5% methyl cellulose (MC) which was applied to semi-solidify the medium to stop the cells from moving around during imaging. For the time course scans (zyt experiment), 50 μl of 24-h old cultures in the above semi-solid medium with 33 μM FM 4-64 were transferred into an 8-well chambered coverslip and incubated at 28 °C using a stage with temperature control.

A SPM2 confocal laser scanning microscope from Leica microsystems with full spectral capabilities was employed to study the emission spectra (zyl), time course (zyt) of FM 4-64 in living *T. reesei*. To study co-staining of the hyphae with FM 4-64 in combination with other organelle stains, an HCX PL APO CS100 × oil immersion objective was used. Confocal microscopy conditions for

![Fig. 1. Molecular structure of FM 4-64.](image)

**Fig. 1.** Molecular structure of FM 4-64.

![Fig. 2. Emission spectrum of FM 4-64 (330 μM) in 0.9% NaCl without T. reesei sample for excitation at 488 nm.](image)

**Fig. 2.** Emission spectrum of FM 4-64 (330 μM) in 0.9% NaCl without *T. reesei* sample for excitation at 488 nm.

![Fig. 3. Emission spectrum of FM 4-64 at 33 μM in living 24-h old culture of *T. reesei* for excitation at 488 nm (green curve represents fluorescent region of interest, purple represents background).](image)

**Fig. 3.** Emission spectrum of FM 4-64 at 33 μM in living 24-h old culture of *T. reesei* for excitation at 488 nm (green curve represents fluorescent region of interest, purple represents background).

![Fig. 4. Comparison of emission spectra of FM 4-64 in methanol (right curve) and *T. reesei* sample (left curve) for excitation at 488 nm. Δ shift = 104 nm.](image)

**Fig. 4.** Comparison of emission spectra of FM 4-64 in methanol (right curve) and *T. reesei* sample (left curve) for excitation at 488 nm. Δ shift = 104 nm.

different dyes and experimental set ups are as shown in Table 1.

The spectral properties of FM 4-64 were investigated by testing the emission spectra for both pure dye solution (330 μM) diluted in 0.9% NaCl and 24-h old *T. reesei* cells stained with 33-μM FM 4-64. No dye emission was obtained at excitation of 488 nm in the FM 4-64 solution without the sample, and the signal intensity was very low (Fig. 2). FM 4-64 in *T. reesei* gave a specific emission curve (Fig. 3) with a similar shape as the pure dye in methanol (see http://www.probes.com). The fluorescence of FM 4-64 in *T. reesei* appeared to have blue-shifted up to 104 nm, with maximum emission at 646 nm (Figs. 3 and 4). The most distinctive emission range was at 560–720 nm for 488-nm excitation (Fig. 4).

Examination of the early dye uptake revealed that plasma membrane staining was immediate (Fig. 5(a)) and staining became more pronounced after 20 min in the dye-containing medium (Fig. 5(b)). Subsequently, light staining of the hyphal cytoplasm could be seen, which increased with time (Figs. 5(c)–(e)). Between 105 and 120 minutes after adding the stain, more fluorescent structures were observed throughout the whole cytoplasm (Figs. 5(f) and (g)). After 135 min, numerous organelle membranes had been stained, and vacuolar membranes were visible as well (Fig. 5(h)). Fluorescence intensity after dye application increased over time.
The signal was discernible immediately after 1.75 min and remained constant during the early staining stage. After 5 min, the fluorescence intensity increased dramatically. From 75 min onwards, the intensity increased relatively slowly until a plateau was reached.

Double-labeling experiments with FM 4-64 and some other organelle-specific chemical dyes were applied to identify co-localization of the fluorescent stains. This work was especially directed towards further visualizing individual components, such as ER and Golgi, which are vital organelles involved in secretion. Therefore, the ER-dye DiOC₆(3) and Golgi-dye BODIPY FL C5 Ceramide were loaded individually to FM 4-64. It should be noted that discrimination between ER and Golgi through their different morphology using confocal microscopy is beyond the limits of the imaging resolution (maximum: 200 nm) of the system employed in this study. A DNA-stain SYTO 40 was applied together with FM 4-64.

