Chapter 7: General Discussion
Upon contact with hydrophilic-hydrophobic interfaces, hydrophobins self-assemble into a 10 nm thick amphipathic membrane (Wösten et al., 1993; 1994a; 1995). As a result, hydrophobins change the nature of a surface, which makes them interesting for use in medical and technical applications (Wessels, 1997). A requirement for successful application of hydrophobins is that they can be produced in large quantities (i.e. grams per liter) and that the assembled membranes meet specific biophysical and biochemical requirements (e.g. a certain hydrophilicity at the exposed side of the membrane). Therefore, the primary aim of the research described in this thesis was to improve the production levels of hydrophobins and to use genetic engineering to modify the properties of hydrophobins. During the course of this work the phenomenon of intron-dependent mRNA accumulation was discovered when Schizophyllum commune was transformed with genes either or not containing introns. In addition, it was shown that in S. commune the GC-content of a gene is crucial for mRNA accumulation.

**Heterologous expression of hydrophobins**

A wild-type strain of S. commune secretes up to 60 mg L\(^{-1}\) SC3 in its culture medium, which is the highest accumulation of a class I hydrophobin in a fungus ever observed. In an attempt to increase production of this hydrophobin, S. commune was transformed with additional copies of the SC3 gene. However, this resulted in silencing of both the introduced and the endogenous genes when more than one copy were introduced (Schuurs et al., 1997). Proteolytic degradation of hydrophobins in the culture medium of S. commune may also hamper production of hydrophobins. This would explain why some of the genetically engineered derivatives of SC3 could not be detected in the medium of liquid shaken cultures, although it was shown that they were secreted into the medium (Appendix to Chapter 6). Production of the dikaryon-specific S. commune hydrophobins SC1, SC4 and SC6 in monokaryotic strains also has proven difficult (Lugones, 1998; van Wetter, 2000; W.S. Kong, unpublished data). mRNA levels similar to that of SC3 were observed when these genes were placed under regulation of the SC3 promoter. However, only small amounts of protein, if any, could be detected in the medium of liquid shaken cultures. This indicates that the proteins are degraded or that production of these hydrophobins is limited at the post-transcriptional level. Attempts to produce hydrophobins in Escherichia coli and Hansenula polymorpha have also been unsuccessful. Production levels of the hydrophobins CU and POH1 in E. coli were low (µg quantities per liter) (Bolyard and Sticklen, 1992; Peñas et al., 1998) while we were unable to express the SC3 in this bacterium (R. Rink, unpublished) and in H. polymorpha (K. Scholtmeijer and E. de Bruin, unpublished). In addition, expression of the hydrophobins SC3 of S. commune (Chapter 2) and ABH1 and ABH3 of Agaricus bisporus (Chapter 3) in strains of Trichoderma reesei and/or Aspergillus niger was examined. These fungi have a high capacity to secrete proteins (up to 30 g L\(^{-1}\)) and are generally regarded as safe (GRAS). Moreover, large-scale fermentation, down-stream processing, mitotic crossing-over and mutagenesis-screening methods are well developed (reviewed by Davies, 1991; Jeenes et al., 1991; van den Hondel et al., 1991; Gwynne and Devchand, 1992; MacKenzie et al., 1993;
Initially, *A. niger* was chosen as a host for heterologous production of hydrophobins because it does not secrete hydrophobins in its culture medium (O.M.H. de Vries, unpublished data), although it produces a hydrophobin that coats conidia. This would simplify purification of the hydrophobin from the medium. SC3 was expressed in *A. niger* behind the *gpd* promoter of *Aspergillus nidulans* or as a fusion with the G2 form of *Aspergillus niger* glucoamylase (Chapter 2). Although mRNA accumulated at relatively high levels (20-40% of that observed in *S. commune*), SC3 levels in the culture medium were less than 1% of the amount secreted by *S. commune*. Heterologous proteins are often degraded in the culture medium of *A. niger* by the action of proteases. When SC3 was added to the medium of *A. niger* it was not degraded. Accordingly, when SC3 was expressed in protease deficient strains levels of SC3 did not increase. Similar results were obtained when ABH3 was expressed in wild-type or protease deficient strains. This indicates that problems with the production of hydrophobins in *A. niger* arise intracellularly and at the post-transcriptional level.

In Chapter 3 a sandwiched-culture technique is described, which can be used to screen filamentous fungi for production of heterologous proteins irrespective of proteases in the medium. This technique involves culturing of transformants between perforated polycarbonate membranes and transfer of this sandwiched-culture to a PVDF-membrane (Wösten et al., 1991). In this way secreted proteins, including proteolytic enzymes and the protein of interest, are immobilized on the PVDF membrane shortly after they have been secreted. This technique thus allows detection (e.g. by antibodies or activity assays) of secreted proteins that otherwise would be degraded. By using this technique it was shown that at least the production of the ABH1 hydrophobin of *A. bisporus* in *A. niger* was hampered by proteolytic digestion in the culture medium. This shows that, apart from intracellular, post-transcriptional problems (Chapter 2), heterologous expression of hydrophobins in *A. niger* may be hampered by degradation of the protein in the medium (Chapter 3).

