Identification of fungal constituents that determine the sensitivity of fungi towards the antifungal protein (AFP) of *Aspergillus giganteus*

vorgelegt von
Diplom-Biologin
Silke Hagen

Von der Fakultät III - Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades
Doktorin der Naturwissenschaften
- Dr. rer. nat. -

genehmigte Dissertation

Promotionsausschuss:

Vorsitzender: Prof. Dr. rer. nat. Roland Lauster
Gutachter: Prof. Dipl.-Ing. Dr. Ulf Stahl
Gutachter: Prof. Dr. Cees van den Hondel

Tag der wissenschaftlichen Aussprache:
23.10.2006

Berlin 2006
D 83

Meinen Eltern

Danksagung

Die vorliegende Arbeit wurde im Zeitraum von Dezember 2002 bis April 2006 im Fachgebiet Mikrobiologie und Genetik des Institutes für Biotechnologie der TU Berlin erstellt.

Mein besonderer Dank gilt Herrn Prof. Dr. Ulf Stahl für die Bereitstellung des Themas, seine großzügige Unterstützung und die stete Bereitschaft zu konstruktiven Diskussionen.

Prof. Dr. Cees van den Hondel danke ich sehr herzlich für die Übernahme des Gutachtens dieser Arbeit.

Mein weiterer Dank gilt Frau Dr. Vera Meyer, in deren Arbeitsgruppe diese Arbeit angefertigt wurde. Sie stand mir als direkte Ansprechpartnerin hilfreich zur Seite und hat viel zum Gelingen dieser Arbeit beigetragen.

Bei Frau Barbara Walewska bedanke ich mich sehr herzlich für die tatkräftige Unterstützung sowie für ihr Interesse und das Engagement in diese Arbeit.

Herrn Dr. Udo Schmidt, Herrn Dr. Dirk Müller, Frau Dr. Jing Wesenfeld, Frau Anja Spielvogel, Herrn Jochen Schmid, Frau Cornelia Luban, Herrn Falk Matthäus, Frau Susanne Engelhardt und Frau Birgit Baumann danke ich für viele anregende Diskussionen - die nicht alle wissenschaftlichen Ursprungs waren, aber häufig dort endeten - und die überaus freundliche Laboratmosphäre.

Mein ausdrücklicher Dank gilt Herrn Prof. Dr. Jens Nielsen, Frau Dr. Isabel Roncero und Frau Dr. Katarina Jernejc für die Bereitstellung der in dieser Arbeit untersuchten Pilzmutanten. Des Weiteren bedanke ich mich bei Herrn Dr. Christian Hoischen für meinen außerordentlich angenehmen Gastaufenthalt am Hans-Knöll-Institut in Jena sowie die Ermöglichung mit bakteriellen L-Formen zu arbeiten.

Allen weiteren Mitarbeiterinnen und Mitarbeitern des Fachgebietes Mikrobiologie danke ich für die nette und kooperative Zusammenarbeit, insbesondere Frau Rita Waggad, Frau Roslin Bensmann und Frau Sonja Leberecht.

Abschließender und überaus herzlicher Dank gebührt meinen Eltern, meinem Bruder Niels und meinem Freund Dirk für deren stete Unterstützung und das unendliche Verständnis auch in schwierigen Zeiten.

Silke Hagen

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List of abbreviations

A	alanine
aa	amino acid
AFP	antifungal protein
Ala	alanine
AMP	antimicrobial peptide
AnAFP	*Aspergillus niger* antifungal protein
approx.	approximately
bp	base pair
C	cysteine
CBD	chitin binding domain
CHS	chitin synthase
Cys	cysteine
Da	Dalton
DNA	deoxyribonucleic acid
D-PDMP	*D*-threo-1-phenyl-2-decanoylamino-3 morpholino-1-propanol
EDTA	ethylenediamine-tetra-acetic acid
e.g.	for example
EUROSCARF	European *Saccharomyces cerevisiae* Archive for Functional Analysis
Fig.	Figure
g	gram
GlcNAc	*N*-acetylglucosamine
Gln	glutamine
h	hour
i.e.	id est
K	lysine
kDa	kilo Dalton
l	liter
Lys	lysine
M	mole
MIC	minimal inhibitory concentration
min	minute
nm	nanometer
NMR	nuclear magnetic resonance
MW	molecular weight
OB-fold	oligonucleotide/oligosaccharide binding-fold
OD	optical density
PAA	polyacrylamide
PAF	*Penicillium* antifungal protein
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
Q	glutamine
RNA	ribonucleic acid
rpm	rotations per minute
S	serine
sec	second
SDS	sodium dodecyl sulphate

T	threonine
Tab.	Table
Thr	threonine
TNM	tetranitromethane
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
Trp	tryptophan
Tyr	tyrosine
V	valine
Val	valine
V_{max}	maximum velocity
W	tryptophan
w/v	weight per volume
Y	tyrosine

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1 Antimicrobial peptides and their transcriptional regulation

1.1 Introduction

The demand for novel agents with antimicrobial activity is high: Rising numbers of immuno-suppressed patients constitute risk groups which are particularly prone to pathogen infection (McKee et al., 2000; Ratnam and Goh, 1994). At the same time, more and more pathogenic microorganisms become resistant to conventional drugs due to intrinsic or acquired resistance (Sefton, 2002). Attractive alternatives to chemically-derived agents constitute the large group of antimicrobial peptides (AMPs), which combine important aspects such as high potency and stringent specificity (Reddy et al., 2004a). Due to their beneficial characteristics, AMPs are also considered as ‘nature’s antibiotics’ (Wang and Wang, 2004). They are now regarded as basic elements for the generation of novel drugs to treat bacterial and fungal infections (De Lucca, 2000; Hancock, 2000).

1.2 Structure and classification

Many different species are known to express AMPs including bacteria, fungi, plants, insects, vertebrates and invertebrates. AMPs are gene-encoded, low-molecular weight proteins, generally consisting of less than 100 amino acids (Ganz, 2005). Their astonishing diversity in structure and chemical nature makes classification a difficult task. However, AMPs are categorised, rather arbitrarily, according to their biochemical or structural features (Tossi and Sandri, 2002). Besides anionic peptides, aromatic dipeptides, and many different processed forms of proteins, the cationic peptides constitute the largest group of AMPs. They can be divided into three subgroups: The linear peptides forming helical structures; the cysteine-rich and open-ended peptides, which contain one or several disulfide bridges; and the peptides rich in specific amino acids.

1.3 Function

Antimicrobial peptides form the first line of innate host defence in higher eukaryotes, in which they are essential factors in repelling pathogen attack (Zasloff, 2002). In contrast to the adaptive immune system - which may take days or weeks until it successfully responds to invasive attack - the innate immune system provides a rapid means to combat pathogen infection right from the start (Clark and Kupper, 2005). A general model describing fundamental aspects of the innate immune response is illustrated in Fig. I.

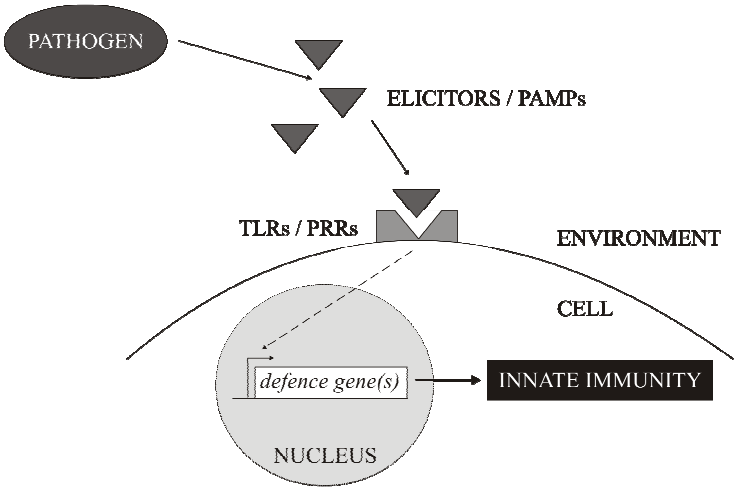

Figure I. Model outlining fundamental aspects in the innate immune response. Elicitors or pathogen-associated molecular patterns (PAMPs) are released by the invading pathogen. Defined receptors, such as the pattern recognition factors (PRRs) in plants or the Toll-like receptors (TLRs) in animals, perceive these pathogen-derived signals, subsequently resulting in defence gene transcription.

Distinguishing feature of the innate immunity comprises of pattern recognition receptors (PRRs) in plants or Toll-like receptors (TLRs) in animals, which usually exhibit a broad range of specificity. These extracellular receptors are able to recognize many related molecular structures referred to as pathogen-associated molecular patterns (PAMPs) (Nurnberger et al., 2004; Zipfel and Felix, 2005). PAMPs generally show little variance. They typically consist of polysaccharides and polynucleotides exclusively present in the invading pathogen. Translation of signals finally results in the transcriptional activation of defence-related genes (Fliegmann et al., 2004). Interestingly, no memory of prior exposure to a certain pathogen is required for PRR expression, which explains the rapidness by which the innate immune response is able to respond to invasive attack.

The effective range of AMPs was found to be not exclusively restricted to the innate immune system. These peptides were also shown to trigger and to interact with the adaptive immune response. Administration of defensins with antigens to mice was e.g. shown to enhance cellular as well as humoral cytokine production and immune response (Oppenheim et al., 2003). In metabolic terms, innate host defence is a greatly economic means of responding to pathogen attack, because only a limited amount of energy is invested into the expression of a basic defence machinery.

Although prokaryotes and lower eukaryotes are devoid of any kind of immune response, they are also known to produce peptides with antimicrobial potential (Cheigh and Pyun, 2005). In contrast to higher eukaryotes, however, these AMPs serve to defend assigned ecological niches against nutrient competitors. *Lactococcus lactis*, for instance, produces an AMP referred to as nisin, which belongs to the group of lantibiotics with antimicrobial activity against Gram-positive bacteria (Breukink and de Kruijff, 1999). Nisin was shown to effectively inhibit the growth of *Listeria monocytogenes*, a nutrient competitor which shares the same habitat as *L. lactis* (Bhatti et al., 2004).

1.4 Mechanism of action

Although they exhibit a rather wide range of variance, it is impressive to note that AMPs seem to operate *via* the same fundamental mode of action comprising of the permeabilisation of microbial membranes (Park and Hahm, 2005; Tossi and Sandri, 2002). Underlying prerequisite for the membrane perturbing effect is their small size, their cationic net charge and their amphipathic character (De Smet and Contreras, 2005).

A model for linear helical peptides with amphipathic character proposes that these molecules interact with membranes, resulting in an increase in membrane permeability. This can be effected either by the interaction of positively charged peptides with anionic lipids in the target membrane, or by the destabilisation of the membrane by lipid displacement. For cysteine-rich peptides, such as defensins, a similar mechanism has been suggested which implies the formation of ion-permeable pores in lipid bilayers (De Lucca and Walsh, 1999; Matsuzaki, 1999; Zasloff, 2002). However, membrane permeabilisation as just described does not sufficiently account for AMP specificity. Thus, it is suggested that a receptor-mediated mechanism is involved in membrane permeabilisation (Thevissen et al., 1997; Thevissen et al., 2000). For nisin it was described that it specifically attaches to Lipid II, a membrane-bound factor in peptidoglucan synthesis, which was shown to constitute an important

component in nisin-induced pore formation (Breukink and de Kruijff, 1999). In addition it was demonstrated that defensins from insects and plants interact with the fungal membrane lipid glucosylceramide (Thevissen et al., 2004).
Other peptides, such as the short, proline-rich drosocin and pyrrhocoricin, were shown to bind to the bacterial heat shock protein DnaK, which is correlated with antimicrobial activity. It was documented that binding permanently closes the cavity of DnaK, consequently inhibiting chaperone-assisted protein folding (Kragol et al., 2001). Hypothetical mechanisms of action could involve activation of hydrolases, disturbance of membrane functions and damage to intracellular targets (Zasloff, 2002).

1.5 Gene regulation of antimicrobial peptides

The high attractiveness of AMPs for medicine or applied biotechnology can be attributed to their biological origin, high sustainability, broad diversity and appealing range of specificity. In order to perfectly exploit these peptides, detailed knowledge has to be gathered concerning their tertiary structure, their site of action and the regulation of AMP-encoding genes.
Many efforts have been undertaken to gain more insight into the regulation of AMP-encoding genes. Whilst expression patterns for genes from mammals, insects and plants are rather well-investigated, comparatively little is known about defence gene expression in bacteria and fungi.

1.5.1 Plants

Plants produce a whole battery of AMPs and proteins, all intended to protect the organism against pathogen attack. Interestingly, the encounter between plant and pathogen can follow two different ways of progression - the so-called susceptible or the resistant route of interaction. During the resistant route of interaction, which is also referred to as the hypersensitive response, the plant aims to literally trap the pathogen within necrotic plant tissue and layers of decomposing cells (Greenberg and Yao, 2004). The susceptible route of interaction implies that the infection can initially ensue (Lay and Anderson, 2005). In this case, the plant has to fall back on its repository of defence-related cell responses, among which the expression of AMPs is included.

Both, biotic and abiotic stresses can trigger a network of individual signalling pathways, which all seem to be extensively interconnected with each other (Pieterse and Van Loon, 2004). Obviously, this cross-talk is very sensible, because it enables the organism to fine-tune and to coordinate its defence responses in accordance with the individual stress applied. In plants, three major signalling pathways are described: The salicylic acid-dependent pathway, the ethylene-dependent pathway, and the (methyl) jasmonate-dependent pathway. These, after induction by pathogen infection or wounding, eventually result in the transcriptional activation of specific defence-related genes (Fig. II). Since the most comprehensive understanding of defence gene regulation is presently achieved in *Arabidopsis thaliana*, subsequent information will exemplarily focus on defence gene expression in this organism.

A. thaliana encodes for the plant defensin PDF1.2, which is expressed as a preprotein containing a putative signal peptide. The mature plant defensin domain shares 92 % sequence identity to the radish antifungal protein 1 (Rs-AFP1). The PDF1.2-encoding gene was found to be induced by exogenous treatment with either ethylene or methyl jasmonate but not by salicylic acid. The opposite was observed for the pathogenesis-related protein 1 (PR-1), which is a small, cationic AMP in *Arabidopsis* (Penninckx et al., 1998). Exogenous application of jasmonic acid or its analogue methyl jasmonate also causes induction of the thionin-encoding gene *thi2.1*. The pathogenesis-related genes *pr-3* and *pr-4*, encoding a basic chitinase (PR-3) and hevein-like protein (PR-4), were shown to underlie the same signalling induction pattern as *pdf1.2*. This implies that *pr-3*, *pr-4* and *pdf1.2* are repressed by the wound-response inducing transcription factor AtMYC2. While repressing pathogenesis-related response genes, AtMYC2 is concomitantly involved in the up-regulation of wound response genes such as *thi2.1*.

Regarding the genetic organisation of *pdf1.2*, a so-called GCC-box was identified in the promoter region of this gene. Deletion or mutation of the GCC-box resulted in substantially lower responses to jasmonate, which suggested that the GCC-box is an essential prerequisite for jasmonate-dependent induction of *pdf1.2* expression (Brown et al., 2003).

The coronatine-insensitive 1 (COI1) gene of *Arabidopsis* encodes an F-box protein. F-box proteins carry an F-box motif of approx. 50 amino acids in length. Upon interaction with other proteins, these complexes are then targeted to ubiquitin-mediated proteolysis (Kipreos and Pagano, 2000). COI1 was shown to be involved in the ubiquitin-proteosome pathway and is required for response to jasmonates (Devoto et al., 2002).

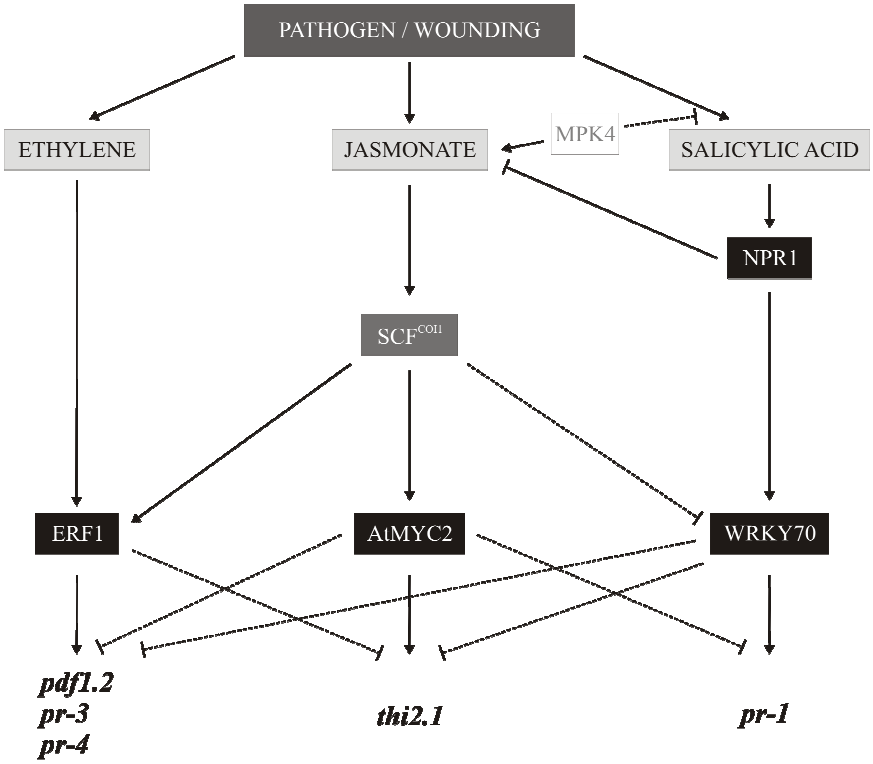

Figure II. Model outlining the interaction of key players in *Arabidopsis* defence gene induction. Arrows indicate positive interaction, while dashed lines represent negative interaction. For further explanation see text.

Recently, it has been found that COI1 associates with other proteins to form ubiquitin-ligase complexes, designated as SCF^{COI1}. These complexes constitute an intermediary module between jasmonate and ethylene signalling *via* the activation of the ethylene response factor 1 (ERF1) (Devoto et al., 2002; Lorenzo et al., 2003). The connection between SCF^{COI1} and ERF1 constitutes, why concomitant induction of the ethylene and the jasmonate response pathways are required for transcriptional induction of *pdf1.2*.

A further regulator of the pathogen defence response is the MAP kinase 4 (MPK4). It was shown to be required for both, the repression of salicylic acid-dependent resistance and for the activation of jasmonic acid-dependent defence gene expression. The identification of the MPK4 substrate 1 (MKS1) is proposed to contribute to MPK4-regulated defence gene activation by coupling the kinase to specific WRKY70 transcription factors (Andreasson et al., 2005). A protein referred to as nonexpressor of PR gene 1 (NPR1) suppresses the jasmonate-dependent pathway downstream of MPK4 (Liechti et al., 2006), while it was shown to positively interact with WRKY70 of the salicylic acid-dependent pathway. Owing to this highly complex system of interconnected signalling pathways, the plant is able to choose the right set of genes according to situational demand.

1.5.2 Insects

The fruit fly *Drosophila melanogaster* is by far the genetically best characterised insect to date. Since the majority of defence gene studies are carried out in *D. melanogaster*, subsequent paragraphs will summarise defence gene regulation in this organism.

In *D. melanogaster*, two distinct signalling pathways are known to regulate the expression of AMPs: While the Toll-pathway is predominantly triggered by fungal and Gram-positive bacterial infection, the immune deficiency (Imd) pathway was shown to be additionally induced by Gram-negative bacterial strains (Fig. III).

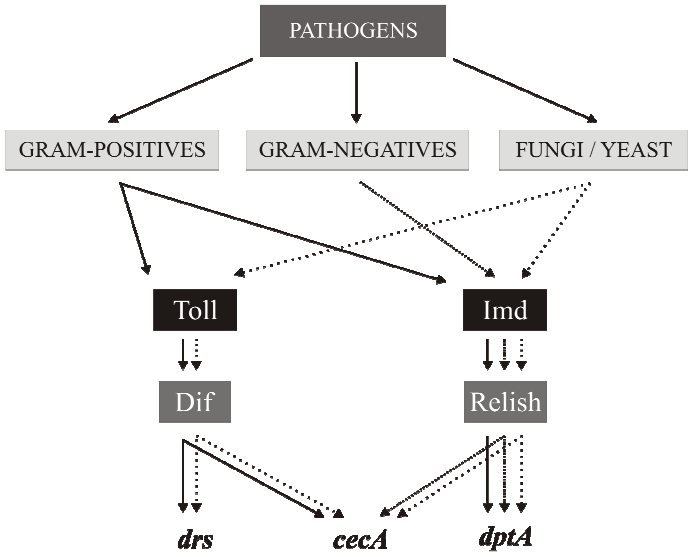

Figure III. Simplified model illustrating the network of defence gene induction in *D. melanogaster*. Differently illustrated lines represent differently triggered pathways (i.e. Gram-positive bacteria, Gram-negative bacteria, fungi and yeast). For further explanation see text.

Microbial recognition acting upstream of the Toll and Imd pathways is achieved, at least partially, through peptidoglycan recognition proteins (Ramet et al., 2002). These allow the discrimination between Gram-positive and Gram-negative bacteria (Leulier et al., 2003).

Toll-pathway induction involves a sequence of events, finally leading to the release of transcription factor Dif. In analogy, the Imd pathway results in cleavage and activation of transcription factor Relish. Both factors, Dif and Relish, can subsequently activate the expression of AMP-encoding genes (Royet et al., 2005). Expression of genes encoding for cecropins (CecA) were shown to be activated by Gram-positive and Gram-negative bacteria as well as by fungi. In analogy, expression of the diptericin A (DptA) encoding gene is

triggered by the same three groups of organisms, while the drosomycin (Drs) encoding gene is transcriptionally activated only upon attack by Gram-positive bacteria and fungi (Hedengren-Olcott et al., 2004).

The Rel family of transcription factors include proteins such as Dif and Relish. These contain the Rel homology domain, which is composed of two immunoglobulin-like ß-barrel subdomains that grip DNA at the major groove (Kabrun and Enrietto, 1994). Interestingly, the Rel domain is not exclusively responsible for DNA binding. Jia et al. (2002) could demonstrate that the Rel domain is also involved in regulated nuclear import of transcription factors. Gene expression of AMPs relies on the translocation of Rel transcription factors into the nucleus. Upon entry, Dif and Relish can bind to κB-like motifs in the promoter region of inducible defence genes to subsequently initiate gene expression.

A further putative promoter element referred to as region 1 (R1) has been identified in *D. melanogaster*. Site-directed mutagenesis of the R1 site has yielded in diminished cecropin A1 gene expression in transgenic larvae and flies. It was proposed that R1 and κB motifs are targets for distinct regulatory complexes that act in concert to promote high levels of antimicrobial peptide gene expression in response to infection (Uvell and Engstrom, 2003).

Although it is generally assumed that the expression of AMP encoding genes in *D. melanogaster* is exclusively regulated by the Toll and Imd pathways, it has been stated that aging, circadian rhythms and mating also seem to influence AMP expression. In mated females, the expression of metchnikowin (Mtk), a proline-rich peptide with antibacterial and antifungal properties, was found to be strongly stimulated during the first six hours after mating. A male seminal peptide referred to as sex-peptide (SP) was shown to be transferred during copulation, which acts as the major agent eliciting transcription of the Mtk-encoding gene. Both, the Toll and the Imd pathways were shown to be involved in SP-induced gene expression (Peng et al., 2005).

1.5.3 Mammals

To date, a wide variety of peptides with antimicrobial activity has been isolated from mammals (Braff et al., 2005). Particularly human AMPs attract much scientific interest, which is explained by the enormous demand for new medical strategies in the battle against human infection (Chen et al., 2005).

The induction of defence-related genes in mammals relies on the same basic mechanism as in *D. melanogaster* (Fig. IV). Ten different Toll-like receptors (TLRs) have been identified in

mammals to date, which all are involved for the detection of different PAMPs (Janssens and Beyaert, 2003). The extracellular detection of fungi, for instance, is achieved by dectin-1, a lectin family receptor for ß-glucans. This receptor mediates cellular responses to the fungal cell wall component *β*-glucan through its interaction with specific TLRs (Gantner et al., 2003).