When stained with DiOC₆(3), the T. reesei ER appeared as a fluorescent strand-like network within the cytoplasm (Fig. 7(a)). A superimposed image of the dual-labeled hypha (Fig. 7(c)) showed apparent co-localization of the FM 4-64-stained membranes with ER. Plasma membrane was stained only with FM 4-64 (Figs. 7(a)–(c)). The association between Golgi and FM 4-64-labeled membranes gave similar results, indicating co-localization of Golgi and FM 4-64-labeled membranes in the cytoplasm but not in plasma membrane (Figs. 8(a)–(c)). There was no overlapping staining using a DNA-specific SYTO 40 and FM 4-64 (Figs. 9(a)–(c)).

The spectral properties of FM 4-64 appeared to change depending on the microenvironment provided by different cell types and growth media because of its aromatic nuclei (Fig. 1). Thus, emission spectra in pure solutions can sometimes be misleading when working with living cells. FM 4-64 has an excitation peak at 505 nm with an emission peak at 750 nm in methanol. No significant emission curve was observed for FM 4-64 in 0.9% NaCl solution, hence confirming that it emits significant fluorescence only when on a lipid-rich membrane.
Therefore, there was no need to wash the dye out of the medium in order to study hyphal staining. This will provide an excellent platform for the next stage of work where a target protein will be traced through the secretory pathway. The emission spectrum of FM 4-64 varies between different cell types. For instance, emission peaks of FM 4-64 at 640 nm and 670 nm have been reported in yeast and plant cells respectively. We found the maximum emission of FM 4-64 in T. reesei to occur at 646 nm indicating its potential to be combined with GFP which is normally fluorescent between 470-560 nm with emission peak at 509 nm. We routinely excite FM 4-64 using 488-nm laser line and image its fluorescence between 560 and 720 nm. GFP provides an excellent candidate for genetic tagging of a secreted protein.

In fungal or other eukaryotic cells, the successive staining of the cell plasma membrane followed by the membranes of different intracellular structures is time-dependent and it is believed that the intake of the dye happens by an active endocytic internalization process rather than unfacilitated diffusion. A membrane-crossing activity for FM 4-64 is suggested because of the action of a flippase enzyme. Once the dye has been translocated to the inner leaflet of the plasma membrane by flippase activity, lipid transfer proteins may then transport dye molecules to the cytosolic face of the membranes of other organelles. Owing to the water solubility of these dyes and reversible incorporation into many membranes, they could enter the cytosol from the cytoplasmic face of the plasma membrane and then label the external leaflet of the organelle membrane. FM 4-64 is assumed to follow this pathway from plasma membrane to target organelles such as Golgi and finally to vacuolar membranes. Evidence also supports that it recycles back to the plasma membrane. Our observations of the time-dependent sequence of FM 4-64 staining indicate that the pathway of dye distribution in living T. reesei closely resembles the pathway discussed above. This also confirms that FM 4-64 is a powerful experimental tool to label components involved in the secretory pathway.

One of the current debates regarding FM 4-64 staining in live cells is organelle specificity. No staining of nuclear membranes by FM 4-64 has been reported. Our findings with living T. reesei support this claim. ER or Golgi membranes have not been previously shown to be stained with FM 4-64 and on two exceptions, a study on Fucus cells and on a BY-2 cell line. Our results indicate co-localization of FM 4-64 with ER and Golgi as well, therefore providing another positive example to add to the two cases mentioned above. Further evidence may be obtained by studies involving genetic tagging of ER and Golgi with a fluorescent marker and FM 4-64 staining of the cells.

In conclusion, FM 4-64 can be used as a powerful experimental tool for visualizing membrane-based structures in living T. reesei, hence providing a basic organelle map to trace genetically tagged proteins traveling along the secretory pathway. However, the complexity of the staining pattern of intracellular membrane structures has to be taken into account when identifying organelles such as ER and Golgi.