In contrast to *A. niger*, *T. reesei* secretes high amounts of class II hydrophobins (HFBI and HFBII) in its culture medium (Nakari-Setälä et al., 1996; 1997). If hydrophobin production is dependent on the presence of specific proteins such as foldases or chaperones in the submerged mycelium, *T. reesei* would be a preferred host for hydrophobin production. Indeed, when SC3 was expressed in *T. reesei* behind the promoters of HFBI or HFBII (Chapter 2), levels of SC3 in the culture medium were similar or even higher than in *S. commune* (60 mg L\(^{-1}\)). Importantly, the protein could be purified from the medium of *T. reesei* by a procedure similar to that used to purify SC3 from the medium of *S. commune*; the presence of the class II hydrophobins HFBI and HFBII did not interfere. Production of SC3 in these strains may be further increased by (i) introduction of extra copies of the expression vector, (ii) targeting the vector to the cellobiohydrolase I (*CBH1*) locus, (iii) mutagenesis or (iv) by growing these strains under more optimized conditions (i.e. in a fermentor).
Furthermore, (v) the SC3-gene may be fused to a highly expressed endogenous gene (e.g. CBHI or EGI) in a similar way as was attempted for expression of SC3 in A. niger by fusion with the glucoamylase gene (Chapter 2). At the moment we are testing whether T. reesei is a generic host for production of hydrophobins.

Intron-dependent mRNA accumulation in S. commune

In an attempt to produce ABH1 heterologously in S. commune, the phenomenon of intron-dependent mRNA accumulation was observed (Lugones et al., 1999a; Chapter 4). When the genomic and the cDNA coding sequences of ABH1 of A. bisporus were introduced in S. commune, mRNA accumulated only in transformants containing the genomic ABH1 sequence. A similar effect was observed with the homologous hydrophobin genes SC6 and SC3. The presence of one intron within the coding sequence or upstream or downstream of the translational unit of the SC3 cDNA was sufficient to obtain SC3 mRNA levels similar to those obtained with the genomic coding sequence naturally containing 5 introns. Addition of an intron to a cDNA of the GFP-gene of Aequorea victoria was necessary to produce this protein in S. commune. Run-on analysis showed that the presence of introns in SC6 only affected mRNA accumulation and not the rate of transcription, showing that intron-dependent mRNA accumulation in S. commune is a post-transcriptional event. The fact that an artificial intron (containing consensus splice-and branch sites in an otherwise randomly generated sequence) could substitute for a native intron and that an intron only increased mRNA accumulation when inserted in the right orientation argues against the role of the introduced sequence as an enhancer.

The phenomenon of intron-dependent mRNA accumulation was previously shown to occur in mammalian and plant cells (Simpson and Filipowicz, 1996; Koziel et al., 1996 and references therein). The results presented in this thesis are in agreement with the general idea that the dependency of mRNA accumulation on introns is a post-transcriptional event (Simpson and Filipowicz, 1996; Koziel et al., 1996 and references therein). However, its mechanism is not clear. Not all genes of plant or mammalian origin contain or require introns for efficient mRNA accumulation and the requirement of introns in plants is species dependent. The mechanism involved in intron-dependent mRNA accumulation may depend on subtle differences in the specificity of association of ribonucleoproteins or spliceosomal proteins with the mRNA and the influence of splicing or spliceosome assembly on other events like capping, polyadenylation, turnover and transport into the cytoplasm.

Previously, short aberrant RNA’s were observed upon introduction of prokaryotic genes (e.g. the hph resistance cassette) in S. commune (Schuren and Wessels, 1994; 1998). It was suggested that this was due to AT-rich regions within the coding sequences of these genes. Indeed, truncation of transcripts in Aspergillus niger (Gouka et al., 1996) and Cryptococcus curvatus (J. Springer, unpublished data) was prevented by increasing the GC-content of AT-rich stretches. To examine whether introns could protect against truncation of transcripts with AT-rich stretches, S. commune was transformed with a construct containing the hph-gene with one or two introns outside the translational unit (Chapter 5). This,
however, did not result in accumulation of full-length \textit{hph} transcripts and in resistance to hygromycin B. Introduction of a modified \textit{hph}-gene in which an AT-rich sequence was replaced by a more GC-rich sequence, without affecting the encoded amino acid sequence, resulted in accumulation of full-length \textit{hph} transcripts and resistance to hygromycin B. In fact, the modified \textit{hph}-gene appeared to be as efficient for selection of transformants as the \textit{phleomycin} resistance cassette and is now the second available selection marker in \textit{S. commune}. In addition, mRNA levels could be increased by introducing a single intron in the modified \textit{hph}-gene, either upstream of the startcodon or downstream of the stopcodon. When both introns were present, mRNA levels were even further increased.