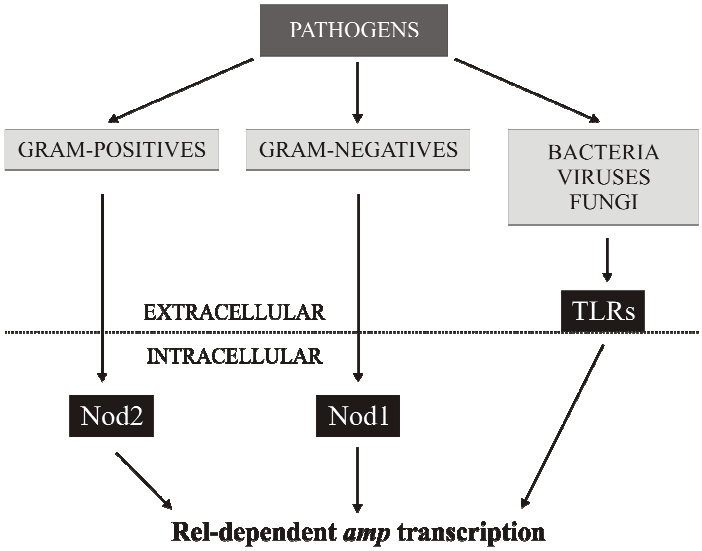

Figure IV. Simplified model illustrating the network of defence gene induction in mammals. Interactions are indicated by arrows. Toll-like receptors (TLRs) are located extracellularly, while nucleotide-binding oligomerisation domain (Nod) proteins 1 and 2 are situated inside the cytoplasm. For further explanation see text.

Detection of bacteria can either be achieved extracellularly by membrane-bound TLRs or intracellularly by nucleotide-binding oligomerisation domain (Nod) proteins. Nod1 and Nod2 recognise distinct motifs of peptidoglycan. While the former plays an important role in the sensing of Gram-negative bacteria (Girardin et al., 2003c), the latter was shown be implicated as a general sensor for both Gram-positive and Gram-negative prokaryotes (Girardin et al., 2003b).

In the Toll-mediated signalling pathway, binding of PAMPs lead to the activation of TIR, the most conserved protein motif within TLRs (Beutler and Rehli, 2002). A signal complex is formed consisting of MyD88, a cytoplasmic adapter protein, IRAK, an interleukin-1 receptor-associated kinase, and tumour necrosis factor receptor-associated factor 6 (TRAF6). IL-1 receptor signalling, finally, leads to the activation of the NF-κB/Rel family of transcription factors, which are required for transcriptional activation of AMP-encoding genes (Girardin et al., 2003a; Medzhitov et al., 1997).

1.5.4 Filamentous fungi

Small, cationic and cysteine-rich AMPs from filamentous fungi include the *Penicillium* antifungal protein (PAF) of *Penicillium chrysogenum* and the antifungal protein (AFP) of *Aspergillus giganteus*. Considerable information has been accumulated regarding the transcriptional regulation of the AFP- and the PAF-encoding genes. However, the molecular mechanisms are still not elucidated to completion. Since the majority of data is available for *A. giganteus*, the main focus will in the following reside on the transcriptional regulation of the AFP-encoding gene.

The 5'-upstream regions of *afp* and *paf* promoters contain several putative regulatory elements (Table I), which are assumed to be involved in the transcriptional regulation of respective genes.

Table I. Putative regulatory elements in the 5'-upstream region of *afp* (Meyer and Stahl, 2002) and *paf* (Marx, 2004) promoters. Number of regulatory elements identified in respective promoter regions is given.

	afp	***paf***
TATA-box	1	1
HAP-like complex binding site	2	2
CreA-binding site	1	4
STRE element	5	2
Heat-shock motif	1	-
GATA factor binding site	2	2
PacC binding domain	2	4

These include a TATA-box and two HAP-like complex binding sites consisting of the consensus sequence 5'-CCAAT, which both are involved in the transcriptional initiation of eukaryotic genes.

A CreA-binding site, with the consensus sequence 5'-SYGGRG, appears in the promoter region of *afp* once and in *paf* four times. This site represents the binding sequence for the CreA protein, which is involved in catabolite repression. While *afp* transcription was shown to be independently regulated from carbon catabolite repression (Meyer et al., 2002), the opposite effect is suggested for the *paf* gene (Marx, 2004).

The presence of five (*afp*) i.e. two (*paf*) STRE-elements with the consensus 5'-CCCCT as well as one a heat-shock motif (5'-NTTCNNGANTTCN) exclusively present in *afp*, may account for stress-related gene expression. For the *afp* gene, it was in fact demonstrated that its expression is strongly up-regulated by excess sodium chloride or ethanol, under carbon starvation conditions as well as after heat-shock induction (Meyer et al., 2002). No heat-shock related gene expression has been observed for *paf* (Marx, 2004). The induction or repression of *afp* transcription resulting from co-cultivation of *A. giganteus* with different fungi was shown to be strongly substrate-specific (Meyer and Stahl, 2003).

Two GATA factor binding sites with the consensus sequence 5'-HGATAR occur in the promoter regions of either gene. GATA motifs represent sites that are recognised by the GATA family of transcription factors (Ko and Engel, 1993). These include the transcriptional activator AreA, which mediates de-repression in the absence of ammonium (Muro-Pastor et al., 1999). Meyer et al. (2002) could show that *afp* transcription is not subjected to nitrogen metabolite repression. The *paf* gene, in contrast, is transcriptionally induced by sodium nitrate, while glutamine was found to repress gene transcription (Marx et al., 1995), indicating an AreA-dependent regulation.

The *pacC* gene encodes a zinc finger transcription factor, which is activated by proteolytic processing in response to ambient alkaline pH (Mingot et al., 2001). Putative members of a signalling cascade involved in ambient alkaline pH sensing are the *pal* genes. Their sole function is to promote the proteolytic activation of PacC (Denison, 2000). The AFP-encoding gene contains two PacC binding domains with the consensus sequence 5'-GCCARG. Both sites were shown to be specifically recognised by the PacC protein of *A. nidulans in vitro* (Meyer and Stahl, 2002). However, it was recently reported that *afp* transcription is not mediated by transcriptional activation through PacC. It rather appears that the calcineurin signalling pathway is implicated in controlling the *in vivo* activation of the *afp* promoter by alkaline pH (Meyer et al., 2005). Calcineurin is a eukaryotic calcium/calmodulin-dependent protein phosphatase, which is involved in the regulation of protein kinases, protein phosphatases, transcription factors, motor proteins and cytoskeletal components. Activated calcineurin dephosphorylates and activates the transcriptional factor Crz1p/Tcn1p, which enters the nucleus to subsequently activate a set of responsive genes by binding to calcineurin-dependent responsive elements (Fernandes et al., 2005). Crz1p in *Saccharomyces cerevisiae* was shown to be required for survival during several environmental stresses, including high salt concentrations and alkaline pH (Heath et al., 2004). The *afp* promoter contains five putative Crz1 sites (V. Meyer, personal communication), thus it can be

speculated that environmental stress may be involved in the transcriptional activation of *afp* expression. In addition, it was found that external phosphate strongly inhibits AFP production on the transcriptional level (Meyer and Stahl, 2002), an effect which was not observed for the *paf* gene (Marx, 2004).

The here summarised data clearly shows that it is not possible to postulate a universal model by which the transcriptional regulation of antimicrobial peptides in filamentous fungi is achieved. No interconnection of different signalling pathways has been observed to date. In addition, intra- or extracellular receptors involved in signal recognition and processing still await their identification. However, expression studies performed with *afp* (Meyer et al., 2002) and *paf* (Marx, 2004) indicate that both proteins yield the highest expression levels during stationary phase, which suggests that they may bestow the producing strain with a selective advantage over nutrient competitors sharing the same habitat or ecological niche as *A. giganteus* or *P. chrysogenum*.

1.6 Application of antimicrobial peptides

Expectations regarding an ample application of new antibiotics in fields such as agriculture, food industry and medicine are rising steadily. However, the breakthrough of AMP application in biotechnology still has not been realised yet. This may partially be due to problems associated with the large-scale production of AMPs. Furthermore, the stabilisation of secondary structure elements to improve receptor-ligand recognition may also constitute a serious obstacle (Tossi, 2005). However, a prime example for the successful application of AMPs in biotechnology is nisin, which has been used as a food preservative for over 50 years now (Reddy et al., 2004b). Only recently, nisin was also shown to exhibit spermicidal properties, and was subsequently suggested to serve as a safe vaginal contraceptive for future therapeutic interventions in sexually transmitted infections (Yedery and Reddy, 2005).

In crop protection, the great advantage of disease resistance strategies is that AMPs can substantially reduce the chemical input into the environment. Application of this alternative approach may contribute to a sustainable and environmentally-safe form of agriculture. Numerous publications broach the issue of novel plant engineering methodologies, all of them aiming at the receipt of fruitful transgenic plants. Examples include the macadamia antimicrobial peptide 1 (MiAMP1), which was successfully expressed in transgenic lines of *Brassica napus* (http://www.regional.org.au/au/gcirc/4/508.htm). The heterologous expression of the *afp* gene from *A. giganteus* in transgenic wheat (Oldach et al., 2001) and rice plants

(Coca et al., 2004) resulted in increased resistance to fungal pathogens. Likewise, the external application of AFP to rice protoplasts (Vila et al., 2001) and tomato seedlings (Theis et al., 2005) was also shown to efficiently prevent fungal infections. Specifically in Europe, the reservation against the use of transgenic crops is eminent. However, the external application of AMPs is expected to be well accepted by consumers. Although the use of conventional crop-protecting agents is in general denounced as harmful, the biological alternative of AMP application in agriculture has still not succeeded yet - despite the advantages that AMPs have to offer: These highly potent peptides originate from biological sources and are therefore associated with comparatively cheap costs of production. Their wide spectrum of therapeutic potential also suggests that AMPs provide powerful tools in the treatment of cancer (Tanaka et al., 2001), viral (Chernysh et al., 2002) or parasitic infection (Vizioli and Salzet, 2002). In fact, several biotechnological companies report about efforts in bringing AMPs to an applicable stage. However, despite the euphoric prognoses about their advantages in practical applications, only polymyxin B and gramicidin S have reached the state of clinical use so far (Bradshaw, 2003).

1.7 What more is to come?

Although the demand for new antibiotics is rising continuously, AMPs still have not found their way into the broad sectors of biotechnology. Rather surprisingly, since the vast amounts of different antimicrobial molecules seem to be highly competitive alternatives to conventional antibiotics. Prerequisite for the putative application of peptides with antimicrobial potential is, however, the analysis of their mode of action: The determination of host-ranges as well as the identification of specific AMP targets is absolutely required. Furthermore, factors such as optimisation of production cost, toxicity against eukaryotic cell types and development of allergic reactions against these peptides have to be also considered (Bradshaw, 2003).

Numerous AMPs have been expressed in transgenic plants, but only some of them have proven successful in protecting the plant against pathogen attack. However, *in silico* modelling is a comparatively new method that provides sophisticated possibilities to optimise AMP characteristics. The application of this method promises the economic production and successful expression of novel drugs customised to the individual demand of consumers (Monk and Harding, 2005).

1.8 Concluding remarks

Information regarding signalling pathways leading to transcriptional regulation of AMP-encoding genes is substantial. Different pathogens, wounding and/or abiotic factors are perceived by organisms through different receptors, ultimately leading to the transcriptional activation of different defence-related or AMP-encoding genes. The correlation between environmental stimulus and defence gene expression may be diverse. However, AMPs are a potent means of providing their host with a selectional advantage - be it either the protection against pathogen invasion or the defence of an ecological niche against putative nutrient competitors.

Interesting to note is that the innate immune response in insects and mammals bear some striking similarities. Here, the Toll pathway gives rise to the Rel-dependent transcription of defence-related genes. It arises the assumption that both immune response pathways must have evolved from one ancestral system. Due to their similarity, it is possible to initially perform research on signal transduction and defence gene activation in the insect model. This harbours the advantage of short generation times and provides a means for mutant analyses. Subsequently, the mammalian system can be screened for the transferability of data. In contrast, transcription of AMP-encoding genes in filamentous fungi does presently not seem to follow a general pattern. However, maximum expression levels of the *afp* gene from *A. giganteus* and the *paf* gene from *P. chrysogenum* is observed during the stationary phase of cultivation, suggesting an ecological advantage for the producing organism in the competition for nutrients. In insects and plants, different signalling pathways are tightly interconnected to yield in the transcriptional activation of specific genes. Obviously, this cross-talk is very sensible and enables the organism to fine-tune and coordinate its defence responses in accordance to individual demand.

In conclusion, we still seem distant from understanding the whole complexity by which organisms are capable of reacting towards life-endangering situations. In order to bring AMPs a step further towards their application in medicine or biotechnology, many more efforts have to be undertaken to scrutinise the molecular aspects involved in defence gene transcription. Numerous AMPs may provide valuable templates for the design of novel, highly effective antibiotics, thus it is considered worthwhile to invest the highest efforts into their analysis.

2 Subject description

Antimicrobial peptides (AMPs) are ubiquitously produced throughout the taxonomic kingdoms. Although AMPs vary considerably in terms of structure and amino acid composition, they commonly adopt a compact structure, show high thermal stability and exhibit strong antimicrobial activity. The majority of AMPs have been isolated from insects and plants, however, filamentous ascomycetes are also known to express and secrete proteins of this group. One of them is the antifungal protein (AFP) of *Aspergillus giganteus* first described by Olson and Goerner (1965).

2.1 The antifungal protein of *Aspergillus giganteus*

A. giganteus belongs to the group of imperfect filamentous fungi and is characterised by its ability to generate long conidiophores. It is furthermore known to secrete two basic proteins, the ribosome-inactivating protein α-sarcin and the AFP (Olson and Goerner, 1965). The *afp* gene encodes a preproprotein with 94 amino acids in length. While the presequence (aa 1 - 26) is considered to act as a secretion signal, the prosequence (aa 27 - 43) is thought of to serve as a precursor for an inactive form of AFP, which upon secretion into the surrounding environment is hydrolytically cleaved off (Wnendt et al., 1994). The mature AFP consists of 51 amino acids, resulting in a molecular weight of 5.8 kDa. Due to the relatively high content in lysines (12 residues), AFP exhibits a pI of 8.8 at neutral pH (Nakaya et al., 1990). AFP adopts a small and compact ß-barrel composed of five highly twisted antiparallel ß-strands (Campos-Olivas et al., 1995), a structure which was demonstrated to share characteristic features of oligonucleotide/oligosaccharide binding (OB)-fold proteins. This configuration is suggested to be implicated in the antifungal activity of AFP, since it was demonstrated to promote DNA binding and condensation *in vitro* (Martinez Del Pozo et al., 2002). Four intramolecular disulfide bridges (Fig. V) result in the tight folding of AFP, which is reflected by a remarkable resistance to heat and protease degradation (Lacadena et al., 1995). Incubation of AFP at 80 °C for one hour was shown to not result in reduced protein activities (Theis et al., 2005).

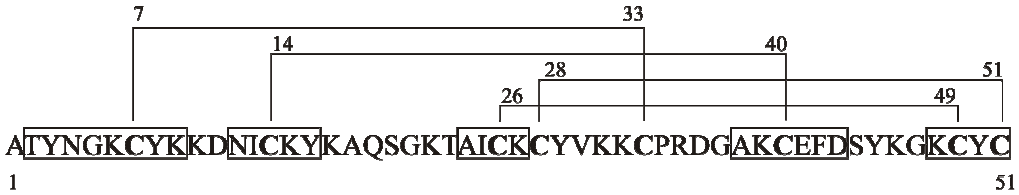

Figure V. Amino acid sequence of AFP and localisation of intramolecular disulfide bridges. Boxed sequences represent ß-sheet structures, cysteine residues are marked in boldface, disulfide bridges are indicated as lines (Campos-Olivas et al., 1995).

Campos-Olivas et al. (1995) reported that AFP contains two surface-exposed protein domains, the cationic site and the hydrophobic stretch as illustrated in Figure VI. In combination, they account for the amphipathic character of AFP.

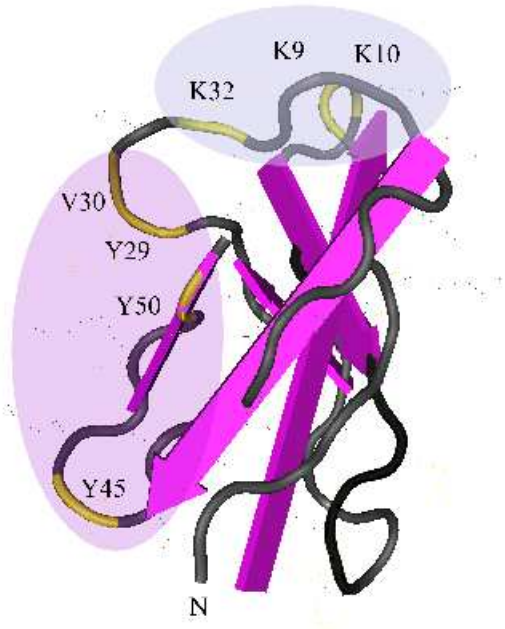

Figure VI. Structural conformation of the *A. giganteus* AFP. Depicted in blue is the cationic site, illustrated in pink is the hydrophobic stretch. N marks the amino-terminal end of AFP, ß-sheet structures are shown as arrows. The image was illustrated using the Cn3D 4.1 software on the basis of the AFP structure published by Campos-Olivas et al. (1995).

Amphipathicity is one of several features contributing to the attachment and the insertion of AMPs into membrane bilayers (Park and Hahm, 2005). AFP was shown to induce aggregation of acidic phospholipid vesicles *in vitro*, leading to the assumption that the cationic site and the hydrophobic stretch may constitute a phospholipid-binding site

(Lacadena et al., 1995). Theis et al. (2003) demonstrated that AFP induces plasma membrane permeabilisation in AFP-sensitive fungi - an effect that was not observed in AFP-resistant strains. Previously, AMPs were suggested to operate *via* a two-step mechanism: Firstly, the binding to specific membrane-localised receptors; secondly, the receptor-induced insertion of AMPs into microbial membranes, which may subsequently result in pore formation (Thevissen et al., 1997). However, for AFP no such receptors have been identified to date.

AFP was demonstrated to exclusively inhibit the growth of several filamentous fungi, in particular strains belonging to the genera *Fusarium* and *Aspergillus* (Table II and Table A, Appendix). No effect was observed when yeast or bacteria were cultivated in the presence of AFP (Theis et al., 2003). Furthermore, application of AFP to mammalian cell types neither induced cytotoxic reactions nor activated the immune response (Szappanos et al., 2006), underlining its excellent potential for the targeted control of fungal pathogens.

Table II. AFP susceptibility of selected human- and plant pathogenic microorganisms (Theis et al., 2003). Abbreviations: MIC = minimal inhibitory concentration; NE = no effect.

Organism	**Characteristics**	**AFP susceptibility MIC (µg/ml)**
Fusarium oxysporum	plant pathogen	1
Aspergillus nidulans	human pathogen	200
Aspergillus fumigatus	human pathogen	10
Aspergillus oryzae	plant pathogen	1
Candida albicans	human pathogen	NE
Escherichia coli	human pathogen	NE

Transmission electronic microscopy analyses revealed that AFP induces membrane alterations exclusively in AFP-sensitive fungi. In addition, it was reported that AFP predominantly localises to the cell wall-attached ‘outer layer’ and accumulates within defined areas of the cell wall in AFP-sensitive fungi (Theis et al., 2005). These results suggest that the cell wall may harbour putative AFP targets. However, no such targets have been identified as yet.

Due to its positive characteristics, AFP seems suitable for future application in biotechnology and other sectors. Fields that could benefit from the antifungal activity of AFP is the food industry, agriculture or medicine. Indeed, it was previously shown that application of AFP to

rice plants (Vila et al., 2001) and tomato seedlings (Theis et al., 2005) successfully prevented infection with *Magnaporthe grisea* and *Fusarium oxysporum*, respectively. The heterologous expression of the *afp* gene in transgenic wheat (Oldach et al., 2001) and rice plants (Coca et al., 2004) also proved successful in inhibiting pathogen infection, indicating that a large potential market could be awaiting AFP commercialisation. Although the public opinion is in general against the use of transgenic plants, it may be worthwhile considering whether the extrinsic application of AFP, e.g. as solution, ointment or powder, may be realised in the foreseeable future.

2.2 Aim of the thesis

This thesis is aimed at elucidating the mode of action of AFP from *A. giganteus*. AFP is a naturally-derived molecule that combines various excellent characteristics, making it an attractive candidate for applications in the medical or biotechnological field. However, prior to the putative application of AFP as an antifungal agent, the molecular mode of action of AFP has to be uncovered. With this prerequisite in hand, it may be possible to furthermore optimise the potency and the specificity of AFP to individual demands. In this work, factors that may influence AFP susceptibility shall be considered:

1. Which domains / amino acids may be involved in the mode of action of AFP?
   Lysines and tyrosines may constitute residues, which may be essential for AFP-induced growth inhibition (Campos-Olivas et al., 1995). Since no *afp* deletion strain of *A. giganteus* is available to date and heterologous AFP expression in *Escherichia coli* strains (BL21(DE3) LysE and Rosetta-gami) have failed (Meyer, 2003), chemically modified AFP versions will be generated. These shall consist of an acetylated and a nitrated AFP derivative, affected in cationic net charge and the hydrophobic protein domain, respectively. Furthermore, cysteine bridges in AFP will be disrupted to investigate whether alterations in protein configuration may influence the potency of AFP. A shortened AFP version, consisting of amino acids 1 to 33 and lacking a functional hydrophobic stretch, will also be analysed regarding its antifungal activity.

2. May chitin be involved in determining AFP susceptibility?
   Fungal cell walls differ from yeast and bacterial cell walls considerably in that they contain relatively large amounts of chitin. Thus, it seems possible that chitin and/or chitin biosynthesis may represent putative AFP targets. In order to gain proof for this hypothesis, *in vitro* chitin binding assays will be carried out in which binding affinities of AFP and chemically modified AFP versions are going to be determined. Conduction of *in situ* chitin synthase assays shall demonstrate whether chitin synthesis is influenced by AFP activity. Analyses of cell wall mutants of yeast and filamentous fungi are assumed to contribute to the understanding of how AFP exerts it growth inhibitory effect in a species-specific manner.

3. May sphingolipids be involved in determining AFP susceptibility?

   Fungal plasma membranes characteristically contain sphingolipids (Daum et al., 1998), which are absent in bacteria. Although sphingolipids also occur in plants and animals, they exhibit structural differences among each other (Warnecke and Heinz, 2003). Thus, it can be speculated that fungal sphingolipids may constitute specific constituents required for developing AFP-sensitive phenotypes. In order to gain support for this speculation, susceptibility studies of different bacterial protoplasts will be carried out. Furthermore, glucosylceramide levels in sensitive fungi will be depleted. If these subsequently exhibit reduced susceptibilities, it would be an indication that sphingolipids i.e. glucosyl-ceramides may represent AFP targets. Analyses of yeast and filamentous fungi affected in membrane lipid composition will provide additional information regarding the involvement of distinct lipid classes in AFP susceptibility.

4. Are nucleic acids involved in the mode of action of AFP?

   In order to demonstrate whether *in vitro* binding of AFP to DNA is of specific character, mobility shift assays will be carried out in which the binding affinity of native and heat-inactivated AFP to nucleic acids will be analysed. Chemically modified AFP versions will also be included in this study. In case that binding affinities of AFP to analysed samples do not differ significantly, it would be an indication that AFP-nucleic acid interactions are of unspecific nature. This, in turn, would suggest that nucleic acids are not (directly) involved in AFP-induced growth restriction.

5. How stable is AFP in gastrointestinal environments and can putative fields of AFP application be expanded?

   Although it exhibits many characteristics that would speak in favour for a putative application in the food industry, it is so far unknown whether AFP is readily degraded upon human ingestion. Conduction of *in vitro* biodegradability assays will provide valuable information whether AFP is degraded in acid or bile solutions that aim at simulating the gastrointestinal environment of man. Susceptibility assays with acid- or bile-treated AFP will be carried out in succession to analyse whether AFP retains its antifungal potential. Screening of major wood decaying fungi will indicate whether AFP offers potential to also be used as a putative antifungal agent in the building industry.