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References
Fluorescence labelling

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Fluorescence labelling has become a technique of increasing importance in modern biotechnology which is increasingly underpinned by advances in materials science. In this paper we describe our contributions to this area. In order to expand the understanding of in vivo fluorescence labelling we carried out the staining of membrane-based structures in effectively secreting Trichoderma reesei using the fluorescent dye FM 4-64 and their confocal microscopy studies. We also describe the observation of efficient fluorescence upconversion in Sm-doped Gd₂O₃ nanopowders synthesised by the spray pyrolysis method. This result indicates the potential for Sm-doped Gd₂O₃ to perform as a fluorescent label excited in red, yellow and green and emitting in blue. Finally, we report a simple approach for synthesizing water-soluble CdS nanoparticles by using ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) as a stabilizer.

Keywords: fluorescence labelling, europium doped Gd₂O₃, samarium doped Gd₂O₃, upconversion, cadmium sulfide nanoparticles.

1. Introduction to fluorescence labelling

The technique of fluorescence labelling has revolutionized modern life sciences. Fluorescent molecules or nanoparticles can be designed to bind with biological macromolecules where they offer scope for quantitative analysis of biochemical reactions. Many fluorescent dyes localise within specific organelles including cytoskeleton, mitochondria, Golgi apparatus, endoplasmic reticulum and nucleus making it possible to establish a detailed picture of cellular environment and its processes. Thanks to environmental sensitivity the fluorescent labels can be used to monitor dynamic processes and localised environmental variables such as: concentration of metallic ions, pH, reactive oxygen species, membrane potential. A significant body of work exists concerning monitoring of cellular integrity, endocytosis, exocytosis, membrane fluidity, protein trafficking, signal transduction and enzymatic activity. Finally, through advanced fluorescent assays and technologies such as gene chips, fluorescent labels facilitate genetic mapping and chromosome analysis in molecular genetics.
It is worthwhile to establish the length scales relevant for biological labelling. Small dye molecules such as fluorescein (FITC) are about 1 nm in size. Fluorescent proteins such as green fluorescent protein (GFP) and its variants, that play a crucial role in modern biology because they can be genetically targeted, are around 10 nm in size. A typical bacterial cell is about 1 micrometer in diameter, while animal cells typically range from several micrometers to few tens of nm, but some can be much larger. These relationships emphasise that fluorescent labelling is well suited for most biological applications.

A wide variety of fluorescent molecules are now available for fluorescent labelling, and catalogues of companies such as Invitrogen list a very broad range of fluorophores to suit most applications. They are often characterized by high quantum efficiencies, but most molecular probes suffer from a narrow optimum excitation region, relative proximity of the excitation and emission regions being very close, relatively broad emission limiting their applicability as multiple labels and sensitivity to photobleaching. In response to these concerns semiconductor quantum dots (QD) have been developed. Most of the QDs available commercially are II-VI nanocrystals, whose optical properties are dictated by quantum confinement. Their spectrally narrow emission characteristics are accompanied by high brightness comparable to fluorescent dyes and the ability to excite multiple colours with a single excitation wavelength. The latter is their unique feature, beneficial for multiple labelling.

The semiconductor QDs presently used are mostly core-shell structures with the core of one II-VI material and the shell from another, often with higher bandgap, such as CdSe/ZnS pair. In order to be used for fluorescent labelling the QDs must be made water soluble and able to be conjugated to biomolecules. An ultrathin polymer layer and a covalently attached outer layer of streptavidin ensure easy access to chemical groups that facilitate binding. The final size of the QD is in the order of 10–15 nm, which is comparatively large with respect to the size of pores in cellular membranes. Consequently, applications of QDs in fluorescent labelling as detection reagents in microscopy must rely on special strategies to introduce these large objects to the cells. Such strategies sometimes exploit the internal cellular transport mechanisms. Other applications of QDs, for example, in DNA chips, flow cytometry and immunoassays experience less constraints and rapid progress is being made. QDs and QD-encoded beads are presently used as platforms for highly multiplexed assays in proteomics, genotyping and gene expression. Future applications for in vivo imaging not only in cells but also in tissues and living organisms are also under development.