The results thus show that intron-dependent mRNA accumulation and truncation of transcripts are independent events that both can affect expression of the \textit{hph}-gene in \textit{S. commune}. It was shown that for \textit{SC3} (411 bp) addition of one intron outside the translational unit was sufficient to restore mRNA accumulation to wild-type levels (Chapter 4), while one intron in the \textit{hph}-gene (1020 bp) was not sufficient to reach maximal accumulation. This suggests that more than one intron is needed for efficient expression of genes encoding long transcripts.

The data also suggest that other prokaryotic genes not expressed in \textit{S. commune}, like β-glucuronidase and β-galactosidase (Schuren and Wessels, 1998), can be successfully expressed in \textit{S. commune} by replacing AT-rich stretches in the coding sequence with more GC-rich sequences in the presence or absence of one or more introns. This strategy may also result in increased efficiency of transformation of the commercially important basidiomycetes \textit{Agaricus bisporus} (common mushroom) and \textit{Pleurotus ostreatus} (oyster mushroom), which have proven recalcitrant to protoplast transformation using plasmids with prokaryotic antibiotic resistance genes. However, hygromycin B resistance has been achieved in \textit{A. bisporus} using an \textit{Agrobacterium tumefaciens} DNA transfer system (de Groot \textit{et al.}, 1998). In this case too, transformation efficiency may be increased by using the modified hygromycin B resistance gene and introduction of an intron.

\textit{Genetic engineering of the SC3 hydrophobin of \textit{S. commune}}

To examine structure-function relationships and to see whether properties of hydrophobins can be changed, derivatives of the SC3 hydrophobin were made: (i) a derivative of SC3 in which Cys130 (which is the 8th cysteine residue of SC3) was replaced by a glycine residue (CG-SC3), (ii) in which the region between the third and the fourth cysteine residue was replaced by the corresponding sequence of cerato ulmin (CU-SC3) and (iii) from which the codons for amino acids 76-86 of SC3 were deleted (HW-SC3). However, these modified proteins could not be detected in the culture medium (see Appendix to Chapter 6). This was probably caused by degradation of CG-SC3 at the intracellular level and of CU-SC3 and HW-SC3 at the extracellular level. Derivatives of SC3 with a modified \textit{N}-terminus could be produced in \textit{S. commune} (Chapter 6). In TrSC3 25 out of 31 amino acids preceding the first cysteine residue of mature SC3 were deleted. In addition, the cell-binding domain of human fibronectin (RGD) was introduced at the \textit{N}-terminal end of mature
SC3 (RGD-SC3) and TrSC3 (RGD-TrSC3). Biophysical characterization of the SC3 derivatives showed that the gross properties of the hydrophobins were unaffected. Self-assembly, hydrophobicity of the hydrophobic side and surface activity after assembly were unaffected. They also formed the characteristic rodlet layer, although the rodlets formed by the truncated forms of SC3 were smaller (about 8-10 nm in diameter) than those of SC3 and RGD-SC3 (10 nm in diameter). However, the biophysical properties of the hydrophilic side of the assemblage did change. TrSC3 (θ 69 ±2) and RGD-TrSC3 (θ 57 ±4) had a decreased wettability at their hydrophilic side compared to that of SC3 (θ 36 ±1) and RGD-SC3 (θ 46 ±8). The changes at this side of the membrane were shown to affect biocompatibility. Growth of fibroblasts (a tumor cell-line) on SC3-coated Teflon was similar to that on bare Teflon but was considerably increased by coating with RGD-SC3 or TrSC3. In the near future the biocompatibility of RGD-TrSC3 will be examined. Recently, it was shown that the presence of the RGD-tripeptide at the surface of a bone implant resulted in an enhanced and accelerated tissue integration (Schaffner et al., 1999). In these experiments the RGD-tripeptide was cross-linked to bovine serum albumin that had been adsorbed to the implant. Alternatively, a polymethylmethacrylate (PMMA) surface was functionalized via acrylic acid coupling. The use of hydrophobin derivatives in coupling the RGD-tripeptide to (hydrophobic) surfaces may offer advantages over these methods. Hydrophobic surfaces can be uniformly coated with hydrophobin independent of reactive groups at the surface. In addition, in contrast to for instance BSA, hydrophobins bind strongly (not being replaced by blood proteins) and in a preferred orientation to the surface while the use of genetically engineered hydrophobins eliminates the need for subsequent cross-linking steps.

Our results obtained with the derivatives of the SC3 hydrophobin indicate that engineered hydrophobins may be used in a broad range of medical and technical applications (e.g. biosensor or implant technology). Hydrophobins with altered biophysical properties (e.g. the wettability of the hydrophobic side of the assemblage) or exposing certain functional groups (e.g. peptides, proteins or antibodies) could be engineered. Using these engineered hydrophobins or blends of different kinds of hydrophobins may create surfaces that specifically bind certain molecules or cells.