3 Material and Methods

3.1 Material

3.1.1 Equipment

Autoclave	1651; Fedegari, Italia
Centrifuge	Sorvall RC-5B; DuPont, Bad Homburg
Clean bench	uvub 1200 Uniflow
Electrophoresis chamber	Wide Mini Sub Cell
	Mini Protean (16 x 14 x 0,15 cm); Bio-Rad, München
Fluorescence reader	Cytofluor 2300; Millipore, Schwalbach
Fraction collector	2110; Bio-Rad, München
Gel drying film	Promega, Mannheim
Gel filtration column	(16 x 700 mm); Amersham Biosciences, Freiburg
Incubator	Certomat IS; Sartorius, Göttingen
Liquid scintillation counter	Wallac 1409 DSA; Perkin Elmer Wallac GmbH, Freiburg
Orbital shaker	Duomax 1030; Heidolph Instruments, Schwabach
Photographic equipment	Camedia Digital Camera C-4000 Zoom; Olympus, Hamburg
	Image Station 440 cf; Kodak, Stuttgart
	Olympus, OM-2 with 50 mm objective; Dunco, Berlin; INTAS
Pipetting equipment	P10, P20, P100, P1000; Abimed, Langenfeld
Power supply	PowerPack Basic; Bio-Rad, München
	Phero-Stab 500; Biotec Fischer, Reiskirchen
Spectrophotometer	Uvikon 860; Kontron Instruments, Neufahrn
	MikroTek DS; Kontron Instruments, Neufahrn
Transilluminator	INTAS; Göttingen
Ultrafiltration chamber	Model 8050; Millipore, Schwalbach
Ultrafiltration membrane	YM1 (44.5 mm); Millipore, Schwalbach
Vacuum equipment	Rotary Slide pump; Heraeus, Hanau
Water bath	Grant LTD; Thermomix 1460 Braun, Melsungen
	"thermed" 5001; GFL, Burgwedel

3.1.2 Enzymes and chemicals

Bile salts	Oxoid, Basingstoke, UK
D-PDMP	Matreya, PA, USA
Generuler	Promega, Mannheim
Glucanex	Novozymes, Bagsvaerd, Denmark
Herring sperm DNA	Promega, Mannheim
Polypeptide SDS-PAGE standards	Bio-Rad, München
Potato dextrose agar	Difco / BD, Heidelberg
UDP-(U-^{14}C)-GlcNAc	Amersham, Buchler
Repelling dichlordimethylsilan	Carl Roth, Karlsruhe
sAFP	SynPep, California, USA
Sephadex G-50	Millipore, Schwalbach
Soluble starch	Difco / BD, Heidelberg

SYTOX-Green	Molecular Probes, Oregon, USA
tRNA	Roche, Basel, Switzerland

All chemicals not listed above were obtained from Merck, Sigma or Boehringer Mannheim and were of analytical grade or better quality.

3.1.3 Strains and cultivars

Alternaria alternata 0203	Institut für Gärungsgewerbe, Berlin
Aspergillus fumigatus 15/0809	Institut für Gärungsgewerbe, Berlin
Aspergillus giganteus 15/0902	Institut für Gärungsgewerbe, Berlin
Aspergillus nidulans 15/1701	Institut für Gärungsgewerbe, Berlin
Aspergillus niger 15/1801	Institut für Gärungsgewerbe, Berlin
Aspergillus niger A395	Jernejc, K., National Institute of Chemistry, Slovenia
Aspergillus niger A493 (DC)	Jernejc, K., National Institute of Chemistry, Slovenia
Aspergillus niger A365 (mcC)	Jernejc, K., National Institute of Chemistry, Slovenia
Aspergillus oryzae A1560	Nielsen, J., Technical University of Denmark, Denmark
Aspergillus oryzae CM100 (*chsB*::*pyrG*)	Nielsen, J., Technical University of Denmark, Denmark
Aspergillus oryzae CM101 (*csmA*::*pyrG*)	Nielsen, J., Technical University of Denmark, Denmark
Aspergillus penicilloides 15/1301	Institut für Gärungsgewerbe, Berlin
Aureobasidium pullulans 0112	Institut für Gärungsgewerbe, Berlin
Bacillus subtilis L170	Hoischen, C., Hans-Knöll-Institute for Natural Products Research, Jena
Ceratocystis moniliformis 25/0101	Institut für Gärungsgewerbe, Berlin
Chaetomium globosum 26/0102	Institut für Gärungsgewerbe, Berlin
Cladosporium cladosporioides 28/0101	Institut für Gärungsgewerbe, Berlin
Cladosporium herbarum 15/1001	Institut für Gärungsgewerbe, Berlin
Cladosporium macrocarpum 28/0101	Institut für Gärungsgewerbe, Berlin
Coniphora puteana 30/0101	Institut für Gärungsgewerbe, Berlin
Escherichia coli K12	Hoischen, C., Hans-Knöll-Institute for Natural Products Research, Jena
Escherichia coli LWF^+	Hoischen, C., Hans-Knöll-Institute for Natural Products Research, Jena
Fusarium oxysporum 39/1201	Institut für Gärungsgewerbe, Berlin
Fusarium oxysporum 4287 (wild-type)	Roncero, I., University of Córdoba, Spain
Fusarium oxysporum Δ*chsV*	Roncero, I., University of Córdoba, Spain
Fusarium solani 39/1001	Institut für Gärungsgewerbe, Berlin
Penicillium brevicompactum 0402	Institut für Gärungsgewerbe, Berlin
Penicillium chrysogenum ATCC10002	Institut für Gärungsgewerbe, Berlin
Penicillium chrysogenum Q176MM	Institut für Gärungsgewerbe, Berlin
Proteus mirabilis LVI	Hoischen, C., Hans-Knöll-Institute for Natural Products Research, Jena
Saccharomyces cerevisiae ALG5	EUROSCARF strain collection
Saccharomyces cerevisiae ALG5	EUROSCARF strain collection
Saccharomyces cerevisiae ALG6	EUROSCARF strain collection
Saccharomyces cerevisiae ANP1	EUROSCARF strain collection
Saccharomyces cerevisiae AST1	EUROSCARF strain collection
Saccharomyces cerevisiae ATG26	EUROSCARF strain collection
Saccharomyces cerevisiae BCK1	EUROSCARF strain collection
Saccharomyces cerevisiae BEM4	EUROSCARF strain collection
Saccharomyces cerevisiae BY4741	EUROSCARF strain collection
Saccharomyces cerevisiae CHO2	EUROSCARF strain collection
Saccharomyces cerevisiae CHS1	EUROSCARF strain collection
Saccharomyces cerevisiae CHS3	EUROSCARF strain collection
Saccharomyces cerevisiae CHS4	EUROSCARF strain collection
Saccharomyces cerevisiae CHS5	EUROSCARF strain collection
Saccharomyces cerevisiae CHS6	EUROSCARF strain collection
Saccharomyces cerevisiae CHS7	EUROSCARF strain collection
Saccharomyces cerevisiae CLA4	EUROSCARF strain collection
Saccharomyces cerevisiae CNB1	EUROSCARF strain collection

Saccharomyces cerevisiae CRZ1	EUROSCARF strain collection
Saccharomyces cerevisiae CWP1	EUROSCARF strain collection
Saccharomyces cerevisiae CWP2	EUROSCARF strain collection
Saccharomyces cerevisiae CYK3	EUROSCARF strain collection
Saccharomyces cerevisiae DEP1	EUROSCARF strain collection
Saccharomyces cerevisiae DIE2	EUROSCARF strain collection
Saccharomyces cerevisiae EDE1	EUROSCARF strain collection
Saccharomyces cerevisiae EKI1	EUROSCARF strain collection
Saccharomyces cerevisiae ELO1	EUROSCARF strain collection
Saccharomyces cerevisiae EMP24	EUROSCARF strain collection
Saccharomyces cerevisiae EPT1	EUROSCARF strain collection
Saccharomyces cerevisiae FAB1	EUROSCARF strain collection
Saccharomyces cerevisiae FKS1	EUROSCARF strain collection
Saccharomyces cerevisiae FKS2	EUROSCARF strain collection
Saccharomyces cerevisiae FPS1	EUROSCARF strain collection
Saccharomyces cerevisiae FRT1	EUROSCARF strain collection
Saccharomyces cerevisiae GAS1	EUROSCARF strain collection
Saccharomyces cerevisiae GSY1	EUROSCARF strain collection
Saccharomyces cerevisiae GSY2	EUROSCARF strain collection
Saccharomyces cerevisiae GUP1	EUROSCARF strain collection
Saccharomyces cerevisiae HXT8	EUROSCARF strain collection
Saccharomyces cerevisiae ILM1	EUROSCARF strain collection
Saccharomyces cerevisiae INP51	EUROSCARF strain collection
Saccharomyces cerevisiae MID2	EUROSCARF strain collection
Saccharomyces cerevisiae MKK1	EUROSCARF strain collection
Saccharomyces cerevisiae MKK2	EUROSCARF strain collection
Saccharomyces cerevisiae MNN9	EUROSCARF strain collection
Saccharomyces cerevisiae MNN10	EUROSCARF strain collection
Saccharomyces cerevisiae MNN11	EUROSCARF strain collection
Saccharomyces cerevisiae MPK1	EUROSCARF strain collection
Saccharomyces cerevisiae MSN5	EUROSCARF strain collection
Saccharomyces cerevisiae MUQ1	EUROSCARF strain collection
Saccharomyces cerevisiae OPI1	EUROSCARF strain collection
Saccharomyces cerevisiae PDR16	EUROSCARF strain collection
Saccharomyces cerevisiae PHO5	EUROSCARF strain collection
Saccharomyces cerevisiae PKR1	EUROSCARF strain collection
Saccharomyces cerevisiae PLC1	EUROSCARF strain collection
Saccharomyces cerevisiae RLM1	EUROSCARF strain collection
Saccharomyces cerevisiae ROM1	EUROSCARF strain collection
Saccharomyces cerevisiae ROM2	EUROSCARF strain collection
Saccharomyces cerevisiae SAC6	EUROSCARF strain collection
Saccharomyces cerevisiae SCS7	EUROSCARF strain collection
Saccharomyces cerevisiae SFH5	EUROSCARF strain collection
Saccharomyces cerevisiae SFK1	EUROSCARF strain collection
Saccharomyces cerevisiae SHE4	EUROSCARF strain collection
Saccharomyces cerevisiae SMI1	EUROSCARF strain collection
Saccharomyces cerevisiae SWI3	EUROSCARF strain collection
Saccharomyces cerevisiae SWI4	EUROSCARF strain collection
Saccharomyces cerevisiae TOR1	EUROSCARF strain collection
Saccharomyces cerevisiae TUS1	EUROSCARF strain collection
Saccharomyces cerevisiae VPS34	EUROSCARF strain collection
Saccharomyces cerevisiae WHI2	EUROSCARF strain collection
Saccharomyces cerevisiae WSC1	EUROSCARF strain collection
Saccharomyces cerevisiae WSC2	EUROSCARF strain collection
Saccharomyces cerevisiae WSC3	EUROSCARF strain collection
Saccharomyces cerevisiae WSC4	EUROSCARF strain collection
Saccharomyces cerevisiae YEH2	EUROSCARF strain collection
Stachybotrys chartarum 87/0101	Institut für Gärungsgewerbe, Berlin
Trichoderma viride	Institut für Gärungsgewerbe, Berlin
Wallemia sebi	Institut für Gärungsgewerbe, Berlin

3.1.4 Media, buffers and solutions

Bile salt solution	1.5 % bile salts in PBS (pH 7.2)
Coomassie staining solution	0.2 % Coomassie brilliant blue R250 (w/v), 30 % methanol, 10 % acetic acid
Coomassie destaining solution	45 % methanol, 10 % acetic acid
Drying solution	10 % glycerol (w/v), 30 % methanol
Gel filtration buffer	0.05 M sodium-acetate, 0.1 M NaCl
Elution buffer	1.5 M NaCl in TE (pH 7.0)
Olson medium	1 % beef extract, 2 % peptone, 2 % soluble starch, 0.5 % NaCl
PBS	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, ad H_20 to 1 litre, adjusted to pH 7.2 with HCl
SDS-PAGE sample buffer (2x)	2 ml 0.5 M Tris-HCl (pH 6.8), 2.4 ml glycerol, 1 ml 10 % SDS, 3 ml ß-mercaptoethanol, 0.1 ml 0.5 % Coomassie G250 (w/v), ad H_2O to 10 ml
SDS-PAGE anode buffer (5x)	121 g Tris, ad H_2O to 1 litre, adjusted to pH 8.9 with HCl
SDS-PAGE cathode buffer (5x)	60.55 g Tris, 89.6 g tricine, 1 g SDS, ad H_2O to 1 litre
Simulated gastric juice	8.3 g proteose peptone, 3.5 g glucose, 2.05 g NaCl, 0.6 g KH_2PO_4, 0.11 g $CaCl_2$, 0.37 g KCl, 0.05 g bile, 0.1 g lysozyme, and 13.3 mg pepsin ad H_2O to 1 litre, adjusted to pH 2.5 with 1N HCl
TAE (5x)	242 g Tris, 57.1 ml acetic acid, 100 ml 0.5 M EDTA, ad H_2O to 1 litre, adjusted to pH 8.0 with HCl
TE buffer	10 mM Tris, 1 mM EDTA, adjusted to pH 7.0 with HCl
YPG medium	0.3 % yeast extract, 1 % peptone, 2 % glucose, adjusted to pH 4.5 or pH 6.0 with HCl or NaOH, respectively

3.2 Cultivation conditions of bacteria and fungi

Filamentous fungi were grown in YPG liquid medium (pH 4.5), except for *A. giganteus*, which was cultivated in Olson medium (see 3.1.4). Yeast strains were grown in YPG liquid medium (pH 6.0) unless otherwise stated. Bacterial L-forms of *Proteus mirabilis* LVI and *Escherichia coli* LWF^+ were cultivated in brain heart infusion (BHI) broth (Sigma), the L-form of *Bacillus subtilis* L170 was cultivated in BHI medium supplemented with yeast extract (30 %) and sucrose (60 %). *E. coli* K12 was grown in BHI. Cultivation temperature of fungi was 28 °C, bacteria were grown at 37 °C. If not otherwise stated, standard cultivations were carried out for 48 h.
Fungal spore suspension was obtained by cultivation of strains in petri dishes containing potato dextrose agar until sporulation commenced. Conidia were taken off using 4 to 6 ml of 0.05 % Triton-X-100 (w/v) per plate. Spore titers were determined microscopically using a Thoma chamber. Spore suspensions were stored at 4 °C for a maximum of one week.

3.3 Protein purification

3.3.1 Preparation of the cation exchange solution

The cation exchange solution was prepared according to the protocol by Theis et al. (2003). In brief, 55 g of carboxymethylcellulose CM 23 (Boehringer Mannheim) were dissolved in 2 litres of 0.5 M NaOH. After 45 min of incubation at room temperature, the cellulose was washed with H_2O to neutral pH. Subsequently, the adsorbent was resuspended in 2 litres 0.5 M HCl and incubated therein for 45 min at room temperature. The cellulose was washed to neutral pH with H_2O. Finally, it was dissolved in 2 litres elution buffer, washed with 2 litres H_2O and resuspended in 2 litres TE buffer (pH 7.0). Fines were discarded by replacement with fresh TE buffer (pH 7.0) and the concentration adjusted to 50 g/l. The cation exchanger was stored at 4 °C for up to three months.

3.3.2 Silanisation of the gel filtration column

The gel filtration column was washed twice with 5 ml toluene (Sigma) and subsequently filled completely with 5 % repelling dichlordimethylsilan (Carl Roth) dissolved in toluene. After 2 h of incubation under a hood at room temperature, the column was washed with a total of 500 ml H_2O, dried and stored at 4 °C until packaging with the gel filtration matrix (see 3.3.3).

3.3.3 Preparation of the gel filtration matrix

For the gel filtration matrix, 20 g of Sephadex G-50 fine grade was dissolved in 500 ml gel filtration buffer (see 3.1.4) and incubated at 4 °C overnight. Subsequently, 400 ml of buffer were discarded. A vacuum was applied for approximately 1 h in order to remove bound CO_2. The solution was adjusted to a volume of 200 ml and carefully poured into the silanised gel filtration column (see 3.3.2) to avoid air bubbles. The column was equilibrated using gel filtration buffer. A flow rate of approximately 0.5 ml/min was adjusted.

3.3.4 Purification of AFP

The isolation of AFP from the culture supernatant of *A. giganteus* was carried out according to the protocol described by Theis et al. (2003). In summary, *A. giganteus* was cultivated at 28 °C, 120 rpm, in 2 l Erlenmeyer flasks containing 500 ml Olsen medium each. After 96 h, the flasks were shifted to a temperature of 37 °C for 17 to 20 h. Basic proteins were isolated from the culture supernatant by cation exchange chromatography. For this purpose, the culture supernatant was adjusted to pH 7.0, separated into three equal volumes of approx. 600 ml and then mixed with a final concentration of 5 g/l cation exchange solution. After 1 h of incubation at 28 °C and 120 rpm, the cation exchange matrix was washed with 2 litres TE buffer (pH 7.0). Cationic proteins bound to the negatively charged matrix were eluted by washing with a total of 100 ml elution buffer. Subsequently, the volume of the eluted protein solution was reduced to 2 - 4 ml by ultrafiltration (see 3.3.5). The protein solution was fractionated twice by Sephadex G-50 gel filtration chromatography (see 3.3.6).

3.3.5 Ultrafiltration

In order to increase protein concentration, the volume of protein-containing buffer was reduced by ultrafiltration. Filtration was carried out in a 50 ml ultrafiltration chamber (Millipore) at a pressure of 4.5 bar. Ultrafiltration was stopped when the protein solution had been reduced to a volume of 3 - 5 ml. Cut-off value of the YM1 membrane applied was 10000 Da. The membrane was used up to 20 times and stored in 10 % ethanol at 4 °C.

3.3.6 Gel filtration chromatography and protein fractionation

Prior to application of protein samples, the prepared gel filtration column (see 3.3.2 and 3.3.3) was equilibrated for 30 min using gel filtration buffer (see 3.1.4). Gel filtration buffer was also used for chromatography. Chromatography was carried out at 4 °C. After application of the sample to the matrix, the fraction collector was started. Fractions were separated in 5 min intervals. Subsequently, the protein content of each fraction was determined spectrophotometrically at an absorbance of 280 nm. All AFP-containing fractions were pooled and ultrafiltrated to reduce the volume to 3 - 5 ml. Gel filtration chromatography was repeated as described, AFP fractions were pooled and ultrafiltrated to finally reduce the sample volume to 1 - 2 ml. After sterile filtration using a 0.2 µm syringe filter, the AFP concentration was determined by SDS-PAGE analysis (see 3.3.7). The AFP solution was stored at 4 °C for up to one year.

3.3.7 Protein determination by SDS-PAGE

The analysis of proteins was carried out on 15 % SDS-PAA gels (Mini Protean, Bio-Rad) according to the Tris-Tricine buffer system (Schagger and von Jagow, 1987). The protein samples were diluted 1:1 in freshly prepared SDS-PAGE sample buffer (see 3.1.4) and incubated at 100 °C for 5 min. The electrophoresis was run at 200 V for 70 min. Gels were incubated in Coomassie staining solution (see 3.1.4) at room temperature and gentle agitation on an orbital shaker (Duomax 1030, Heidolph Instruments) overnight. In order to remove unspecific staining, the gel was subsequently incubated in Coomassie destaining solution (see 3.1.4) at room temperature and gentle agitation on an orbital shaker for 30 - 60 min. Conservation of gels was achieved by incubation in drying solution (see 3.1.4) for 1 h at room temperature. Protein concentrations were determined by using the Polypeptide SDS-PAGE standard (Bio-Rad) with known concentration.

3.4 Chemical modification of AFP

3.4.1 Acetylation of lysines

Acetylation of lysines and proof of modification was carried out according to the protocols by Knopfe (2000). In brief, AFP was diluted in modification buffer (0.05 M Na_2HPO_4/KH_2PO_4; I= 0.5 NaCl, pH 8.0). Over a period of 30 min, a total of 36 µl acetic anhydride was added to 29 ml solution containing 7.25 mg AFP. At a given pH, acetic anhydride specifically targets the ε-amino group of lysine, leading to the masking of amino groups by carbonyl groups. Subsequently, the solution was incubated at room temperature for 1 h. At all times, the solution was constantly stirred and kept at pH 7.7 using 0.1 N NaOH. Finally, the protein was dialysed against AFP standard buffer (0.05 M Na-acetate; 0.1 M NaCl) and quantified by SDS-PAGE analysis (see 3.3.7). The acetylated version of AFP was termed acAFP. Subsequently, the extent of protein modification was determined using an indirect method in which trinitrobenzene sulphonate (TNBS) interacts with free amino groups of proteins. Amino groups that have been blocked due to chemical modification are excluded from the reaction. The absorption of trinitrophenylated reaction products was measured at a wavelength of 340 nm. On average, four of 12 lysines are modified in acAFP. Lysines in AFP are similarly well exposed, therefore they share equal chances of becoming chemically modified by the acetylating reaction. A targeted analysis for directly localising the position of acetylated residues is not possible. Proof of protein stability was shown by subjecting acAFP to SDS-PAGE analyses before and after completion of susceptibility studies.

3.4.2 Nitration of tyrosines

The nitration of tyrosines was carried out according to Walker (2002). AFP was incubated with 105 mM tetranitromethane (TNM) dissolved in acetonitrile. TNM specifically targets tyrosines and results in the reduction from pK_a 10 for tyrosine to pK_a 7 for 3-nitrotyrosine. After an incubation period of 2 h, the reaction was quenched using *ß*-mercaptoethanol. After 15 min at room temperature, PBS was added to the sample. Finally, the protein was dialysed against AFP standard buffer and quantified by SDS-PAGE analysis (see 3.3.7). The nitrated AFP version was termed nAFP. Subsequently, the extent of protein modification was determined by measuring the absorption of the reaction product, 3-nitrotyrosine, at a wavelength of 428 nm. Tyrosine residues residing inside the hydrophobic stretch are exposed towards the surrounding area, thus are considered to be particularly prone to chemical modification. On average, three of six tyrosines are chemically modified in nAFP. Proof of protein stability was shown by subjecting nAFP to SDS-PAGE analyses before and after completion of susceptibility studies.

3.4.3 Disruption of disulfide bridges

The disruption of cysteine bridges in AFP was carried out according to the protocol by Walker (2002). AFP was incubated in modification buffer (0.2 M Tris-acetate, pH 8.0; 6 M guanidine·HCl; 10 mM dithiothreitol) at 37 °C for 2 h. 2-nitro-5-thiocyanobenzoate (NTCB) was added to a 10-fold excess over sulfhydryl groups in protein and buffer. After additional incubation at 37 °C for 20 min, the protein was cleaved by adjusting to pH 9.0 using

NaOH. The cleavage generates a peptide blocked at its N-terminus as the cysteinyl residue is converted to an iminothiazolidinyl residue, thereby preventing the reformation of disulfide bridges. Incubation at 37 °C for a total of 16 h was succeeded by the dialysis of the protein against AFP standard buffer. The protein was subsequently quantified by SDS-PAGE analysis (see 3.3.7). The AFP version with disrupted disulfide bridges was termed cysAFP. It is assumed that the folding of cysAFP is similar to that of AFP, however, most likely to a less compact extent than the native protein. The electrophoretic mobility of cysAFP was compared to that of AFP by applying both of them to a native protein gel (Walker, 2002). Since cysAFP exhibited a significantly slower mobility than AFP, it was concluded that cysteine bridges in the chemically modified protein are disrupted. Proof of protein stability was shown by subjecting cysAFP to SDS-PAGE analyses before and after completion of susceptibility studies.

3.4.4 C-terminal truncation of AFP

The C-terminal truncated AFP version was synthesised by SynPep (Dublin, CA, USA). The short version of AFP consists of amino acids 1 to 33 and was termed sAFP. One of the naturally occurring disulfide bridges, spanning between cysteines residue 7 to 33, was included into sAFP with the aim to provide a means of protein stabilisation. Remaining cysteines were substituted by serines at positions 14, 26, and 28. It is assumed that sAFP adopts a similar conformation as AFP, stabilised by the included disulfide bridge. Proof of protein stability was shown by subjecting sAFP to SDS-PAGE analyses before and after completion of susceptibility studies.