Our research program is concerned with lanthanide doped nanocrystals. Lanthanide ions confer significant advantages to semiconductor nanoparticles that are important for fluorescence labelling. They offer the potential for multi-colour imaging with single wavelength excitation by using different ions. As with all lanthanides upconversion processes can be expected with excitation in NIR and the emission in visible. The NIR excitation offers deeper penetration into tissues and helps eliminate background luminescence. Additionally, upconversion offers higher efficiency than the multiphoton excitation. The lanthanide ions have comparatively long lifetime,
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useful for time-resolved methods and photobleaching important in fluorescent molecules should be strongly reduced.

We are reporting here our preliminary studies of in vivo fluorescent labelling which we carried out in the context of a systems biology program to map out the secretion pathway in a cell. Further we present the results of spectroscopic and laser scanning microscopy investigations of lanthanide doped nanoparticles. Finally, we present the preliminary studies of bulk wet chemical synthesis of such nanoparticles. The paper is divided into three sections describing these three groups of results.

2. In vivo fluorescence labelling using chemical dyes – tagging of membranes and selected organelles in the living fungus Trichoderma reesei

Trichoderma reesei is an industrially important microorganism that is able to secrete abundant quantities of native enzymes, in the order of grams per litre. The fungus can be genetically engineered to secrete valuable gene products such as growth factors, however at much lower yields, due to unidentified secretion roadblocks [1]. In order to be able to trace the secretion of endogenous and foreign proteins through secretory pathways they need to be visualized using fluorescent labelling.

FM 4-64 is one of amphiphilic styryl dyes whose properties are described at http://www.probes.com. We used FM 4-64 to visualize secretory pathways because it is membrane selective, compatible with fluorescent proteins (GFP/YFP) used in fluorescence resonance energy transfer (FRET) experiments, and it has also other advantages such as photostability and low-toxicity [2].

We examined the spectral characteristics of FM 4-64 in T. reesei, shown in Fig. 1. The dye in the living fungus shows the emission extending in the range 560 to 720 nm, with the maximum at 646 nm. The red curve shows the solution of the same dye in methanol. The spectral shift is due to the effect of cellular environment.

Fig. 1. Fluorescence of FM 4-64 in T. reesei cells (left curve). Right curve shows the spectrum of a pure dye solution in methanol. The spectral shift is due to the effect of cellular environment.
methanol, which is spectrally shifted by 104 nm towards longer wavelength. Such large shift is due to the effect of cellular environment on the dye molecules. It should be emphasized that this spectral characteristic is very different from GFP and RFP [3] and thus FM 4-64 can be used in *T. reesei* in conjunction with genetically targeted probes.

We also explored the time evolution of staining with FM 4-64. The intensity of staining progressively increases over a course of about 150 minutes and approaches saturation after that. Further we explored double labelling with organelle-specific dyes in *T. reesei*. Three dyes were used in this study, these were an endoplasmic reticulum (ER) dye DIOC6(3), a Golgi dye BODIPY FL C5 and a nucleus stain SYTO 40. These data are presented in Fig. 2. We observe that apart from the nucleus stain SYTO 40, none of applied staining seemed to be specific to single organelles in *T. reesei*.

### 3. Spectral properties of Gd$_2$O$_3$:Sm and Gd$_2$O$_3$:Eu doped nanoparticles and their potential for fluorescence labelling

The Gd$_2$O$_3$ nanoparticles examined in this study were produced by spray pyrolysis method [4], a simple technology that provides excellent doping control. The nanoparticles were doped with 10% Sm and 10% Eu and they showed fluorescence emission at a range of excitation wavelengths. Selected excitation and emission spectra for Gd$_2$O$_3$ (with the emission excited at 405 nm and excitation detected at 603 nm) are presented in Fig. 3. The spectra show a range of peaks that have been attributed to specific transitions within Sm shown in Fig. 4 [5–11]. A comparative set of data for Eu-doped nanoparticles is presented in Fig. 5. In both excitation curves for Sm and Eu we observe a broad excitation band, in contrast to bulk rare earth doped materials, where the excitation curve is composed of single isolated lines corresponding to appropriate transitions. In both types of nanoparticles we observe these transitions on a broad and intense background, which indicates that rare earth ions in nanoparticles can be excited using a broad variety of excitation wavelengths.