3.5 *In situ* chitin synthase activity assay

A modified protocol described by Choi and Cabib (1994) was applied to prepare spheroplasts from germinated conidia of filamentous fungi. In brief, 1.4 ml 0.1 M EDTA and 24 µ ß-mercaptoethanol were added to each gram of mycelium. The volume was adjusted to 3.5 ml using distilled water and the sample subsequently incubated at 30 °C for 30 min, slightly shaking. Subsequently, mycelium was washed using 5 ml 0.8 M sorbitol. Mycelium was then resuspended in 6.7 ml solution A (0.57 ml citrate-buffer, pH 6.3; 67 µl 0.1 M EDTA; 0.8 M sorbitol). To solution A, 10 - 40 mg/ml Glucanex were added. Incubation at 30 °C for 30 min under constant shaking was succeeded by centrifugation and resuspension of cells in 30 ml chilled 0.05 M Tris/HCl (pH 7.5). Further incubation on ice for 5 min was followed by centrifugation and resuspension of spheroplasts in 1 ml assay buffer (0.05 M Tris/HCl, pH 7.5; 33 % glycerol). Analysis of spheroplasts was carried out by microscopy. Finally, the optical density of spheroplasts was determined at a wavelength of 600 nm. The chitin synthase assay was carried out after the slightly modified protocol described by Crotti et al. (2001). Reaction mixtures containing 32 mM Tris/HCl pH 7.5; 4.3 mM magnesium acetate; 1.1 mM uridine diphospho-N-acetyl-D-[U-^{14}C] glucosamine (^{14}C-GlcNAc); 2 µl trypsin (5 mg/ml); 20 µl spheroplasts in a total volume of 50 µl. Mixtures were incubated at 30 °C for 90 min. 2 µl of soybean trypsin inhibitor solution at a concentration 1.5 times that of the trypsin solution was applied in order to activate zymogenic chitin synthases. After addition of 10 % trichloroacetic acid and filtration, the insoluble chitin formed was assayed by measuring radioactivity using a liquid scintillation counter. The chitin synthase assay was performed in the presence and in the absence of 1 µg/ml AFP. The relative chitin synthase activity was determined by relating the specific enzyme activity (pmol incorporated ^{14}C-GlcNAc/optical density of spheroplasts) to the negative control. Measurements were carried out in triplicates.

3.6 *In vivo* assays

3.6.1 Susceptibility study

Filamentous fungi were cultivated in YPG medium (pH 4.5), yeasts were cultivated in YPG medium (pH 6.0), and bacterial L-forms were grown in BHI broth (*P. mirabilis* LVI, *E. coli* LWF$^+$) or BHI broth supplemented with yeast extract and sucrose (*B. subtilis* L170). *E. coli* K12 was grown in BHI. 1 x 10^3 conidia or cells were added to 150 µl of culture medium containing AFP at different concentrations ranging from 0 - 400 µg/ml. Analyses were carried out in microtiter plate format. After 48 h of incubation, the minimal AFP concentration that prevented growth of a given test organism was determined and declared as the minimal inhibitory concentration (MIC). Growth evaluation was either carried out visually i.e. by microscopy or by measuring the optical density at a wavelength of 600 nm. Measurements were carried out in triplicates.

3.6.2 SYTOX-Green uptake assay

The SYTOX-Green uptake assay was carried out according to the method described by Theis et al. (2003) in microtiter plate format. In brief, 1 x 10^2 conidia were cultivated in 150 µl YPG medium for 20 - 40 h at 28 °C. AFP or chemically modified AFP derivatives and SYTOX-Green were added to final concentrations of 100 µg/ml and 0.2 µM, respectively. Fluorescence was quantified immediately after application. Measurements were carried out over a time course of 210 min using a CytoFluor 2350 fluorescence measurement system (Millipore) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence values were corrected by subtracting the fluorescence value of samples incubated in the absence of AFP or chemically modified AFP derivatives. Measurements were carried out in triplicates.

3.6.3 *D*-PDMP assay

Assays were carried out in microtiter plate format using 1 x 10^3 conidia, which were inoculated in 150 µl YPG liquid medium each. AFP was added to samples in concentrations ranging between 0 - 400 µg/ml as well as 35 µM *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (*D*-PDMP). In controls, *D*-PDMP was replaced by sterile distilled water. Cultivations were carried out at 28 °C for 48 h. Subsequently, growth was assessed by measuring the optical density at a wavelength of 600 nm. Measurements were carried out in triplicates.

3.7 *In vitro* assays

3.7.1 Mobility shift assay

For mobility shift assays, 5 µg herring sperm DNA (Promega) or 7 µg yeast tRNA (Roche) were incubated with 0 - 30µg AFP either at room temperature or at 95 °C for 60 min. Samples were loaded on TAE agarose gels (0.7 %). After electrophoretic separation at 25 V overnight, gels were stained using ethidium bromide and analysed by a transilluminator.

3.7.2 *In vitro* chitin binding assay

The regeneration of chitin (Sigma) was achieved by the procedure described by Koo et al. (2002). In brief, 1 g of chitin was dissolved in 10 ml phosphoric acid (85 %). After stirring for 24 h at 4 °C, aggregates of chitin were thoroughly grinded. The chitin material was subsequently washed by filtration using distilled water. When the flow-through had obtained a neutral pH, the prepared chitin was finally dissolved in TE buffer (pH 7.0). Subsequently, the binding capacity of 100 µl regenerated chitin was determined to consist of 10 µg AFP. Thus, aliquots of 100 µl regenerated chitin were incubated with 10 µg protein to be analysed. The chitin matrix was successively treated with TE washing buffer (pH 7.0) and TE elution buffer (pH 7.0), of which the latter contains 1.5 M NaCl. All solutions applied to the matrix had volumes of 100 µl. After centrifugation, these were taken off thoroughly and retained at – 20 °C until subjection to SDS-PAGE analyses (see 3.3.7). The protein content of individual samples was determined using the Kodak Image Station 440 cf. Proteins identified in samples that were isolated before the application of washing buffer were termed ‘non-bound protein fractions’. Proteins that were identified in samples after the application of elution buffer were defined as ‘bound protein fractions’. Measurements were carried out in triplicates.

3.7.3 Biodegradability of AFP

AFP was applied to simulated gastric juice (see 3.1.4) or bile salt solution composed of 1.5 % bile salts dissolved in PBS (pH 7.2) to a final concentration of 500 µg/ml. Samples were incubated in 1.5 ml reaction tubes at 37 °C and 120 rpm. After 0 h, 3 h, 6h and 24 h, aliquots were taken to be analysed by SDS-PAGE (see 3.3.7). Protein gels were assessed using the Kodak Image Station 440 cf.

4 Results

4.1 Generation of chemically modified AFP versions

Amphipathicity is a general feature of antimicrobial peptides, which in combination with amino acid composition, cationic net charge and size allow them to attach and to insert into membrane bilayers (Park and Hahm, 2005). In agreement with this are the structural features of AFP, among which the cationic site and the hydrophobic stretch account for the amphipathic character of the protein, assumed to be involved in AFP-evoked membrane permeabilisation. The precise involvement of tyrosines located inside the hydrophobic region and cationic net charge of AFP was to be investigated here in more detail.

Initial attempts concerned with the heterologous expression and purification of AFP from *E. coli* have failed (Meyer, 2003). Therefore, chemically modified AFP versions were produced. While the nitrated AFP (nAFP) derivative exhibits a non-functional hydrophobic stretch, the acetylated AFP (acAFP) version carries a modified net charge due to the masking of lysines by carbonyl groups. A short version of AFP (sAFP) was synthesised (aa 1 to 33), which is devoid of the hydrophobic stretch. It furthermore misses tyrosines at positions 45 and 50 usually located inside the hydrophobic stretch. Finally, a derivative of AFP was created, which exhibits disrupted disulfide bridges (cysAFP). Proof of protein stability was shown by subjecting AFP, acAFP, nAFP, cysAFP and sAFP to SDS-PAGE analyses before and after completion of susceptibility assays.

4.1.1 Determination of minimal inhibitory concentrations

The growth inhibitory effect of chemically modified AFP versions was to be assessed. Susceptibility studies were carried out using 10^3 conidia of selected fungal test strains exhibiting resistance (*Penicillium chrysogenum*), moderate sensitivity (*Aspergillus nidulans*, *Fusarium solani*) and high sensitivity (*A. fumigatus*, *A. niger*, *Fusarium oxysporum*) towards AFP. These were incubated in the presence of rising protein concentrations usually ranging between 0 to 400 µg/ml. The MIC was defined as the concentration which resulted in complete growth inhibition. Table 1 summarises the MICs determined for AFP (for reference) and for chemically modified AFP versions, respectively.

Table 1. Minimal inhibitory concentration (MIC) determined for AFP and chemically modified AFP versions. Fungal test strains were cultivated with rising concentrations of AFP or chemically modified AFP derivatives. Experiments were carried out in triplicates. Abbreviations: acAFP = acetylated AFP; nAFP = nitrated AFP; cysAFP = AFP exhibiting disrupted disulfide bridges; sAFP = short AFP; NE = no effect.

	MIC [µg/ml]					
	Penicillium chrysogenum ATCC 10002	*Aspergillus nidulans* IfGB 15/1701	*Fusarium solani* IfGB 39/1001	*Aspergillus fumigatus* IfGB 15/0809	*Aspergillus niger* IfGB 15/1801	*Fusarium oxysporum* IfGB 39/1201
AFP	NE	200	120	10	1	1
acAFP	NE	> 200	> 200	150	120	120
nAFP	NE	> 400	> 400	40	20	20
cysAFP	NE	1	1	> 400	10	1
sAFP	NE	> 400	> 400	> 400	> 400	50

In general, the growth inhibitory effect of chemically modified AFP versions was significantly reduced compared to AFP. This particularly held true for acAFP, nAFP and sAFP, of which the first exhibits a reduced basic net charge and the latter two are devoid of a functional hydrophobic protein domain. Interesting to note is that acAFP exhibited dramatically increased MICs for *A. niger* and *F. oxysporum* (120-fold increase compared to AFP), indicating that the cationic net charge of AFP is essential for provoking growth inhibition. The growth inhibitory effect was less pronounced when the same fungi were cultivated in the presence of nAFP (20-fold increase in MIC), indicating that the hydrophobic protein domain is also required for growth inhibition, however, does not seem to play an equally significant role as the cationic protein charge. Similar results were observed for *A. fumigatus*, where cultivation in the presence of acAFP resulted in a 15-fold increase, while incubation with nAFP caused a 4-fold increase in MIC. In summary, these data suggest that the amphipathic character is important for observing AFP-induced growth inhibition.

The MICs of acAFP for *A. nidulans* and *F. solani* could only roughly be estimated (> 200 µg/ml) since the protein stock concentration was too low to test concentrations above 200 µg/ml. In respect to sAFP, *F. oxysporum* was the only test strain which was restricted in growth (MIC 50 µg/ml), again indicating that deficiency in a functional hydrophobic protein region reduces the antifungal potency of AFP significantly. Remarkably, the growth inhibitory effect of cysAFP was shown to be extremely variable. For *A. niger*, a 10-fold increase in MIC was observed. More dramatically, a 40-fold increase in protein concentration

was not sufficient to restrict growth of *A. fumigatus*. In contrast, no alteration in MIC was observed for *F. oxysporum*, while *A. nidulans* and *F. solani* were shown to be significantly more susceptible to cysAFP than to AFP. This observation points towards the possibility that different fungi may contain different AFP targets. Alternatively, cysAFP may be assumed more flexible than AFP due to disrupted disulfide bridges. This could result in enhanced interactions with putative AFP targets in some fungi, accounting for the dramatically increased sensitivities of *A. nidulans* and *F. solani*. Neither of the analysed proteins resulted in growth restriction of *P. chrysogenum*, demonstrating that chemical modifications of AFP have no influence on the susceptibility of this fungus.

4.1.2 Determination of membrane permeabilising potentials

With the aim to determine the membrane permeabilising potential of chemically modified AFP versions, assays were conducted which make use of the fluorogenic dye SYTOX-Green. SYTOX-Green is an organic compound that fluoresces upon interaction with nucleic acids - however, SYTOX-Green can only penetrate into cells with impaired plasma membranes (Thevissen et al., 1999). In the past, SYTOX-Green uptake assays have proved a valuable tool for determining membrane permeabilisation in filamentous fungi. The membrane permeabilising potential of plant defensins from *Neurospora crassa* was successfully demonstrated by this assay (Thevissen et al., 1999). Likewise, AFP-induced membrane permeabilisation was shown by the application of this technique (Theis et al., 2003). Therein obtained data suggested that the degree of AFP activity can be directly correlated with the extent of fluorescence measured. Subsequently, 10^2 conidia of each selected test strain were grown in 150 µl YPG liquid medium (pH 4.5). After 20 h (*A. niger*) or 40 h (*F. oxysporum* IfGB 39/1201, *F. solani*, *P. chrysogenum*, *A. nidulans*) at 28 °C, 0.2 mM SYTOX-Green and 100 µg/ml AFP or chemically modified AFP versions were added to the samples. Exemplarily illustrated in Figure 1 are the results obtained for *F. oxysporum*. Identical permeabilisation patterns were obtained for *A. niger* (data not shown).

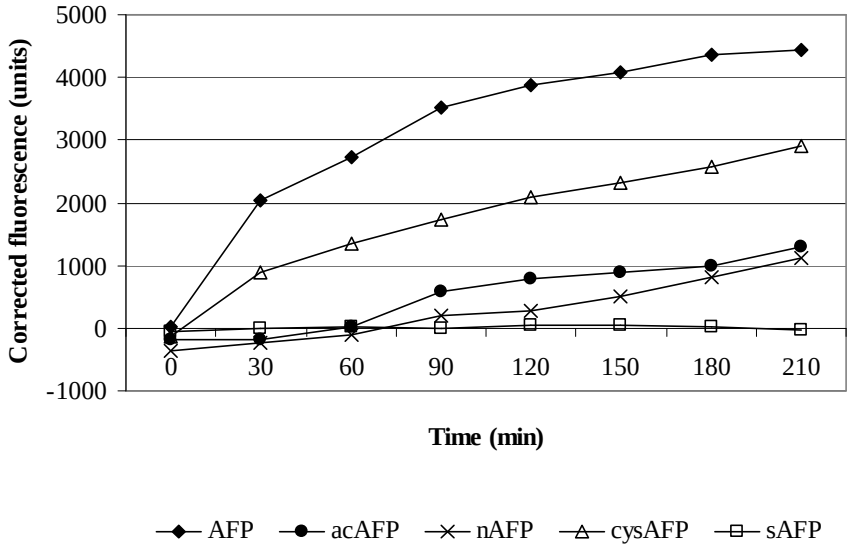

Figure 1. SYTOX-Green uptake assays conducted to analyse plasma membrane permeabilisation in *F. oxysporum* IfGB 39/1201. Incubation of *F. oxysporum* was carried out in the presence of 0.2 µM SYTOX-Green and 100 µg/ml AFP or chemically modified AFP versions over a period of 210 min. Fluorescence values were corrected with the baseline fluorescence, which was determined by incubating *F. oxysporum* in the absence of AFP. Triplicate measurements were carried out. A representative experiment is shown. Abbreviations: acAFP = acetylated AFP; nAFP = nitrated AFP; cysAFP = AFP exhibiting disrupted disulfide bridges; sAFP = short AFP.

Clearly, the most distinct membrane permeabilisation was evoked by AFP. A markedly reduced membrane permeabilising effect was observed for cysAFP, while membrane permeabilising potentials of acAFP and nAFP were even more dramatically affected. No membrane permeabilising effect was observed for sAFP. As expected, neither of the analysed proteins provoked membrane permeabilisation in *P. chrysogenum* (data not shown).

Intrigued by the highly variable results obtained in susceptibility assays of *A. nidulans* and *F. solani* (see 4.1.1), more information was to be gathered concerning the membrane permeabilising effect of cysAFP on respective strains. Interestingly, no significant discrepancies between membrane permeabilising potentials of AFP and cysAFP could be observed (Fig. 2), suggesting that membrane permeabilisation alone does not provoke growth restriction. In consequence, it may be speculated that different fungi contain different i.e. more than one AFP target(s).

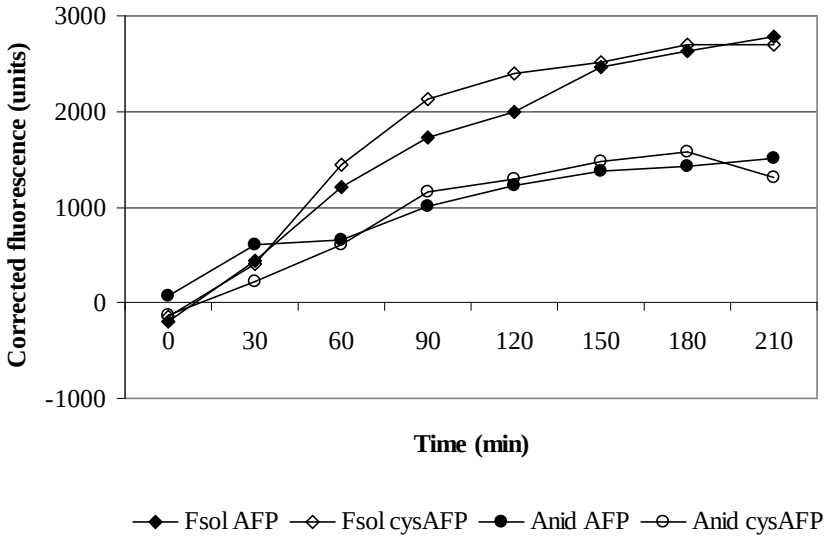

Figure 2. SYTOX-Green uptake assay conducted to analyse plasma membrane permeabilisation in *F. solani* and *A. nidulans*. After addition of 0.2 µM SYTOX-Green and 100 µg/ml AFP or cysAFP to fungal test strains, fluorescence was measured for up to 210 min. Correction of fluorescence values was achieved by subtracting the baseline fluorescence (samples incubated in the absence of AFP or cysAFP). Experiments were carried out in triplicates, a representative experiment is shown. Abbreviations: acAFP = acetylated AFP; nAFP = nitrated AFP; cysAFP = AFP exhibiting disrupted disulfide bridges; sAFP = short AFP; Fsol = *F. solani*; Anid = *A. nidulans*.

4.2 In vitro chitin binding of AFP

Previously published data demonstrated that AFP can be purified by chitin affinity chromatography (Liu et al., 2002). In order to show that AFP does bind to chitin specifically, and to analyse whether the basic net charge, the cationic and/or the hydrophobic protein domains are involved in this process, *in vitro* chitin binding assays were carried out with AFP and chemically modified AFP versions. Proteins were applied to chitin columns and eluates subjected to SDS-PAGE analyses. While proteins removed during washing steps were considered as non-bound AFP fractions, proteins isolated during elution steps were regarded as bound AFP fractions. As Figure 3 reflects, chitin binding affinities of AFP, nAFP, sAFP and cysAFP were nearly identical (91 to 100 %), indicating that the hydrophobic protein domain and correct protein folding is dispensable for efficient chitin binding.

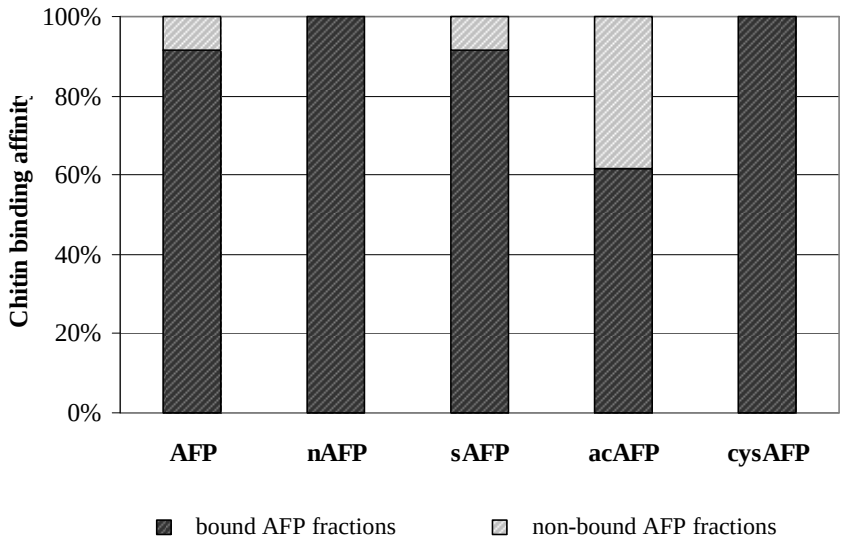

Figure 3. Binding affinity of AFP and chemically modified AFP versions to chitin. Native and chemically modified derivatives of AFP were incubated with chitin. Subsequently, AFP fractions were determined that did bind to chitin (dark grey) and that did not bind to chitin (light grey). Experiments were carried out in triplicates, a representative experiment is shown. Abbreviations: acAFP = acetylated AFP; nAFP = nitrated AFP; cysAFP = AFP exhibiting disrupted disulfide bridges; sAFP = short AFP.

A significant deviation in chitin binding affinity was observed for acAFP. Here, only 61 % of the protein applied did in fact adhere to chitin, proposing that the cationic net charge of AFP may contribute to efficient chitin adherence. However, the residual chitin binding affinity of acAFP also suggested the presence of further protein domains, which may facilitate the attachment of AFP to chitin. Therefore, the amino acid sequence of AFP was compared with several chitin binding proteins. Commonly, these proteins contain a discrete chitin-binding domain (CBD) that mediates adsorption to the substrate. Analyses revealed that AFP shares considerable homology with the AKWWTQ-motif (Fig. 4), a region that is well conserved in bacterial chitin binding domains (Miyamoto et al., 2002). Sequence similarities of 90 % are obtained when comparing AFP with the AKWWTQ-motif of either ApChiA from *Aeromonas punctata* or Cbp1 from *Altermonas* sp. (T-Coffee). No such sequence homologies were observed when AFP was aligned with CBDs from plants, insects, invertebrates or fungi (data not shown).

```
AFP          1        ATYNG----KCYKKDNICKYKAQSGKTAICKCYVKKC    33
ApChiA     772    AWSAGTVYNTNDKVSHKQLVWQAKYWTQGNEPSRTADQWKLV   813
EndoI       39    EWQSDTIYTGGDQVQYNGSAYQANYWTQNNDPEQFS--YAVV    78
Cbp1       435    AWNSTTTYVAGDRVTHQQKVYEAKWWTQGEEPGA-SDVWKAI   475
ChiA       778    TWDRSTVYVGGDRVIHNSNVFEAKWWTQGEEPGT-ADVWKAV   818
ChiC        29    QWQSQQVYTGGDAVTYQSAKYTAKWWTQNQNPAQNSNTYDVW    70
ChiD        35    EWSQSSAYNGGAQVQKSQQAFEAKWWTQADPVTHSGQWDDW     75
SmChiB     455    AYVPGTTYAQGALVSYQGYVWQTKWGYITSAPGSDSA-WLKV   495
```

Figure 4. Amino acid sequence alignment of AFP with bacterial chitin binding proteins. Abbreviations: ApChiA = *Aermononas punctata* chitinase A; EndoI = *Vibrio furnissii* chitodextrinase; Cbp1 = chitin-binding protein of *Alteromonas* sp. strain O-7; ChiA = chitinase A of *Alteromonas* sp. strain O-7; ChiC = chitinase C of *Alteromonas* sp. strain O-7; ChiD = chitinase-like enzyme from *Alteromonas* sp. strain O-7; SmChiB = *Serratia marcescens* chitinase B. Residues that are identical to AFP are indicated in boldface. Boxed sequences indicated the conserved AKWWTQ-motif of bacterial chitin binding proteins.

It is possible to speculate that chitin binding is evoked by the putative CBD. Since sAFP contains the complete sequence assumed to consist of the CBD, it may account for efficient chitin binding as observed for AFP. Structurally, the putative CBD of AFP is localised on a linear stretch of amino acids in direct opposition to the hydrophobic protein domain (Fig. 5). These data demonstrate that AFP binds efficiently to chitin, thereby providing initial support for the involvement of chitin in the mode of action of AFP. Further indication was provided by the revelation of a putative CBD, which may facilitate binding of AFP to chitin. In summary, these results encourage the assumption that chitin and/or chitin biosynthesis could be involved in the mode of action of AFP.

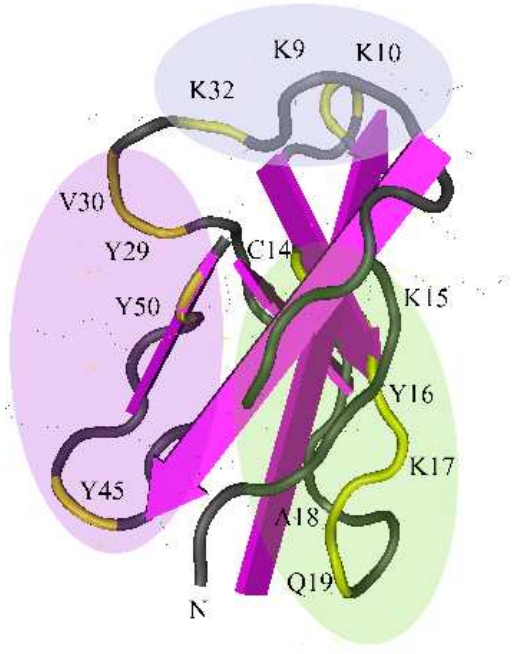

Figure 5. The putative chitin binding domain (CBD) of AFP. The putative CBD is represented by green shading. Indicated in blue is the cationic site, the hydrophobic stretch is represented in pink shading. The amino-terminal end of AFP is indicated by N, ß-sheet structures are shown as arrows. The image was illustrated using the Cn3D 4.1 software on the basis of the AFP structure previously published by Campos-Olivas et al. (1995).

4.3 Putative AFP targets associated with fungal cell walls

Previous results indicated that AFP binds efficiently to chitin, possibly mediated by a putative CBD. In order to obtain further proof for the involvement of chitin and/or chitin biosynthesis in the mode of action of AFP, chitin synthase mutants of filamentous ascomycetes were subjected to susceptibility analyses.

4.3.1 AFP susceptibility of *F. oxysporum* and *A. oryzae* chitin synthase mutants

Filamentous fungi contain distinct classes of chitin synthases (classes III, V and VI; Roncero, 2002) that are absent in yeast. In order to determine whether deficiency in any of these chitin synthase classes would render fungi less susceptible towards AFP, class III and V mutants of AFP-sensitive fungi (*F. oxysporum* 4287 and *A. oryzae* A1560 with MICs of 1 µg/ml) were chosen to be analysed. These were subjected to susceptibility studies (see 3.6.1). As Figure 6

illustrates, the class V mutant (*chsV*) of *F. oxysporum* (Madrid et al., 2003) was considerably less affected by AFP than the corresponding wild-type strain (4287). Apparently, growth inhibition in the *chsV* mutant remained the same (27.31 % at 50 µg/ml AFP and 28.87 % at 100 µg/ml AFP) irrespective of the AFP concentration tested. Even at concentrations of 400 µg/ml AFP, a residual growth was measured that estimated 26.3 % in relation to the negative control (data not shown). These data indicate that deletion of class V chitin synthase in *F. oxysporum* 4287 does indeed result in a significantly reduced AFP susceptibility.

In order to exclude that the obtained results were due to morphological abnormalities which were described for the *chsV* mutant under low osmotic conditions (Madrid et al., 2003), experiments were repeated in growth medium supplemented with 1 M sorbitol. In analogy to previous results, the mutant responded with a similarly reduced AFP susceptibility (data not shown), suggesting that the reduced AFP susceptibility of the *chsV* mutant is not due to morphological deficiencies.

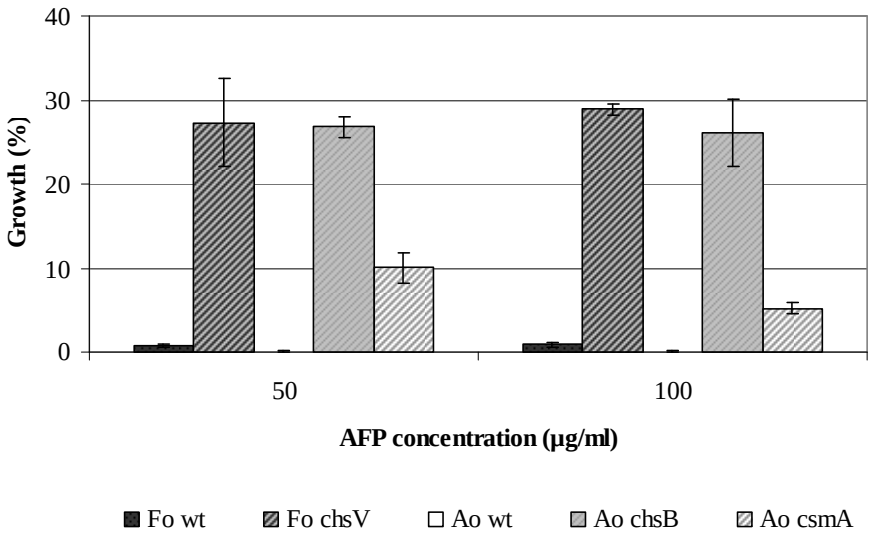

Figure 6. Susceptibility analyses carried out with chitin synthase mutants of *F. oxysporum* 4287 and *A. oryzae* A1560. Corresponding wild-type strains were included in the analyses for reference. Growth is expressed in % in relation to the negative control (not treated with AFP). Experiments were carried out in triplicates. Abbreviations: Fo = *F. oxysporum*; Ao = *A. oryzae*.

Analyses were also carried out with chitin synthase mutants of *A. oryzae* A1560. AFP susceptibilities of a class III (*chsB*) and a class V (*csmA*) mutant (Muller et al., 2002) were analysed and compared with the wild-type sensitivity. Figure 6 shows that at 50 µg/ml and 100 µg/ml AFP, the *chsB* and *csmA* mutants were less susceptible than the wild-type strain. In

comparison to the *F. oxysporum chsV* mutant, it was found that *chsB* and *csmA* mutants exhibited a similarly reduced growth inhibition. However, increasing the AFP concentration to 400 µg/ml resulted in further growth reduction of the *chsB* mutant to 7.3 % (data not shown). In summary, these data show that the *csmA* mutant of *A. oryzae* A1560 is significantly more susceptible to AFP than the *chsB* mutant or the *chsV* mutant of *F. oxysporum* 4287. It can therefore be assumed that class III and V chitin synthases may constitute putative AFP targets.

4.3.1.1 Susceptibility of *F. oxysporum chsV* mutant to chemically modified AFP versions

In order to determine whether *F. oxysporum* 4287 wild-type and *chsV* mutant exhibit deviating susceptibilities towards chemically modified AFP versions from *F. oxysporum* IfGB 39/1201, susceptibility studies were carried out with these fungi (see 3.6.1). Table 2 summarises the MICs of chemically modified AFP versions for the *chsV* mutant and the corresponding wild-type strain (4287) as well as those for *F. oxysporum* IfGB 39/1201.

Table 2. Minimal inhibitory concentration (MIC) of chemically modified AFP versions determined for *F. oxysporum* strains. *F. oxysporum* 4287 and corresponding *chsV* mutant as well as *F. oxysporum* IfGB 39/1201 were cultivated in the presence of AFP or chemically modified AFP versions. Experiments were carried out in triplicates. Abbreviations: acAFP = acetylated AFP; nAFP = nitrated AFP; cysAFP = AFP exhibiting disrupted disulfide bridges; sAFP = short AFP; NE = no effect.

	AFP	**acAFP**	**nAFP**	**cysAFP**	**sAFP**
F. oxysporum IfGB 39/1201	1	120	20	1	50
F. oxysporum 4287	1	> 100	50	1	50
F. oxysporum chsV	NE	> 100	NE	NE	NE

The here presented results show clearly that wild-type strains of *F. oxysporum* (4287 and 39/1201) respond to chemically modified AFP versions in a comparable manner. Neither of the AFP versions tested influenced the growth of the *F. oxysporum chsV* mutant. The corresponding wild-type strain (4287), in contrast, was significantly more susceptible towards AFP than the *chsV* mutant, exhibiting a MIC of 1 µg/ml. The MICs of nAFP and sAFP were 50-fold higher than the MIC of AFP. No difference in MIC was observed between cysAFP

and AFP (1 µg/ml). The MICs of acAFP for the *chsV* mutant and *F. oxysporum* 4287 could only roughly be estimated (> 100 µg/ml), since the protein stock concentration was too low to test concentrations above 100 µg/ml. These results indicate that the *F. oxysporum chsV* mutant is as resistant towards chemically modified AFP versions as it is to AFP. In addition, it was shown that *F. oxysporum* wild-type strains (4287 and IfGB 39/1201) exhibit a comparable susceptibility towards the tested AFP versions.

4.3.2 Determination of AFP-induced plasma membrane permeabilisation in *F. oxysporum* and *A. oryzae* chitin synthase mutants

With the aim to confirm results previously obtained in susceptibility studies (see 4.3.1), the membrane permeabilising potential of AFP on chitin synthase mutants and corresponding wild-type strains of *F. oxysporum* 4287 and *A. oryzae* A1560 was to be analysed. Thus, SYTOX-Green uptake assays were carried out as previously described. As shown in Figure 7, the wild-type strain of *F. oxysporum* and *A. oryzae* became equally well permeated by AFP. In contrast, the *chsV* and the *csmA* mutant seemed not to be affected by AFP at all. Compared to *chsV* and *csmA*, fluorescence of *chsB* appeared to be slightly elevated. However, during the whole course of incubation, no significant increase in fluorescence was observed. These data indicate that plasma membranes in chitin synthase mutants fail to become permeabilised by AFP.

Subsequently, the membrane permeabilising ability of chemically modified AFP versions was to be analysed by subjecting the chitin synthase mutant of *F. oxysporum* 4287 to SYTOX-Green uptake assays. Membrane permeabilisation was shown to be identical to patterns observed in *F. oxysporum* IfGB 39/1201. In agreement with previous studies aimed at determining the susceptibility of *F. oxysporum chsV* towards chemically modified AFP versions, none of these derivatives was shown to be capable of inducing plasma membrane permeabilisation in the *chsV* mutant (data not shown).

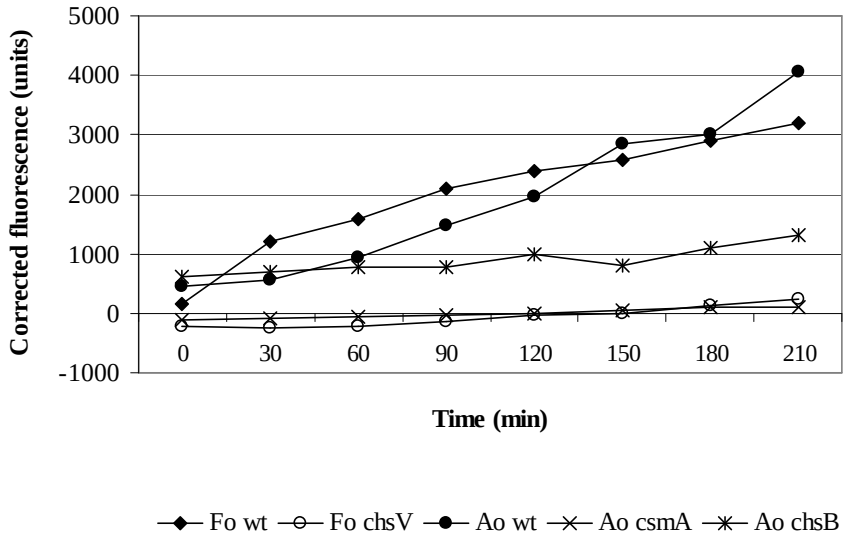

Figure 7. SYTOX-Green uptake assays carried out with chitin synthase mutants of *F. oxysporum* 4287 and *A. oryzae* A1560 to analyse plasma membrane permeabilisation. *F. oxysporum* 4287 and corresponding *chsV* mutant as well as *A. oryzae* A1560 and corresponding mutants *chsB* and *csmA* were incubated in the presence of 0.2 µM SYTOX-Green and 100 µg/ml AFP over a period of 210 min. Fluorescence values were corrected with the baseline fluorescence, which was determined by incubating fungi in the absence of AFP. Experiments were carried out in triplicates. A representative experiment is shown. Abbreviations: acAFP = acetylated AFP; nAFP = nitrated AFP; cysAFP = AFP exhibiting disrupted disulfide bridges; sAFP = short AFP.

4.3.3 Determination of *in situ* chitin synthase activities in the presence of AFP

The chitin synthase mutants analysed in this work were shown to respond to AFP with reduced sensitivities. However, only the *F. oxysporum chsV* mutant exhibits less cell wall chitin (60 %), which suggests that chitin itself does not decide exclusively about AFP susceptibility. In order to analyse whether AFP targets chitin synthases, AFP-resistant and AFP-sensitive fungi were examined in *in situ* chitin synthase activity assays. These consisted of incubating fungal spheroplasts in the presence of ^{14}C-GlcNAc and AFP (see 3.5). From the amount of incorporated radioactivity was concluded whether chitin synthase activities are induced or inhibited by AFP activity. Table 3 shows very clearly that chitin synthase activity in wild-type strains of *F. oxysporum* 4287 and *A. oryzae* were significantly reduced in the presence of AFP by approx. 40 %.

Table 3. Relative chitin synthase activities in the presence of AFP. *A. niger*, *F. oxysporum* 4287 and corresponding *chsV* mutant, *A. oryzae* A1560 as well as *P. chrysogenum* were incubated with ^{14}C-GlcNAc in the presence or in the absence of AFP. The relative chitin synthase activity was determined by relating the specific enzyme activity to the negative control (incubation of fungi in the absence of AFP). Standard deviation of the means is given in parenthesis. Abbreviations: NE = no effect. The respective minimal inhibitory concentrations (MICs) of AFP are given for reference. Experiments were carried out in triplicates.

	Aspergillus niger 15/1801	*Fusarium oxysporum* 4287	*Fusarium oxysporum* 4287 *chsV*	*Aspergillus oryzae* A1560	*Penicillium chrysogenum* ATCC 10002
Relative chitin synthase activity	0.28 (+/- 0.00)	0.62 (+/- 0.02)	1.57 (+/- 0.01)	0.60 (+/- 0.02)	4.6 (+/- 0.00)
MIC AFP [µg/ml]	1	1	> 400	1	NE

Similarly, chitin synthase activity in *A. niger* was significantly reduced by about 70 %. In contrast, AFP-resistant fungi such as *F. oxysporum chsV* mutant and *P. chrysogenum* exhibited remarkably elevated levels of chitin synthase activity (approx. 160 and 460 %, respectively). These data suggest that chitin synthases of classes III and V are indeed targets of AFP. The increased chitin synthase activity in AFP-resistant fungi is suggested to contribute to the integrity of their cell walls in the presence of AFP.

4.4 Putative AFP targets associated with fungal plasma membranes

Subsequently, it was to be examined whether structures other than cell walls may be involved in the mode of action of AFP. Plasma membranes of fungi and bacteria are known to differ in characteristic components, including the fact that prokaryotes lack sphingolipids but rather contain phospholipids (Daum et al., 1998; Dickson and Lester, 1999). Thus, analyses were conducted which focussed on lipid constituents specifically occurring in fungal plasma membranes, aiming at the identification of further putative AFP targets.

4.4.1 Bacterial protoplasts remain AFP-resistant

In order to analyse whether bacterial protoplasts remain AFP-resistant, susceptibility studies were conducted in which cell wall-less L-forms of *Bacillus subtilis*, *Proteus mirabilis* and *E. coli* (Gumpert and Hoischen, 1998) were analysed (see 3.2 and 3.6.1). Although these strains lack a cell wall by mutation, they are easy to handle and stably propagate in brain heart infusion (BHI) broth (*P. mirabilis*, *E. coli*) or BHI broth supplemented with yeast extract and saccharose (*B. subtilis*). An approx. 10-fold increase in MIC was observed when *F. oxysporum* IfGB 39/1201 or *A. niger* were cultivated in BHI broth or BHI broth supplemented with yeast extract and saccharose in the presence of AFP (data not shown). Taking the reduced AFP potency into consideration, susceptibility studies on bacterial L-forms were carried out in BHI broth nonetheless. Pre-cultures of bacterial L-forms were used to inoculate 150 µl of cultivation broth with an OD_{600nm} of 0.05. Finally, AFP was added to samples in concentrations ranging between 0 - 400 µg/ml. After incubation at 37 °C at 200 rpm for 6 h, growth was evaluated microscopically. No differences in growth were observed for samples cultivated in the presence or in the absence of AFP (data not shown). Furthermore, samples of L-forms that were cultivated in the presence of AFP grew equally well as *E. coli* K12, a wild-type strain which served as the positive control (data not shown). From these results can be concluded that bacterial plasma membranes do not contain a putative AFP target. Thus, it is suggested that specific lipid components exclusively present in fungal plasma membranes are involved in determining AFP sensitivity.

4.4.2 Fungal glucosylceramides may account for AFP sensitivity

The resistance of bacterial protoplasts suggested that plasma membrane components exclusively found in fungi may determine AFP susceptibility. Sphingolipids constitute integral components of eukaryotic cell membranes (Dickson and Lester, 1999). Belonging to this large class of membrane lipids are the glycosphingolipids. The observation that numerous AFP-sensitive fungi contain glucosylceramides incited the assumption that these may constitute putative AFP targets (Table 4).

Table 4. Glycosphingolipids in fungi (Warnecke and Heinz, 2003). The minimal inhibitory concentration (MIC) of AFP is given for reference. Abbreviations: GlcCer = glucosylceramide; GalCer = galactosylceramide; NE = no effect.

Fungus	Glycosphingolipid	AFP susceptibility MIC (µg/ml)
Aspergillus niger	GalCer GlcCer	1
Aspergillus fumigatus	GlcCer GalCer	10
Fusarium solani	GlcCer	120
Saccharomyces cerevisiae	GalCer	NE

Since no suitable mutants were available for analyses, it was decided to test the hypothesis that glucosylceramides may constitute putative AFP targets by inhibiting glucosylceramide synthesis in AFP-sensitive fungi by chemical means. It has been demonstrated that an analogue of ceramide, *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (*D*-PDMP), inhibits UDP-glucose:N-acylsphingosine glucosyltransferase (Inokuchi and Radin, 1987), the enzyme required for glucosylceramide synthesis. Thus, application of *D*-PDMP is expected to deplete glucosylceramide levels in fungal plasma membranes. When fungi consequently exhibit reduced AFP susceptibilities, it may be concluded that glucosyl-ceramides may represent putative AFP targets. In accordance with previously described susceptibility studies, 10^3 conidia of selected fungal test strains were incubated in 150 µl YPG liquid medium (pH 4.5) in the presence of rising AFP concentrations (0 - 400 µg/ml) and *D*-PDMP (35 µM). After 48 h at 28 °C without agitation, growth was assessed using a

microplate spectrophotometer. In control cultivations, the *D*-PDMP solution was replaced by distilled water. Figure 8 indicates, that the cultivation of *A. niger* in the presence of *D*-PDMP resulted in considerably reduced AFP susceptibilities. At 0.5 µg/ml AFP, residual growth of 32.0 % in the presence of *D*-PDMP and 18.1 % in the absence of *D*-PDMP was observed. At 2.5 and 4 µg/ml AFP, cultivation in the presence of *D*-PDMP resulted in 2 % and 1.9 % growth, respectively. No growth was observed for cultivations in the absence of *D*-PDMP at respective AFP concentrations. In conclusion, an approx. two-fold increase in growth is observed for cultivations in the presence of *D*-PDMP, suggesting that reduced glucosylceramide levels render fungi more resistant towards AFP.

Identical experiments carried out with *A. fumigatus* provided even more pronounced effects. Here, incubation in the presence of *D*-PDMP rendered the fungus dramatically less AFP-sensitive than in the controls (Fig. 9). In fact, growth of *A. fumigatus* in the presence of *D*-PDMP seemed to remain on a basic level (approx. 23 %), regardless of the AFP concentration applied. In contrast, cultivation in the absence of *D*-PDMP significantly inhibited fungal growth at 5 µg/ml and 10 µg/ml AFP to 14.1 % and 2.2 %, respectively. No *D*-PDMP-induced AFP degradation was observed during the course of experiments (data not shown). In consequence, the here presented data suggest that glucosylceramides may represent putative AFP targets.

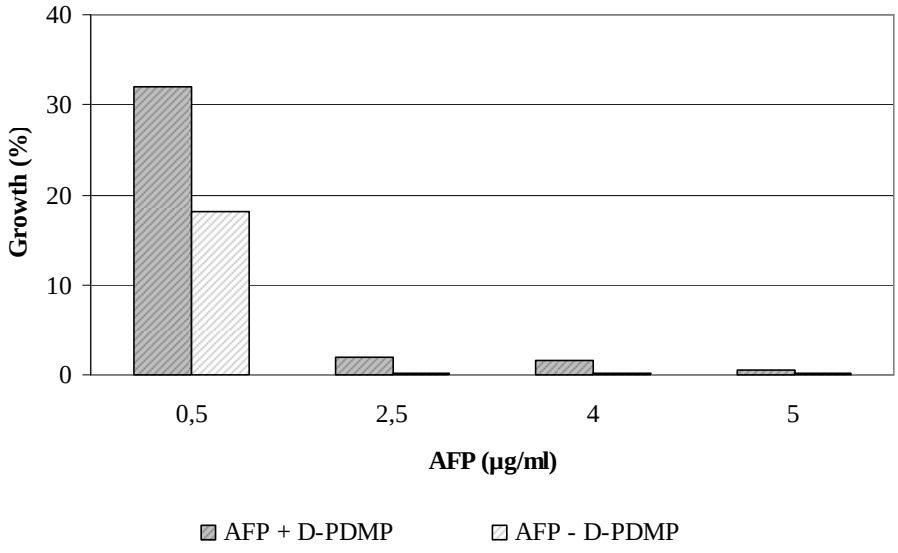

Figure 8. Growth of *A. niger* with AFP in the presence or in the absence of *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (*D*-PDMP). *A. niger* was cultivated in the presence of rising AFP concentrations and 35 µM *D*-PDMP for 48 h at 28 °C in YPG liquid medium. Cultivations in the absence of *D*-PDMP served as reference. Growth is expressed in % in relation to the negative control (not treated with AFP). Experiments were carried out in triplicates.

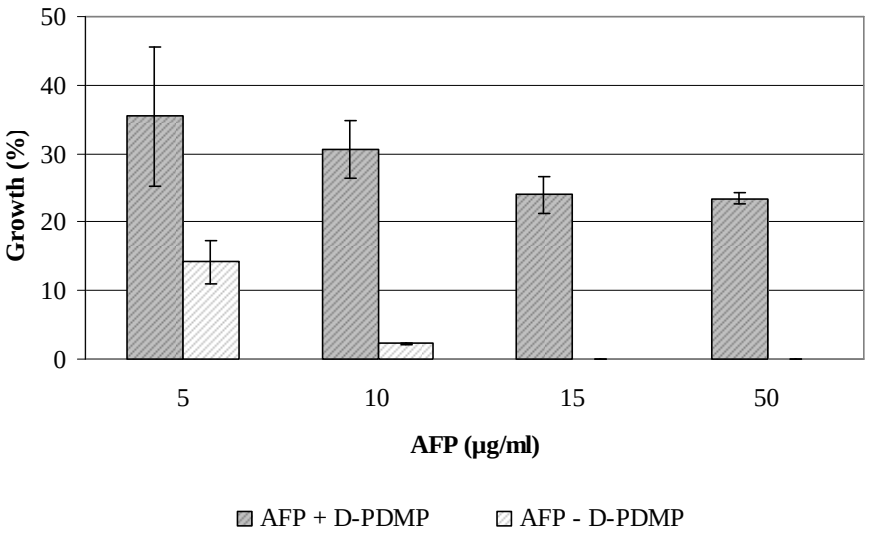

Figure 9. Growth of *A. fumigatus* with AFP in the presence or in the absence of *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (*D*-PDMP). *A. fumigatus* was grown in YPG liquid medium for 48 h at 28°C in the presence of rising AFP concentrations and *D*-PDMP (35 µM), while cultivations in the absence of *D*-PDMP served as reference. Growth is expressed in % in relation to the negative control (fungi cultivated in the absence of AFP). Experiments were carried out in triplicates.