The results presented in Figs. 3 and 5 were taken using a fluorimeter with Xe lamp excitation at a spectral resolution of 2 nm in emission and 5 nm in excitation. The same
nanoparticles were examined using a laser scanning microscope Leica TCS SP2 with full spectral capabilities. The excitation wavelength used in these studies was 405 nm, the same as previously, and the spectral resolution was 5 nm. The main Sm and Eu lines were clearly visible under these conditions (data not shown).

Fig. 3. Selected excitation (a) and emission (b) spectra for Gd$_2$O$_3$ doped with 10% Sm. The emission is excited at 405 nm and excitation detected at 603 nm.

Fig. 4. Schematic diagram of transitions in Sm$^{3+}$. 
Further we explored the spectral properties of these nanoparticles with varying excitation wavelengths in a laser scanning microscope. The results presented here were taken for a Sm-doped sample excited at 710, 633, 594, 561 and 514 nm. We observed a clear fluorescence upconversion, in the form of a blue band that shifted with excitation energy (Fig. 6). The emission takes place at shorter wavelengths when excited at 514 nm and moves towards longer wavelengths with 710 nm excitation. These nanoparticles have also been functionalised [12] and used for fluorescence labelling studies of human lymphoma cells.

Fig. 5. Selected excitation (a) and emission (b) spectra for Gd₂O₃ doped with 20% Eu. The emission is excited at 405 nm and excitation detected at 614 nm.

Fig. 6. Fluorescence up-conversion for excitation at 514, 561, 594, 633 and 710 nm.
4. Synthesis of CdS nanoparticles in the presence of Eu and EDTA

In an effort to produce larger quantities of rare earth doped nanoparticles we attempted their wet chemical synthesis method following reference [13]. It should be noted that large difference in size of host cation and lanthanide ions, charge mismatch between the cations, different coordination numbers in crystallographic structures, and low affinity of lanthanide ions towards sulfur and selenium make it difficult to achieve doping inside of the nanoparticles. We have therefore explored the synthesis of II-VI nanoparticles with a capping layer capable of binding RE ions. Our approach was to use ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) both as a capping agent for synthesis of CdS nanoparticles and a ligand forming complex with Eu\textsuperscript{3+} in non-coordinating solvent ethylene glycol [13].

In the procedure, 532 mg CdAc\textsubscript{2}·2H\textsubscript{2}O is dissolved in 100 ml ethylene glycol (EG) solution at room temperature (solution 1); 78 mg thiourea and 372 mg EDTA are separately dissolved in another 100 ml EG solution at 120°C (solution 2). Under vigorous magnetic stirring, the solution 2 is quickly injected to the solution 1. The mixed solution is clear until it is heated to the EG’s boiling point (200°C); then after about 10 min the solution became milky yellow, indicating the formation of CdS nanoparticles.

The synthesis with addition of EDTA leads to better control of nanoparticle size and agglomeration, but still leads to formation of nanoparticles with broad size distribution and vacancy sulphur defect dominated fluorescence.

Fluorescence of CdS and CdS capped with Eu-EDTA complex nanoparticles is shown in Fig. 7. It is clear that CdS EDTA capped nanoparticles without Eu\textsuperscript{3+} show much weaker fluorescence, dominated by the surface and vacancy sulphur defects. The formation of EDTA–Eu complex increases band gap fluorescence of CdS. No energy transfer between NPs and RE ion was observed. This work is a preliminary

![Fig. 7. Fluorescence of CdS and CdS capped with Eu-EDTA complex nanoparticles excited at 405 nm.](image-url)
In summary, we described here our results of organelle-specific staining using chemical dyes in *T. reesei*, and current status of projects on upconversion and fluorescence labelling using rare earth doped Gd$_2$O$_3$ nanoparticles as well as novel approaches to wet synthesis of rare earth doped II-VI nanoparticles.

**References**


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