4.4.3 AFP susceptibility of cAMP-dependent protein kinase mutants of *A. niger* affected in membrane lipid composition

In an approach to further investigate the influence of lipid composition on AFP-susceptibility, *A. niger* mutants with alterations in lipid content were subjected to SYTOX-Green uptake assays. It was recently shown that disruption or overexpression of genes encoding for either regulatory and/or catalytic subunit of the cAMP-dependent protein kinase (PKA) considerably influences lipid biosynthesis in *A. niger* A395 (Jernejc and Bencina, 2003). Table 5 gives an overview on the here analysed strains with regard to their respective lipid profile.

Table 5. *A. niger* cAMP-dependent protein kinase (PKA) mutants exhibiting altered membrane lipid composition (Jernejc and Bencina, 2003). PKA mutants of *A. niger* A395 used in analyses to determine altered AFP-susceptibilities. Summarised in this table are relative values (approximated) in relation to the *A. niger* A395 (equated = 1). Abbreviation: mcC = multicopy catalytic subunit of PKA; DC = deleted catalytic subunit of PKA.

	Mutant strain mcC	**Mutant strain DC**
PKA activity	increased or uncontrolled	abolished
Total lipids	0.92	1.69
Neutral lipids	0.70	2.00
Phospholipids	0.94	0.61
Glycolipids	3.33	1.13

Concentrations of 10^2 conidia were incubated in sample volumes of 150 µl YPG liquid medium (pH 4.5). After 20 h (wild-type) or 40 h (mutant strains) at 28 °C, 0.2 mM SYTOX-Green and 100 µg/ml AFP were added to each sample in order to examine membrane permeabilisation in wild-type and mutant strains. AFP-induced membrane permeabilisation of *A. niger* mutant mcC (multicopy catalytic subunit of PKA) did not significantly differ from the wild-type situation. Both strains became readily permeated by AFP. However, Figure 10 depicts that the plasma membrane of the DC (deleted catalytic subunit of PKA) mutant was not affected by the presence of AFP.

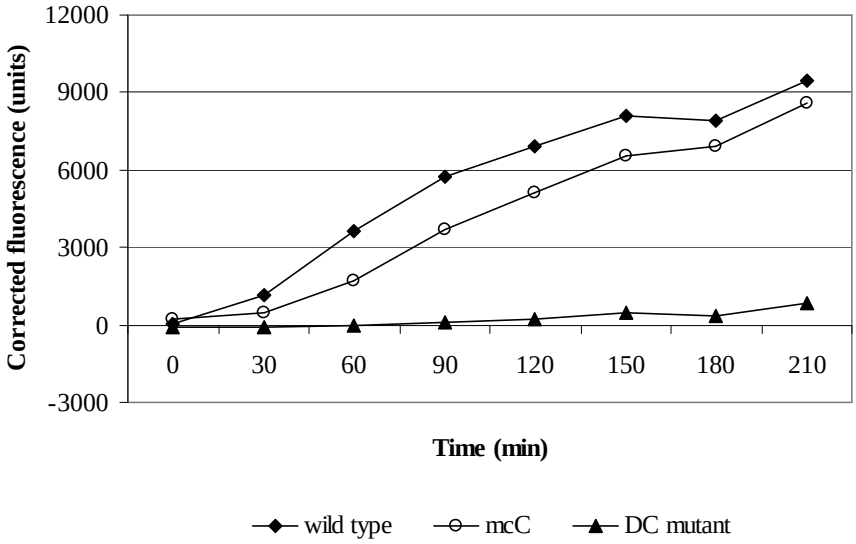

Figure 10. SYTOX-Green analysis of *A. niger* cAMP-dependent protein kinase (PKA) mutants with altered membrane lipid composition. PKA mutants of *A. niger* A395 were incubated in the presence of 0.2 µM SYTOX-Green and 100 µg/ml AFP over a period of 210 min. Fluorescence values were corrected with the baseline fluorescence, which was determined by incubating the fungi in the absence of AFP. Experiments were carried out in triplicates, a representative experiment is shown. Abbreviation: mcC = multicopy catalytic subunit of PKA; DC = deleted catalytic subunit of PKA.

Susceptibility analyses using 2 µg/ml AFP revealed that the DC mutant is still susceptible towards AFP, however, to a minor extent than the wild-type or the mcC mutant (Fig. 11). In summary these data suggest that increased levels of neutral lipids, possibly in combination with an elevated amount of total lipids, may account for the reduced ability of AFP to permeabilise plasma membranes in the DC mutant. However, since PKA activity is also known to be involved in dimorphism (Saudohar et al., 2002), hyphal growth polarity (Bruno et al., 1996) and regulation of triacylglycerol levels (Thines et al., 2000), other aspects underlying PKA signalling may affect AFP susceptibility.

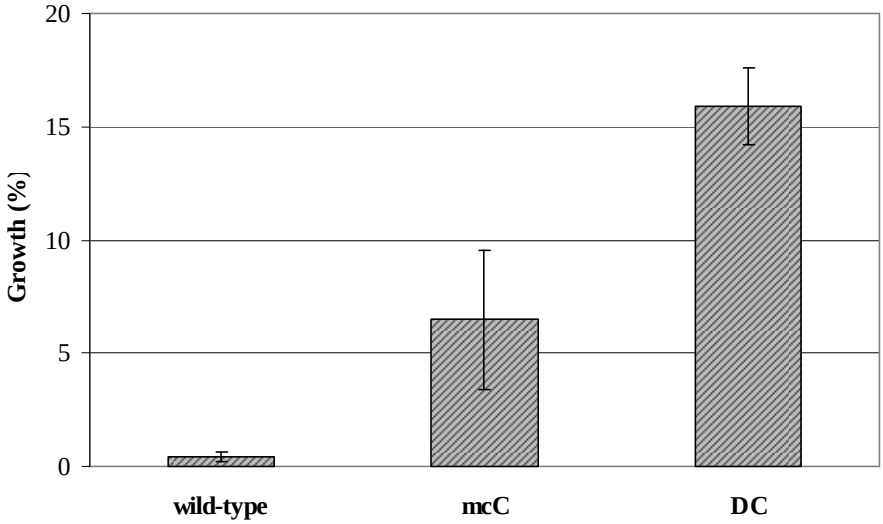

Figure 11. Growth of *A. niger* cAMP-dependent protein kinase (PKA) mutants with altered membrane lipid composition in the presence of 2 µg/ml AFP. Growth of wild-type A395 and corresponding mutants mcC (multicopy catalytic subunit of PKA) and DC (deleted catalytic subunit of PKA) is expressed in % in relation to the negative control, which is the cultivation in the absence of AFP. Experiments were carried out in triplicates.

4.5 AFP susceptibility of *S. cerevisiae* mutants

Based on the data described above, it can be hypothesised that mutations affecting processes involved in cell wall or plasma membrane synthesis could render a resistant organism AFP-sensitive. By means of screening mutants of an AFP-resistant fungus for AFP-sensitive phenotypes, it may be possible to identify distinct genes that could determine if an organism is susceptible towards AFP or not. Using *S. cerevisiae* for this purpose implies the advantages that its genome is fully sequenced and annotated. Furthermore, the availability of mutant libraries provides a valuable means for the functional analysis of many different genes. Thus, a mutant library generated by the European *Saccharomyces cerevisiae* archive for functional analysis (EUROSCARF) was screened for yeast strains of interest. Selected mutants were either affected in cell wall synthesis, cell wall integrity, cell wall integrity signalling or lipid biosynthesis. A description of yeast mutants analysed in the course of this work is given in Table B, Appendix.

In susceptibility studies, pre-cultures were used to inoculated 150 µl YPG liquid medium (pH 6.0) with 10^3 cells. Each sample was cultivated in the absence or in the presence of 400 µg/ml AFP. For reference, the corresponding wild-type strain (BY4741) was also included in the analysis. Cultivations were carried out at 28 °C for 28 h at 120 rpm. Growth was determined

using a microplate spectrophotometer. Figures 12, 13 and 14 confirm that the wild-type strain of *S. cerevisiae* is resistant towards AFP, while mutants WSC1, SMI1 (Table 12) and SFH5 (Table 14) exhibited moderate AFP sensitivities. Slightly moderate AFP sensitivities were observed in strains MPK1, CRZ1, SWI4 (Table 12), TOR1, CWP2, CHS4 (Table 13), CHO2, INP51, ALG5, DIE2 and GSY1 (Table 14). Mutant strains CHS1 (Table 13) and VPS34 (Table 14) displayed the most pronounced AFP-induced growth restrictions. These data indicate that deletion of distinct cell wall or plasma membrane-associated genes may indeed render a resistant organism susceptible towards AFP. Rather surprisingly, mutants FKS1, GAS1 (Table 13) and FPS1 (Table 14) were observed to grow significantly better in the presence of AFP, suggesting that AFP may also administer a growth enhancing effect under specific circumstances.

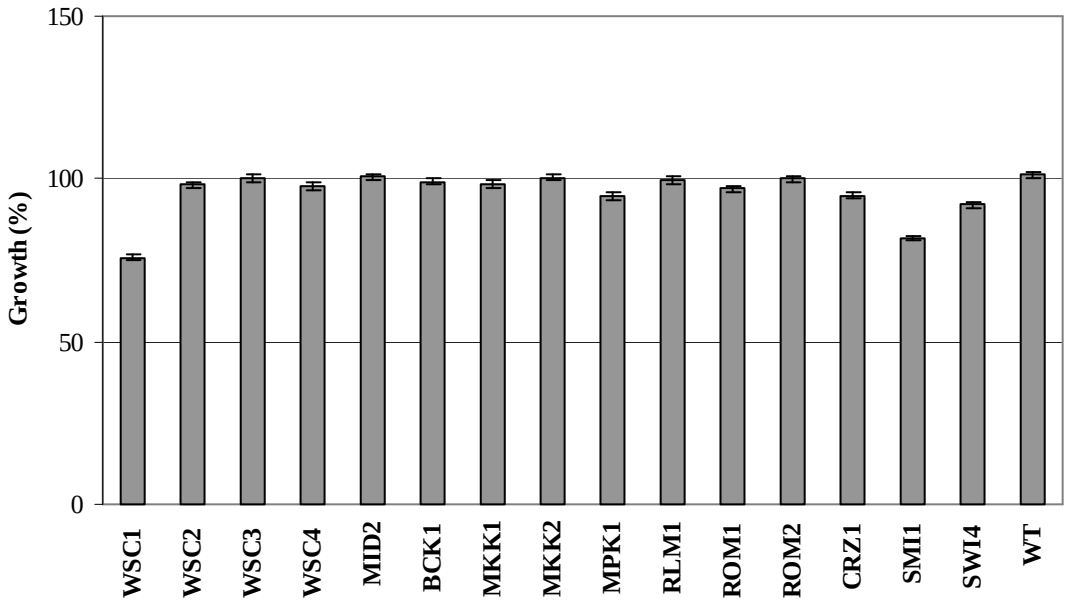

Figure 12. AFP susceptibility of *S. cerevisiae* mutants affected in cell wall integrity signalling. Concentrations of 10^3 cells were incubated at 28 °C for 28 h. Growth was determined spectrophotometrically and expressed in % in relation to the negative control (cultivation in the absence of AFP). Experiments were carried out in triplicates. Mutant characteristics are listed in Table B, Appendix.

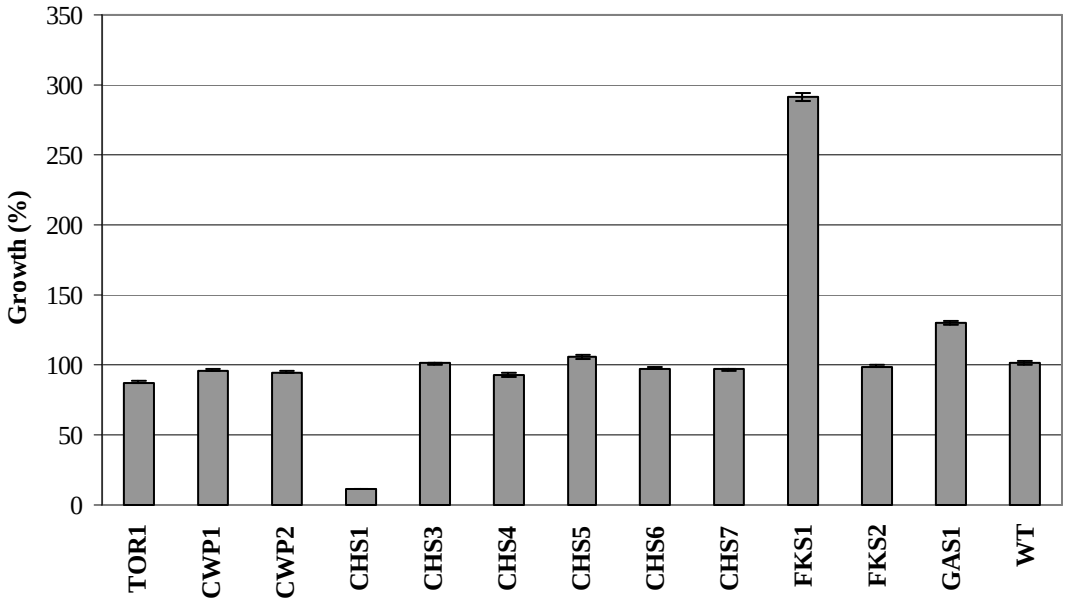

Figure 13. AFP susceptibility of *S. cerevisiae* mutants affected in cell wall synthesis. 10^3 cells were incubated in YPG liquid medium (pH 6.0) at 28 °C for 28 h. Subsequently, growth was determined spectrophotometrically and expressed in % in relation to the negative control (incubation of yeast in the absence of AFP). Experiments were carried out in triplicates. For characteristics of mutants see Table B, Appendix.

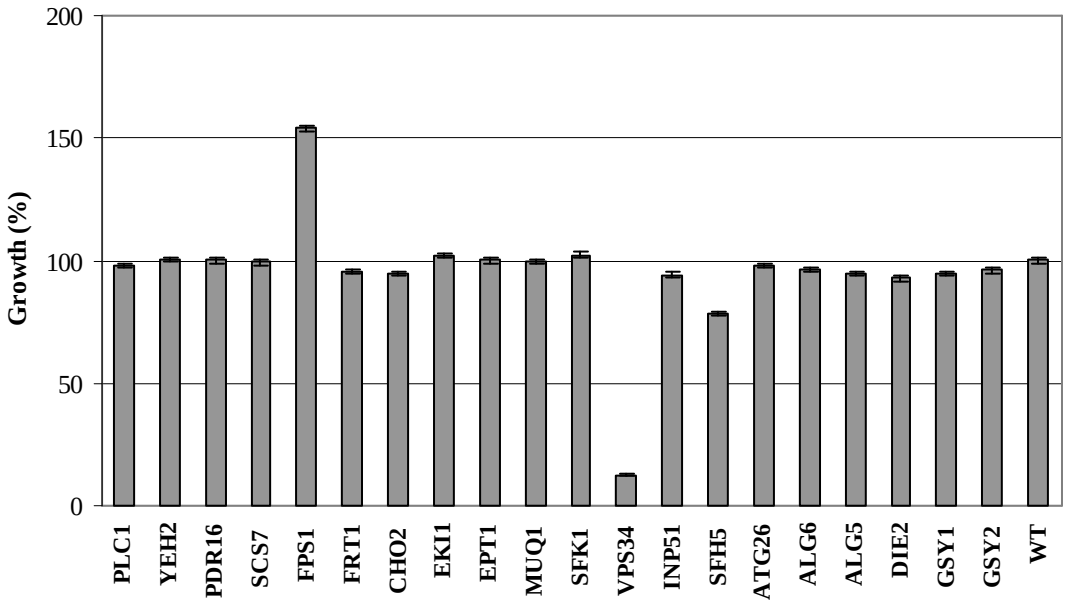

Figure 14. AFP susceptibility of *S. cerevisiae* mutants affected in lipid biosynthesis and/or membrane composition. Yeast strains were cultivated as described in Figure 13. Results are expressed in % in relation to the negative control, which is the cultivation without AFP. Experiments were carried out in triplicates. Mutants are described in Table B, Appendix.

In order to confirm that the amount of cell wall chitin does indeed not influence AFP susceptibility, 26 *S. cerevisiae* mutants were selected which exhibit altered chitin levels in comparison to the wild-type strain (see Table C, Appendix). Mutants with up to eight-fold higher chitin levels than the wild-type strain remained AFP-resistant. Similarly, strains with reduced chitin levels did not become more resistant towards AFP (data not shown). These data indicate that the amount of chitin does not seem to determine AFP susceptibility.

4.6 Putative AFP targets localised inside the cell

AFP is a cationic protein, thus it is imaginable that it could adsorb to almost any kind of negatively charged cell component. Martinez Del Pozo et al. (2002) reported the interaction of AFP with DNA, demonstrating that AFP binds to DNA and promotes DNA condensation. They suggested that AFP exhibits an OB-fold topology by which the protein is capable of interacting with oligonucleotides. However, it was omitted to provide information whether heat-inactivated AFP would behave in a similar fashion. Subsequent experiments were aimed to identify whether AFP with reduced or abolished biological activity shows the same binding affinity to nucleic acids as native AFP.

4.6.1 Binding affinity of AFP to DNA

In order to determine DNA binding affinities of AFP and heat-inactivated AFP, *in vitro* mobility shift assays were conducted. Theis et al. (2005) reported that treatment of AFP at 100 °C for 15 min resulted in approx. 90 % loss of antifungal activity. In agreement with this was the finding that incubation of AFP at 95 °C for 1 h resulted in a similar loss of antifungal activity (data not shown). Samples of 5 µg herring sperm DNA and 0 µg, 3 µg, 10 µg or 30 µg AFP and subjected to mobility shift assays (see 3.7.1). Results confirm previously published data from Martinez Del Pozo et al. (2002) that AFP binds to DNA (Fig. 15). A remarkable shift is observed when DNA was incubated with 3 µg AFP. With rising AFP concentrations (10 µg and 30 µg) the shift became more distinct, reflected by the observation that respective samples remained inside the wells. However, identical results were obtained for heat-inactivated samples of AFP, demonstrating that biologically inactive AFP has the same DNA binding affinity as native AFP samples.

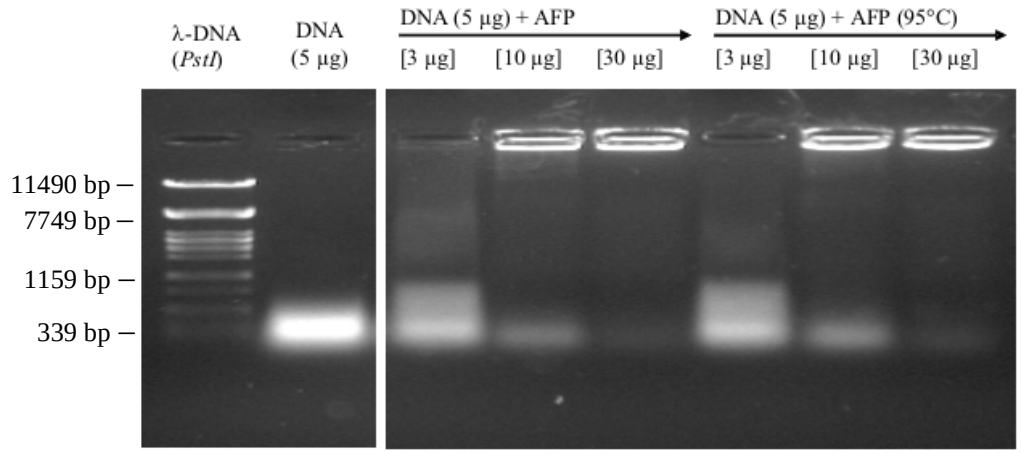

Figure 15. Binding affinity of AFP and heat-inactivated AFP to DNA. Incubation of 3, 10 and 30 µg AFP in the presence of 5 µg herring sperm DNA was either carried out at room temperature or at 95 °C for 1 h. Herring sperm DNA alone was applied for reference. *PstI*-restricted λ-DNA served as size marker. All samples were electrophoretically separated on a TAE agarose gel (0.7 %). Experiments were carried out in triplicates.

4.6.2 Binding affinity of AFP to RNA

In order to determine RNA binding affinities of AFP and heat-inactivated AFP, *in vitro* mobility shift assays were conducted using 7 µg yeast tRNA and 0 µg, 3 µg, 10 µg or 30 µg AFP, respectively. In agreement with DNA mobility shifts carried out (see 3.7.1), AFP is also capable of binding to RNA (Fig. 16).

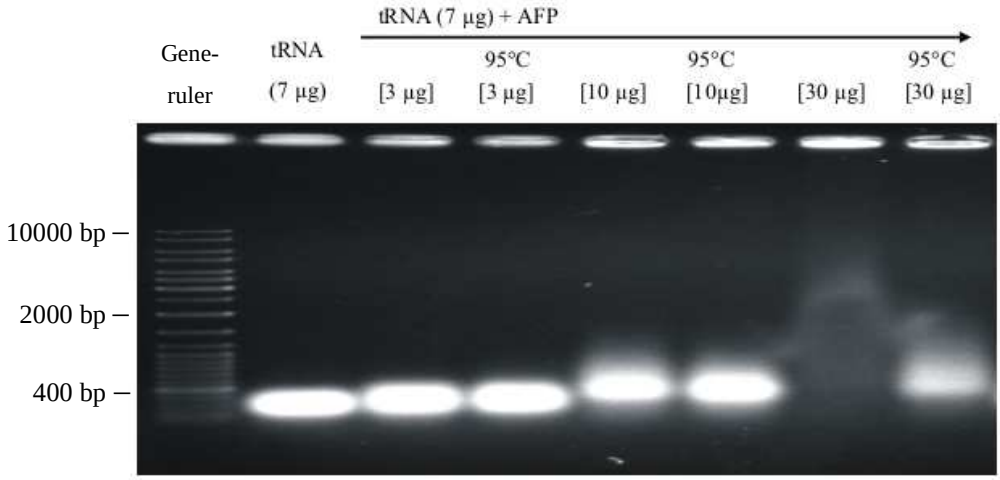

Figure 16. Binding affinity of AFP and heat-inactivated AFP to tRNA. 3 µg, 10 µg and 30 µg AFP incubated in the presence of 7 µg yeast tRNA at room temperature or at 95 °C for 1 h. tRNA alone was applied for reference. The Generuler served as size marker. All samples were electrophoretically separated on a TAE agarose gel (0.7 %) and stained with ethidium bromide. Experiments were carried out in triplicates.

With rising concentrations of AFP and heat-inactivated AFP, the shifts became more pronounced. However, incubation of 30 µg native AFP in the presence of yeast tRNA resulted in a most dramatic shift, consequently causing the sample to remain inside the well. This effect was not observed for the same concentration of heat-inactivated AFP. Nevertheless, a shift was here observed anyhow.

In a subsequent approach, mobility shift assays were also carried out with cysAFP or sAFP. Since concentrations of cysAFP were too low to permit the analysis of higher protein concentrations, the assays were carried out with 3 µg and 10 µg cysAFP in the presence of 7 µg yeast tRNA only. Again, binding activities of untreated and heat-inactivated proteins were determined. Figure 17 illustrates a significant shift for untreated samples of cysAFP. At concentrations of 3 µg and 10 µg, the shift was so dramatic that samples remained inside wells.

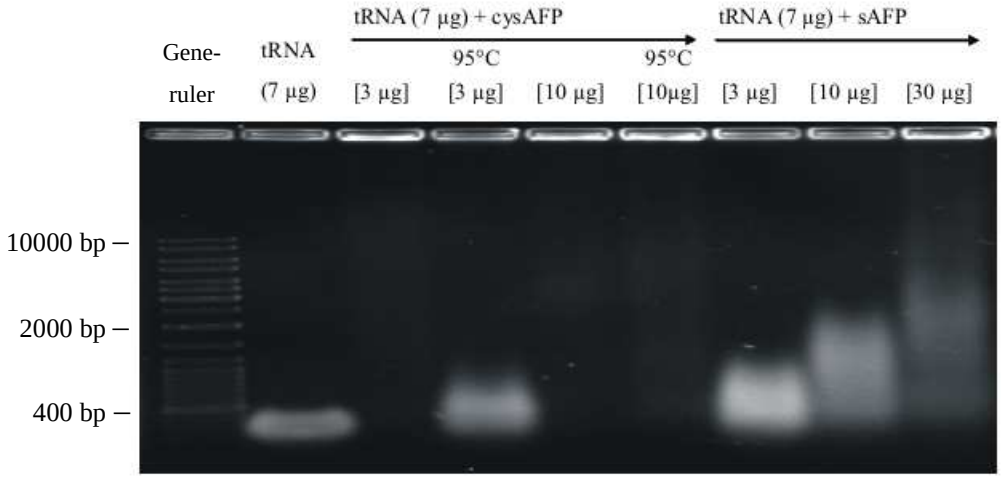

Figure 17. Binding affinity of sAFP, cysAFP and heat-inactivated cysAFP to tRNA. Incubation of 3 µg, 10 µg and 30 µg sAFP with 7 µg yeast tRNA. Incubationswere carried out at room temperature. Incubation of 3 µg and 10 µg cysAFP was either carried out at room temperature or at 95 °C for 1 h. tRNA alone was applied for reference. As size marker served the Generuler. All samples were electrophoretically separated on a TAE agarose gel (0.7 %). Experiments were carried out in triplicates. Abbreviations: cysAFP = AFP exhibiting disrupted disulfide bridges; sAFP = short AFP.

A similar effect was observed for 10 µg heat-inactivated cysAFP, in contrast to the same concentration of untreated protein, which exhibited a comparably moderate shift. Likewise, the incubation of 3 µg, 10 µg, and 30 µg sAFP with 7 µg yeast tRNAresulted in significant shifts. The higher the protein concentration applied, the more distinct were the shifts observed. Since sAFP was shown not to exhibit any antifungal activity on the whole, heat inactivating procedures were here omitted. In summary, these data suggest that nucleic acids do not constitute a primary target of AFP.

4.7 Biotechnological application of AFP

With the perspective of applying AFP as a putative antibiotic agent in fields such as the food industry, the question arose whether AFP becomes degraded during passage through the gastrointestinal tract of man. The gastrointestinal tract constitutes a harsh environment, where low pH and enzyme cocktails contribute to the degradation of ingested food. The here applied *in vitro* assays aim to simulate the most significant aspects of human digestion.

4.7.1 Biodegradability of AFP

Simulated gastric juice (Beumer et al., 1992) was used to imitate the environment normally found in the human gastrointestinal tract. It was formulated using proteose peptone, glucose, salts, bile, lysozyme and pepsin. AFP was applied to simulated gastric juice or buffer solution (control) to a final concentration of 500 µg/ml. Samples were incubated at 37 °C. After 0 h, 3 h, 6h and 24 h, aliquots were taken to be analysed by SDS-PAGE (see 3.7.3). Protein gels were assessed using the Kodak Image Station 440 cf. Figure 18 summarises the results.

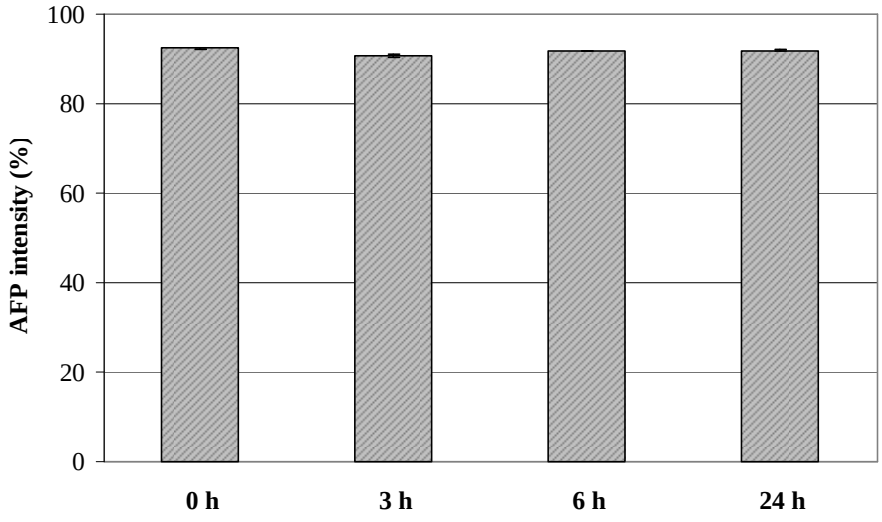

Figure 18. Stability of AFP after incubation in simulated gastric juice. Samples were taken after 0 h, 3 h, 6 h and 24 h of incubation at 37 °C. AFP intensity is expressed in % in relation to the negative control (incubation of AFP in buffer solution). Experiments were carried out in triplicates.

The data suggests that AFP is stably maintained in simulated gastric juice even after an incubation period of 24 h at 37 °C. AFP intensities determined at time points 0 h, 3 h, 6 h and

24 h all ranged between 90 % and 92 %. Acid-tolerance of AFP was furthermore confirmed by visual inspection of protein gels. No AFP degradation could be observed (data not shown).
In the upper intestinal tract bile is secreted into the gut (Chou and Weimer, 1999). In order to determine bile tolerance of AFP, bile salt solution was formulated. Since the concentration of bile is variable and difficult to predict, 1.5 % bile salts from ox gall were dissolved in PBS (pH 7.2) simulating bile concentrations in the jejunum (Marteau et al., 1997). Finally, AFP was applied to bile salt solution or buffer solution (control) to a final concentration of 500 µg/ml. Figure 19 demonstrates that AFP incubation in bile salt solution of up to 24 h at 37 °C does not cause a significant decrease in AFP intensities.

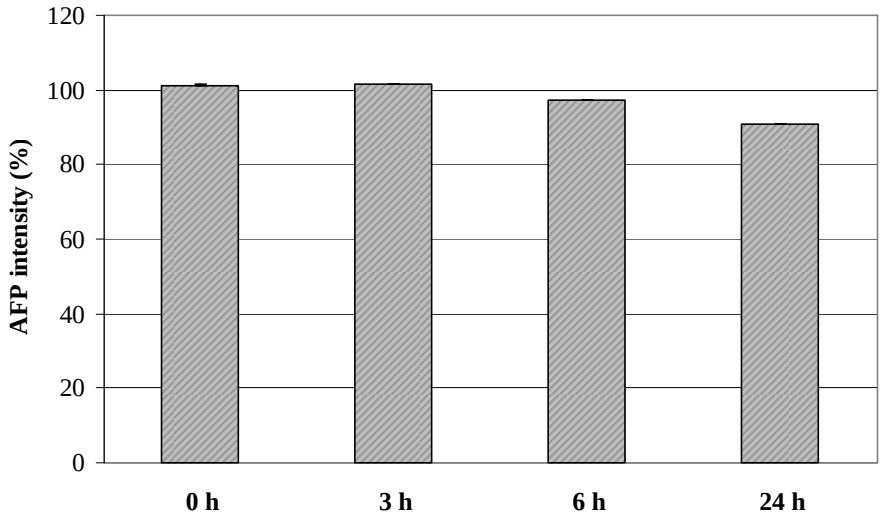

Figure 19. Stability of AFP after incubation in 1.5 % bile salt solution. Aliquots were taken for analyses after 0 h, 3 h, 6 h and 24 h, incubation was carried out at 37 °C. AFP intensity is expressed in % in relation to the negative control, which is the incubation of AFP in buffer solution. Experiments were carried out in triplicates.

Further proof for the bile-tolerance of AFP was provided by the visual inspection of protein gels. No protein degradation could be observed (data not shown).
In an approach to determine whether simulated AFP has retained its full antifungal activity, susceptibility studies were conducted using wild-type strains of *F. oxysporum* (IfGB 39/1201) and *A. niger* (IfGB 15/1801). Conidia of fungi were incubated with AFP samples obtained from 24 h-incubations at 37 °C in simulated gastric juice or 1.5 % bile salt solution. Growth was assessed after 48 h at 28 °C using a microplate spectrophotometer. Interestingly, incubation periods of 24 h at 37 °C in gastric juice-simulating medium or bile salt solution (1.5 %) did not reduce the antifungal activity of AFP. The MICs of 1 µg/ml remained the same (data not shown), indicating that AFP retains its full antifungal activity.

4.7.2 AFP susceptibility of wood decaying fungi

Many fungi are known to thrive on cellulose-containing materials. Cellulose is abundantly distributed - not only in nature, but also in the building industry. Fungal infestation of wood or wall paper, for instance, may impose serious problems for premises and man. Therefore, AFP was tested against a number of well-established wood-decaying fungi in order to analyse whether the fields of AFP application may possibly be extended. Susceptibility studies were carried out using selected fungal strains (Table 6). Strains, such as *P. brevicompactum*, *Alternaria alternate*, *Wallemia sebi*, *Trichoderma viride*, *Cladosporium macrocarpum* and *Coniphora puteana* were difficult to cultivate in YPG liquid medium (pH 4.5), thus growth - even in the absence of AFP - was rather poor. Hence, the determination of AFP susceptibilities for these strains was not possible. However, remaining strains, such as e.g. *A. penicilloides*, *Chaetomium globosum* or *Coniphora puteana*, were readily inhibited in their growth by AFP. From the results can be concluded that AFP may constitute an attractive antifungal agent, which may also find its application in the building industry and affiliated fields.

Table 6. Selection of major wood decaying fungi analysed in AFP susceptibility studies. Fungi are ranked in order of succession on cellulose-containing substrate (from top to bottom). Incubation was carried out in the absence or in the presence of 400 µg/ml AFP. Experiments were carried out in triplicates. Abbreviations: + = yes; n.d. = not determined i.e. fungi did not grow in standard cultivation medium.

Strain	Remark	AFP susceptible
Penicillium brevicompactum	prefers cellulose-containing substrates	n.d.
Aspergillus penicilloides	occurs frequently in museums	+
Cladosporium herbarum	prefers cellulose-containing substrates	+
Cladosporium cladosporioides	prefers cellulose-containing substrates	+
Alternaria alternata	prefers cellulose-containing substrates	n.d.
Wallemia sebi	prefers cellulose-containing substrates	n.d.
Chaetomium globosum	indicator for dampness	+
Trichoderma viride	indicator for dampness	n.d.
Stachybotrys chartarum	indicator for dampness	+
Ceratocystis moniliformis	causes ‘Stammholzbläue’	+
Cladosporium macrocarpum	causes ‘Schnittholzbläue’	n.d.
Aureobasidium pullulans	causes ‘Anstrichbläue’	+
Coniphora puteana	brown rot fungus	n.d.

5 Discussion

Subject of this thesis is the identification of fungal cell wall and plasma membrane components that contribute to the susceptibility of filamentous ascomycetes towards AFP. Obtained data is expected to provide to a more detailed understanding of the molecular mechanism by which AFP exerts its growth inhibitory effect. Results indicate that AFP targets components localised in cell walls and plasma membranes of sensitive fungi, possibly causing cell wall stress and the induction of the cell wall integrity pathway. A model will be postulated, which attempts to depict the mode of action of AFP.

5.1 AFP shares considerable similarity to chitin-binding proteins

In vitro chitin binding studies proved that AFP adheres to chitin very efficiently, thereby confirming previous results that AFP can be purified using chitin affinity chromatography (Liu et al., 2002). Chitin binding analyses performed with chemically modified AFP versions singled out acAFP, which had lost ~ 38 % of its binding capacity, suggesting that the basic net charge of AFP may be required for efficient chitin binding. However, AFP is proposed to contain a putative chitin binding domain (CBD) that consists of the AFP sequence CKYKAQ (aa 14 to 19), sharing considerable homology with the conserved chitin binding motif AKWWTQ of bacterial CBDs. As previously stated, it is unknown which individual lysine residues in acAFP have been targeted by the acetylating reaction. It is therefore tempting to speculate that lysine residues at positions 15 and/or 17, both residing in the assumed CBD of AFP, were chemically modified. The masking of lysines inside this putative domain could alter the character of the chitin binding motif, possibly resulting in reduced chitin binding affinities. Presuming that the identified sequence in AFP does constitute a CDB, the reduced chitin binding affinity of acAFP may be attributed to chemically modified lysine(s) located within this domain. This, in turn, would indicate that the assumed CBD of AFP is required for efficient chitin binding.

Notably, all AFP residues localised within the putative CBD are arranged to form a linear stretch, suggesting that this specific configuration facilitates the attachment to chitin chains or microfibrils rather than to single GlcNAc units. A similarly arranged chitin binding site was reported for the AVR4 elicitor from *Cladosporium fulvum* (van den Burg et al., 2004). AVR4 is supposed to effectively shield chitin on the fungal cell wall, thereby preventing its cell wall

from being degraded by plant chitinases. The elicitor was furthermore demonstrated to exclusively interact with $(GlcNAc)_3$ repeats. Hevein, a lectin-like protein from the rubber tree *Hevea brasilliensis*, contains only a small binding pocket, assumed to provide enough contact to sustain interaction with GlcNAc units alone (van den Burg et al., 2004).

Binding to chitin oligosaccharides may be supported by the finding that AFP shares striking characteristics with oligonucleotide/oligosaccharide binding (OB)-fold proteins (Martinez Del Pozo et al., 2002). The amino acid sequences of these proteins show no significant similarity, however, OB-fold proteins adopt a common fold comprising of a five-stranded *ß*-sheet coiled to form a closed *ß*-barrel (Murzin, 1993). Characteristically, OB-fold proteins bind to oligonucleotides, oligosaccharides, proteins, metal ions or catalytic substrates (Arcus, 2002). The bacterial AB_5 toxins were shown to either bind to oligosaccharide moieties of gangliosides in the cell membrane or to glycosylated proteins on the cell surface (Merritt and Hol, 1995). Beta-strands 2 and 3 are localised in the centre of the molecule and constitute the fold-related binding face (Arcus, 2002). Despite the fact that the short AFP (sAFP) exhibits a C-terminal truncation and therefore lacks *ß*-strands 4 and 5, its composition can still be traced back to the remaining strands 1 to 3 – assuming that sAFP adopts the same folding pattern as AFP. To assist correct folding of sAFP, one native disulfide bridge spanning between cysteine residues 7 and 33 has been retained. Mispairing is tried to be prevented by displacing residual cysteines with serines. According to Figure 20, sAFP has retained the fold-related binding face (*ß*-strands 2 and 3), of which strand 2 and part of loop 2 harbour the putative CBD. Using chitin binding analyses, it was demonstrated that sAFP binds equally well to chitin as native AFP, indicating that the OB-fold topology of AFP may contribute to chitin binding. Chitin binding may be enforced by the solvent-exposed localisation of the putative CBD of AFP, which was described for chitin-binding sites of tachycitin from the horseshoe crab *Tachypleus tridentatus* and hevein (van den Burg et al., 2004). Although AFP shares no sequence homology, it exhibits structural identities with chitin-binding proteins from other organisms, such as e.g. from crustaceans (Osaki et al., 1999) or plants (Leah et al., 1991). These similarities include low molecular weight, cationic net charge, high number of *ß*-sheets, high potency, presence of several disulfide bridges and proteolytic resistance (Theis and Stahl, 2004). In summary, these aspects may further support the assumption that AFP is a chitin-binding protein.

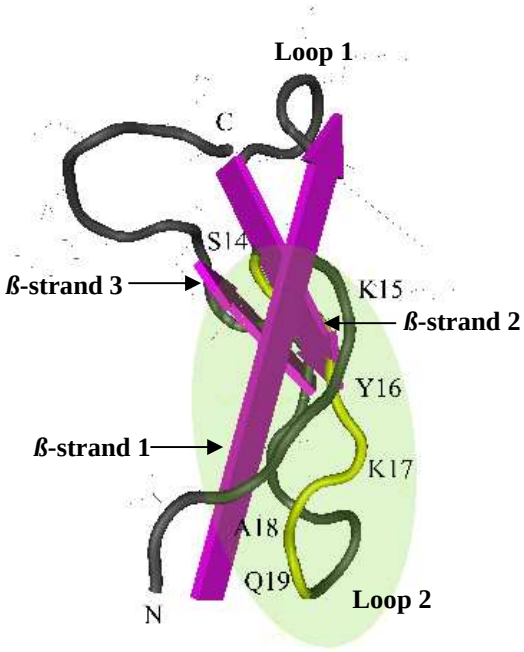

Figure 20. Localisation of the putative chitin binding domain (CBD) in sAFP. The putative CBD is localised on *ß*-strand 2 and part of loop 2, and is represented by green shading. Beta-sheet structures are shown as arrows. The amino- and carboxy-terminal ends of AFP are indicated by N and C, respectively. The assumed folding pattern of AFP is depicted, however, there is no proof for the actual configuration of AFP (for further information see text). The image was illustrated using the Cn3D 4.1 software based on the AFP structure published by Campos-Olivas et al. (1995).

The OB-fold topology was recently suggested to be involved in the antifungal activity of AFP, comprising of nucleic acid binding to subsequently promote condensation and charge neutralisation of DNA (Martinez Del Pozo et al., 2002). This hypothesis is called into question, however, as the here presented data indicates that interaction between AFP and nucleic acids appears to be of unspecific nature: Both, AFP and heat-inactivated AFP were demonstrated to bind equally well to DNA and RNA. A comparable binding behaviour was observed for sAFP, which was shown to be dramatically impaired in its biological activity. This is reflected by a reduced or even abolished potency and its inability to permeabilise plasma membranes in sensitive fungi. Data supporting the opinion that binding of AFP to nucleic acids seems to occur in an unspecific manner were contributed by Theis et al. (2005), who studied the cellular distribution of AFP in AFP-sensitive *A. niger* hyphae by transmission electron microscopy. They could show that no nuclear or mitochondrial localisation of AFP was observed when *A. niger* was incubated with the respective MIC. Only at concentrations 300-fold higher than the MIC, AFP was found to localise inside necrotic cells of *A. niger*

(Theis et al., 2005). An interaction between AFP and nucleic acids alone would furthermore not explain the species-specificity by which AFP exerts its growth inhibitory effect on certain fungi. In addition, if nucleic acids were the primary target of AFP, bacteria and other AFP-resistant organisms are assumed to also exhibit AFP-induced growth restrictions. However, at this point it cannot be ruled out that AFP is able to interact with nucleic acids. Although a direct involvement appears unlikely, the binding to DNA or RNA could still have an impact on the antifungal activity of AFP.

5.2 Putative AFP targets associated with chitin biosynthesis and cell wall integrity signalling

Yeast mutants with elevated cell wall chitin content were described to be more sensitive towards the antifungal drug amphotericin B than strains with low chitin content (Bahmed et al., 2003). Screening of yeast mutants with significantly elevated chitin levels indicated, however, that there is no relationship between cell wall chitin content and susceptibility to AFP. Hence, it is proposed that chitin itself must assign a more concomitant role in AFP functionality. Interestingly, sAFP still accomplishes the attachment to chitin in spite of its significantly reduced bioactivity. Thus, chitin binding and perturbation of cell integrity are obviously two independent events, suggesting that the adherence of AFP to chitin may enable the protein to get into and/or be maintained in close proximity to additional AFP targets.

Polyoxins and nikkomycins are the most widely studied agents interfering with chitin biosynthesis. It is assumed that they operate by binding to the catalytic site of chitin synthases (Ruiz-Herrera and San-Blas, 2003). For AFP, a similar mechanism affecting chitin biosynthesis could be thinkable. The synthesis and the deposition of chitin is a complex network of biochemical and biophysical events, in which membrane-bound chitin synthases play a central role (Cohen, 2001). Most fungi contain multiple chitin synthases and their coding genes have been divided into six classes according to their sequence similarity (Roncero, 2002). Different chitin synthases perform distinct cellular functions during fungal growth und underlie the regulation of gene products that are ultimately involved in their temporal regulation and spatial localisation (Selvaggini et al., 2004). While yeast lack chitin synthase gene classes III, V and VI, filamentous fungi are highly dependent on them since they seem to play a significant role in the polarised synthesis of chitin (Takeshita et al., 2006). In order to find out whether a lack of these specific chitin synthase classes would result in

reduced AFP susceptibilities, a class V mutant of *F. oxysporum* 4287 (*chsV*) and a class V and class III mutant of *A. oryzae* (*csmA* and *chsB*, respectively) were analysed. Susceptibility studies showed that the chitin synthase mutants tested in this work are indeed considerably less sensitive towards AFP than the corresponding wild-type strains. Reduced sensitivities are also reflected in the inability to permeabilise mutant plasma membranes with the AFP concentrations tested. In consequence, this suggests that chitin synthase classes III and V may serve as AFP targets, rendering strains which lack respective chitin synthase classes moderately sensitive or even AFP-resistant. However, reduced AFP susceptibilities of class III and class V mutants could also be due to remodelled cell walls, aimed at complementing for arisen defects. This reorganisation could certainly affect the accessibility of putative AFP targets, thereby resulting in reduced susceptibilities of mutants.

Takeshita et al. (2006) reported that all filamentous ascomycetes whose genomes have been made public contain genes encoding class V and VI chitin synthases. The former contain a C-terminal chitin synthase domain and an N-terminal myosin motor-like domain (MMD), while the MMDs of class VI chitin synthases are slightly smaller by comparison. *A. nidulans* chitin synthases CsmA (class V) and CsmB (class VI) were both shown to localise to the hyphal apex, and were suggested to perform some type of compensatory role that is essential for hyphal tip growth. In this context, it may be possible that the basal AFP susceptibility of the class V chitin synthase mutants may be ascribed to the putative activity of class VI chitin synthases. A similar effect could also hold true for class III chitin synthases.

Measurement of *in situ* chitin synthase activities provided additional indication that AFP targets chitin biosynthesis. Wild-type strains of AFP-sensitive fungi, such as *A. niger*, *F. oxysporum* 4287 and *A. oryzae* all exhibit reduced chitin synthase activities in the presence of AFP. Although not investigated into the least detail, chitin synthesis in known to comprise of a succession of events commencing with the trafficking of zymogenic chitin synthase clusters to the plasma membrane *via* microvesicles termed chitosomes (Bracker et al., 1976; Ruiz-Herrera et al., 1977). Upon fusion with the plasma membrane, the inserted chitin synthase units are proteolytically activated. Newly synthesised chitin chains are subsequently translocated across the plasma membrane, where they finally coalesce to form microfibrils.

Two mechanisms have been proposed to explain the incorporation of new cell wall material into pre-existing structures. One suggests that cell wall expansion comprises of a continuous process relying on the dynamic balance between lytic- and ligase-type activities. Newly formed building blocks are inserted at growth sites in which bonds in the recently assembled cell wall are cleaved. Although this mechanism receives increasing support, proof of concept

is still missing. The alternative assumption states that cell wall polymers, which at the growing points may not be completely solidified by cross-links, slide apart to provide an area of incorporation for newly synthesised chitin filaments (Cid et al., 1995). Due to the complexity and the lack of in-depth knowledge about chitin synthesis and deposition (Cohen, 2001), AFP-induced reduction in chitin synthase activity may be explained by multiple possibilities. These include that AFP may i) interfere with the transport of chitosomes towards the hyphal tip, ii) hinder the release of chitin synthases from chitosomes, iii) prevent the proper localisation and anchoring of chitin synthases into the plasma membrane, iv) interfere with the proteolytic activation of zymogenic chitin synthases, v) inhibit chitin synthase activity by either adhering to chitin precursor molecules or to chitin chains, vi) block chitin synthase activity, e.g. by binding to the catalytic site of chitin synthases and/or vii) affect the incorporation of chitin microfibrils into the cell wall, possibly by preventing cross-linkage with other cell wall components i.e. ß-glucans.

It has previously been shown that cell wall damage can trigger compensatory reactions that aim to ensure cell wall integrity. Application of antifungal drugs specifically interfering with chitin or ß-glucan synthesis in *A. niger* were described to lead to the induced expression of *α*-1,3-glucan synthase encoded by the *agsA* gene (Damveld, 2005). In *S. cerevisiae*, deletion of the catalytic subunit Fks1p of ß-1,3-glucan synthase or the ß-1,3-glucanosyltransferase Gas1p, involved in ß-1,3-glucan remodelling, result in significantly reduced levels of ß-1,3-glucan in the wall and in the formation of viable but swollen cells. These mutants were shown to display an increase in chitin content and in the expression of the alternative subunit of the yeast ß-1,3-glucan synthase, Fks2p, proposed to be involved in ensuring cell wall integrity (de Nobel et al., 2000).

Cell wall integrity signalling, also referred to as the protein kinase C (PKC) signalling pathway, has been described in much detail for *S. cerevisiae* (Fig. 21). Briefly, it comprises of a family of membrane-localised sensors (Wsc1-4p and Mid2p) coupled to the guanine nucleotide exchange factor Rom2p, which modulates activity of the GTPase Rho1p (Bickle et al., 1998). Rho1p regulates the cell wall synthesising enzyme ß-1,3-glucan synthase *via* its catalytic subunit Fks1p. Rho1p is also required for protein kinase C (Pkc1p) regulation, which in turn mediates signals through a MAP kinase cascade. A key regulator of cell wall integrity is the MAP kinase Mpk1p whose activity is controlled by the upstream kinases Mkk1/2p and Bck1p. These in turn are regulated by Pkc1p, which responds to upstream signalling from Rho1p (Atienza et al., 2000). The Rho1p-activated Fks1p is an integral membrane protein involved in ß-1,3-glucan synthase activity. Activation of Mpk1p results in stimulation of

Rlm1p and Swi4p transcription factors to drive transcription of cell wall-related genes (Levin, 2005).

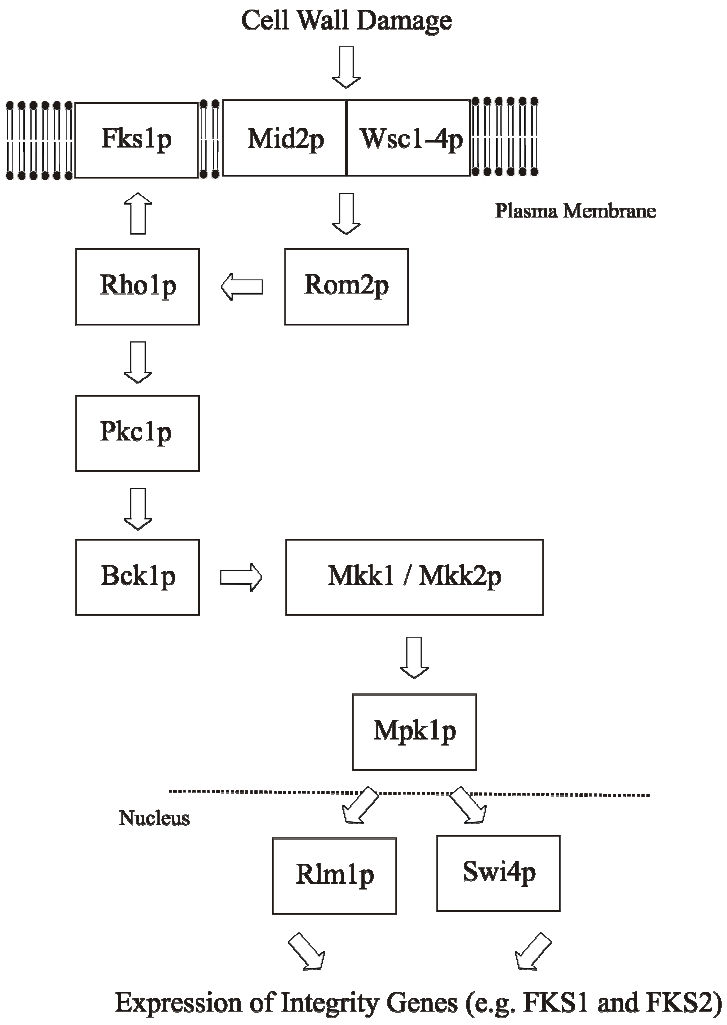

Figure 21. Schematic illustration of the fungal cell wall integrity pathway. Basic components are depicted. Plasma-membrane localised protein sensors (Wsc1-4p and Mid2p) perceive cell wall damage and transfer signals through the nucleotide exchange factor Rom2p to the GTPase Rho1p. Rho1p regulates the cell wall synthesising enzyme *ß*-1,3-glucan synthase *via* its catalytic subunit Fks1p. Rho1p is also required for protein kinase C (Pkc1p) regulation, which in turn mediates signals through a MAP kinase cascade (Bck1p, Mkk1p and Mpk1) to transcription factors Rlm1p and Swi4p. These downstream regulators up-regulate transcription of genes involved in cell wall biogenesis, such as the two alternate catalytic subunits of *ß*-1,3-glucan synthase, Fks1p and Fks2p.

Elevated chitin synthase activities, as observed for AFP-resistant strains such as the *F. oxysporum chsV* mutant and *P. chrysogenum*, suggests that AFP triggers the fungal cell wall integrity pathway. In agreement with this is the finding that treatment of *S. cerevisiae* with calcofluor white, an inhibitor of chitin assembly, was reported to result in increased chitin synthase activities (Roncero et al., 1988). AFP has indeed been shown to provoke cell

wall stress and to induce the cell wall integrity pathway in a reporter strain of *A. niger* comprising of the *agsA* promoter fused to a nuclear targeted green fluorescent protein (V. Meyer, unpublished data). The α-1,3-glucan synthase gene *agsA* in *A. niger* was shown to underlie RlmA transcription and is involved in cell wall remodelling in response to cell wall stress (Damveld et al., 2005). Consequently, it is possible to speculate that AFP-resistant fungi are capable of counteracting AFP-induced cell wall damage by the induced expression of additional chitin synthase-encoding genes or other cell wall-related genes.

The hypothesis that cell wall integrity signalling may be involved in counteracting AFP-induced cell wall stress was corroborated by susceptibility analyses of *S. cerevisiae* mutants. However, the observation that these strains generally exhibit only moderate AFP susceptibilities indicates that the presence of specific AFP targets, e.g. classes III and V of chitin synthase genes, may be required for developing clearly AFP-sensitive phenotypes. Nevertheless, since deviations of measurements were extremely small in all yeast mutants tested in this work (in general below 1 %), strains are considered as conspicuous when displaying at least 5 % growth deviation in comparison to the wild-type strain. Table 7 summarises mutants of *S. cerevisiae* affected in cell wall integrity i.e. PKC signalling, which were identified to exhibit deviating AFP susceptibilities in comparison to the wild-type strain. The moderate to slightly moderate AFP susceptibilities of strains lacking Wsc1p, Swi4p and Mpk1p indicate that cell wall damage reception and downstream signalling to integrity-associated genes is essential for successfully counteracting AFP-induced cell wall damage, and stands in agreement with the hypothesis that AFP triggers the cell wall integrity pathway in fungi.

Table 7. Mutants of *S. cerevisiae* affected in protein kinase C (PKC) signalling that exhibit AFP-induced growth restriction. Growth reduction in the presence of AFP is expressed in % in relation to the control (cultivation in the absence of AFP). Mutant phenotype, molecular function and biological processes in which individual proteins are implicated are given as far as known (www. yeastgenome.org). For further information see Table B, Appendix.

Protein	Molecular function	Biological process	Mutant phenotype	AFP-induced growth reduction (%)
Wsc1p	Transmembrane receptor.	Rho1p protein signal transduction, actin cytoskeleton organisation and biogenesis, cell wall organisation and biogenesis, endocytosis, establishment of cell polarity. Part of the PKC signalling pathway.	Increased resistance to caspofungin.	24.1
Swi4p	Transcription factor.	G_1/S transition of mitotic cell cycle and transcription. Cell wall organisation. Part of the PKC signalling pathway.	Normal chitin levels, decreased resistance to calcofluor white.	7.9
Mpk1p	MAP kinase.	Cell wall organisation and biogenesis, protein amino acid phosphorylation and signal transduction. Part of the PKC signalling pathway.	Decreased levels of chitin, decreased resistance to calcofluor white.	5.3

The analysis of yeast mutants has also shown that strains affected in cell wall biosynthesis and/or organisation also exhibit AFP-induced growth reductions (Table 8). Interestingly, it is the CHS1 mutant which exhibits a dramatic growth inhibition (approx. 11 % residual growth). Since Chs1p is required for repairing the chitin septum after cell separation (Lesage et al., 2005), the severe growth restriction could be attributed by an unprotected birth scar, which may be particularly prone to AFP-induced cell damage.

Table 8. Mutants of *S. cerevisiae* affected in cell wall biosynthesis or organisation that exhibit AFP-induced growth restriction. Growth reduction in the presence of AFP is expressed in % in relation to the cultivation in the absence of AFP. As far as known, mutant phenotype, molecular function and involvement of proteins in biological processes is given (www. yeastgenome.org). For further information see Table B, Appendix.

Protein	Molecular function	Biological process	Mutant phenotype	AFP-induced growth reduction (%)
Chs1p	Chitin synthase.	Cell budding and cytokinesis (completion of separation).	Increased levels of chitin, normal resistance to calcofluor white.	88.8
Smi1p	Function unknown.	Cell wall organisation and biogenesis, ß-1,3-glucan biosynthesis.	Increased levels of chitin, decreased resistance to calcofluor white.	18.2
Tor1p	Phosphatidyl-inositol 3-kinase and protein kinase.	G_1 phase of mitotic cell cycle, cell wall organisation and biogenesis, regulation of cell growth, regulation of progression through cell cycle.	Increased glycogen accumulation.	12.4
Chs4p	Enzyme activator.	Cell wall chitin biosynthesis, cytokinesis and response to osmotic stress. Activator of Chs3p.	Decreased levels of chitin, increased sensitivity to caspofungin.	7.9
Cwp2p	Cell wall protein.	Cell wall organisation / biogenesis and regulation of pH.	Increased sensitivity to Congo red, calcofluor white and Zymolyase.	5.1
Crz1p	Transcription factor.	Calcium-mediated signalling, regulation of DNA-dependent transcription. Stress response, regulates chitin synthesis.	-	5.1

The moderate to slightly moderate AFP susceptibilities of *S. cerevisiae* strains lacking Smi1p, Tor1p, Cwp2p, Chs4p and Crz1p may be due to defects in chitin or ß-1,3-glucan biosynthesis and/or cell wall organisation. The CWP2 mutant has indeed been shown to exhibit a strongly reduced electron-dense layer on the outside of the cell wall, indicating that Cwp2p is involved in stabilising fungal cell wall composition (van der Vaart et al., 1995). In summary, these results suggest that the susceptibility of fungi towards AFP may also be connected to deficiencies in cell wall composition and/or stability.

5.3 Sphingolipids may represent putative AFP targets

Biomembranes have several important functions in that they serve as diffusion barriers, catalyse selective transport processes and harbour receptors that contribute to recognition processes. Major lipids of fungal plasma membranes are phospholipids, sphingolipids and sterols (Daum et al., 1998), while prokaryotic plasma membranes are mainly composed of phospholipids and lack the latter two components. The finding that bacterial protoplasts remain AFP-resistant indicates that phospholipids do not represent putative AFP targets. Further support that fungi-characteristic plasma membrane lipids are involved in AFP-induced growth inhibition was supplied by the revelation that defensins, which are structurally related to AFP, interact with fungal glucosylceramides (Thevissen et al., 2004). Glucosylceramides represent the simplest member of the large group of glycosphingolipids. Although glucosylceramides are common to all eukaryotes, they exhibit specific differences. Fungi synthesise a characteristic consensus glucosylceramide structure which features three variations, including a galactosyl or a glucosyl head group, C_{16} and C_{18} fatty acids and a trans-desaturation at carbon 3 of the fatty acid. These structures do not occur in animals or plants (Warnecke and Heinz, 2003). Based on literature data, it was noted that AFP-susceptible fungi tend to contain glucosylceramides that exhibit a trans-desaturation at carbon 3 i.e. Δ3-desaturated fatty acids (Table 9). From this observation was subsequently suggested that Δ3-desaturated glucosylceramides may be implicated in determining AFP sensitivity.

Table 9. Glucosylceramides in fungi as described by Warnecke and Heinz (2003). Boldface indicates that ceramide backbones of respective glycosylceramides were confirmed to consist of C_{16} or C_{18} hydroxy fatty acids. The minimal inhibitory concentration (MIC) of AFP is given for reference. Abbreviations: GlcCer = glucosylceramide; GalCer = galactosylceramide; NE = no effect; + = yes; - = no.

Fungus	Glycosphingolipid	Δ3-desaturated fatty acid	AFP susceptibility MIC (µg/ml)
Aspergillus niger	**GalCer**	+	1
	GlcCer	+	
Aspergillus fumigatus	**GlcCer**	+	10
	GalCer	+	
Fusarium solani	**GlcCer**	+	120
Aspergillus nidulans	**GlcCer**	-	200
Candida albicans	**GlcCer**	-	NE
Saccharomyces cerevisiae	GalCer	-	NE

Indeed, it was shown that depletion of cellular glucosylceramide levels in strains of *A. niger* and *A. fumigatus* provoked significantly reduced AFP susceptibilities (see 4.4.2), underlining the putative involvement of glucosylceramides in AFP activity. In agreement with this finding is the observation that glucosylceramides of *Fonsecaea pedrosoi* are targeted by antifungal antibodies that directly inhibit fungal development (Nimrichter et al., 2004). In addition, it was demonstrated that plant defensin-sensitive wild-type strain of *Neurospora crassa* contains mainly Δ3-desaturated glucosylceramides, while a plant defensin-resistant mutant of *N. crassa* was shown to lack fatty acid Δ3-desaturation (Park et al., 2005). Possibly, Δ3-desaturated glucosylceramides may be required for AFP-induced destabilisation and consequent permeabilisation of plasma membranes. However, proof is still missing for this hypothesis. Alternatively, AFP could also interact with glucosylceramides to subsequently trigger cell wall integrity signalling: Glucosylceramides belong to the large group of sphingolipids, which are known to occur in dynamic assemblies situated inside the exoplasmic leaflet of lipid microenvironments, also referred to as lipid rafts. Lipid rafts take part in the process of signal transduction and harbour a given set of proteins that can change size and composition in response to intra- or extracellular stimuli. Specific protein-protein interactions are favoured by lipid raft structures, resulting in the activation of different signalling cascades including stress response (Cheng et al., 2003; Simons and Toomre, 2000).

Delom et al. (2006) recently reported the connection between sphingolipid expression and cell wall stress. They showed that treatment of fungal cell walls with the antifungal compound calcofluor white resulted in subtle changes in the expression of Pil1p and Lsp1p, two sphingolipid long-chain base-responsive inhibitors of protein kinases involved in cell wall integrity signalling pathways and Rho1p.

Interestingly, sphingolipids were furthermore described to be involved in intracellular vesicle transport (Holthuis et al., 2001) and were shown to be essential for the establishment and the maintenance of cell polarity by controlling the actin skeleton (Cheng et al., 2001). It is well established that fungal hyphae grow by apical extension, which implies that materials and machinery required for plasma membrane and cell wall synthesis are carried *via* vesicle-mediated transport processes to the right place on the cortex (Momany, 2002). This includes that vesicles initially undergo long-range transport along polarised arrays of microtubules. Having reached the tip region, vesicles form aggregates termed the Spitzenkörper, which mediate the actin-dependent dispersal of these structures to their final destination (Li et al., 2006). The scaffold protein SepA of *A. nidulans* was shown to participate in two actin-mediated processes, namely septum formation and establishment of polarized hyphal growth (Sharpless and Harris, 2002). Notably, the stable recruitment of SepA to hyphal tip stands in interrelation to sphingolipids (Pearson et al., 2004), being in line with the observation that sphingolipids are required for cell polarity in *A. nidulans* (Cheng et al., 2001). Only recently, the heat-stable antifungal factor (HSAF) of the bacterial biocontrol agent *Lysobacter enzymogenes* C3 was reported to dramatically affect polarised growth of *A. nidulans* hyphae (Li et al., 2006), proposing that antifungal activity of AFP could possibly result in similar effects. In agreement with this are recent analyses that indicate that application of AFP to *A. niger* germlings results in polarised growth arrest, causes swellings of hyphal tips and provokes apical and subapical branching. These data furthermore indicate that AFP causes cell wall stress mainly at the hyphal apex and inhibits hyphal polarity maintenance (V. Meyer, unpublished data).

Susceptibility analyses of *S. cerevisiae* lipid mutants indicate that vesicle transport, and hence cell polarity, may in fact stand in interrelation with AFP activity (Table 10). Yeast mutants devoid of Vps34p, Inp51p or Sfh5p exhibit AFP susceptibilities ranging from high to slightly moderate sensitive. Interestingly, the Sfh5p homologue Sec14p was shown to be required for vesicle-dependent membrane trafficking of secretory glycoproteins from the late Golgi and cell viability (Bankaitis et al., 1990; Skinner et al., 1995). Homologues of Inp51p, termed Inp52p and Inp53p, were demonstrated to be directly implicated in the regulation of actin

polymerisation and cell growth in response to stress (Ooms et al., 2000). Thus, AFP susceptibilities of Sfh1p or Inp51p mutants are suggested to be due to defects in vesicle trafficking i.e. polarity establishment.

Table 10. Mutants of *S. cerevisiae* affected in vesicle transport and cell polarity exhibiting AFP-induced growth restriction. Growth reduction in the presence of AFP is expressed in % in relation to the control (cultivation in the absence of AFP). Mutant phenotype, molecular function and biological process in which individual proteins are implicated are given as far as known (www. yeastgenome.org). Further information is given in Table B, Appendix.

Protein	Molecular function	Biological process	Mutant phenotype	AFP-induced growth reduction (%)
Vps34p	Phosphatidyl-inositol 3-kinase activity and protein kinase.	Inositol lipid-mediated signalling, phosphoinositide phosphorylation, protein phosphorylation and targeting to vacuole, vacuolar transport and vacuole inheritance.	Defective vacuolar protein sorting.	87.4
Sfh5p	Phosphatidyl-inositol transporter.	Phospholipid transport.	-	21.9
Inp51	Inositol-polyphosphate 5-phosphatase.	Cell wall organisation and biogenesis, endocytosis, inositol lipid-mediated signalling and phosphoinositide dephosphorylation.	Abnormal vacuoles.	5.7

The pronounced AFP susceptibility of the VPS34 mutant (~ 12 % residual growth) indicates that interference with sorting and delivery of soluble vacuolar proteins may indeed have a significant influence on developing AFP-sensitive phenotypes. Deletion of VPS34 was previously reported to result in missorting of vacuolar and perhaps other proteins (Bulik et al., 2003). In addition, it was suggested that Vps-containing cytosolic protein complexes may affect sorting of Chs3p into chitosomes or trafficking of chitosomes to the plasma membrane (Shiflett et al., 2004).

The observation that some yeast mutants exhibited elevated growth levels in the presence of AFP was rather surprising (Table 11). Why strains deficient in Fks1p, Fps1 and Gas1p grow significantly better (~ 291, 153 and 130 % growth, respectively) in the presence of AFP is presently not known. Common characteristics of Fks1p and Gas1p mutants is that both exhibit slow growth, contain increased levels of chitin and are affected in cell wall organisation and biogenesis.

Table 11. Mutants of *S. cerevisiae* exhibiting elevated growth levels in the presence of AFP. Growth in the presence of AFP is expressed in % in relation to the control (cultivation in the absence of AFP). Mutant phenotype, molecular function and biological process in which individual proteins are implicated are given as far as known (www. yeastgenome.org). See Table B (Appendix) for further information.

Protein	Molecular function	Biological process	Mutant phenotype	AFP-induced growth increase (%)
Fks1p	Beta-1,3-glucan synthase activity.	Cell wall organisation and biogenesis, ß-1,3-glucan biosynthesis and endocytosis.	Increased levels of chitin, decreased resistance to calcofluor white, increased sensitivity to caspofungin. Slow growth. Affected in cell wall organisation, biogenesis and vesicle trafficking.	291.7
Fps1p	Glycerol transporter activity and transporter activity.	Conjugation with cellular fusion, glycerol transport.	Normal chitin levels, decreased resistance to calcofluor white.	153.8
Gas1p	Beta-1,3-glucanosyltrans-ferase activity.	Cell wall organisation and biogenesis and ß-1,3-glucan synthesis.	Increased levels of chitin, decreased resistance to calcofluor white, exhibits cell wall defects. Slow growth. Affected in cell wall organisation and biogenesis.	130.1

Interestingly, the FKS1 mutant is known to be affected in vesicle trafficking, which is proposed to be involved in Chs3p recycling from the plasma membrane to chitosomes (Ortiz and Novick, 2006). Although speculative, it may be assumed that chitin synthases may remain inside the plasma membrane of FKS1 mutants throughout the cell cycle, being immediately able to counteract AFP-induced cell wall damage. Since Gas1p mutants are described to be affected in cell wall organisation and biogenesis as well as filamentous growth, it may be hypothesised that this strain may also be affected in vesicle trafficking at some point, resulting in a similar phenotype as the FKS1 mutant. However, no connection between FPS1 deletion and vesicle transport has been reported to date.

Plasma membrane composition does, nevertheless, influence AFP susceptibility, as the analyses with cAMP-dependent protein kinase (PKA) mutants of *A. niger* indicates. PKA consists of a catalytic and a regulatory subunit. In the absence of cAMP, the catalytic subunit combines with the regulatory subunit to form the inactive PKA holoenzyme (Walsh et al., 1968). PKA and cAMP regulation are involved in many different pathways, such as hyphal growth polarity (Bruno et al., 1996), dimorphism (Saudohar et al., 2002) or phospholipid biosynthesis (Kinney et al., 1990). Deletion of PKA activity was described to result in a 1.6-times elevated level in total lipids with two-times more neutral lipids and a decrease in phospholipids (DC mutant strain). In consequence, the lipid profile of the DC mutant consists of nearly 90 % neutral lipids and 10 % phospholipids and glycolipids (Jernejc and Bencina, 2003). Thus, it may be concluded that the moderately sensitive phenotype of the DC mutant can be ascribed to the high proportion of neutral lipids, which seem to dramatically reduce AFP-induced plasma membrane permeabilisation. Moreover, the lack of PKA activity in the DC mutant may possibly be also be connected to a reduction in glucosylceramide levels, which were shown to be involved in developing AFP-sensitive phenotypes (see 4.4.2). Thus, a rather challenging hypothesis would be to assume that the DC mutant lacks Δ3-desaturated glucosylceramides, which were above postulated to play a role in determining AFP-sensitive phenotypes. However, proof is still missing to support this theory.

5.4 Working model proposed for the mode of action of AFP

The here and previously accumulated data (Theis et al., 2005; Theis et al., 2003) indicates that AFP susceptibility seems to be exclusively determined by cell wall and plasma membrane constituents occurring in AFP-sensitive filamentous fungi. In the sequence of events, extracellular AFP is postulated to bind to fungal cell wall chitin (Fig. 22), possibly by binding to chitin *via* the putative chitin binding domain. Chitin binding itself was demonstrated to not affect fungal growth, thus it is proposed that chitin binding may enable AFP to get into and/or remain in close proximity to additional AFP targets. These may consist of chitin synthases of the classes III and V as well as of glucosylceramides. AFP treatment of resistant fungi was shown to result in increased levels of chitin synthase activity, which suggests that AFP induces the cell wall integrity pathway (PKC signalling pathway) in fungi. Stress-induced recruitment of chitin synthases from chitosomes to the cell surface may result in elevated synthesis of chitin.

In addition, increased glucan synthesis may also serve to counteract for AFP-induced cell wall damage. Although speculative, it is proposed that AFP-resistant fungi lack distinct glucosylceramide variants (i.e. Δ3-desaturated glucosylceramide) inside lipid rafts. This situation could make them insensitive towards AFP-provoked plasma membrane destabilisation and consequent permeabilisation (see B), and may prevent AFP-induced disruption of hyphal polarity axes. In contrast, application of AFP to sensitive fungi was shown to result in reduced levels of chitin synthase activity. Here, AFP is supposed to interact with specific glucosylceramides (e.g. Δ3-desaturated glucosylceramide) present in lipid raft structures of these strains. This interaction may then result in plasma membrane destabilisation and subsequent permeabilisation (see A) as well as disruption of hyphal polarity axes. The assumed interference of AFP with vesicle-mediated transport processes, including the delivery of class III and V chitin synthases to the plasma membrane (e.g. by hindrance of chitin synthase release from chitosomes, chitosome transport and/or prevention of proper localisation and anchoring of chitin synthases into plasma membranes), may render AFP-sensitive fungi incapable of complementing AFP-provoked cell wall damage.

Alternatively, AFP may also affect the proteolytic activation of zymogenic chitin synthases, may block chitin synthase activity, could interfere with the incorporation of chitin microfibrils into cell walls and/or may inhibit chitin synthase activity by adhering to chitin precursors or to chitin microfibrils. In consequence, growth is assumed to stall and fungi will eventually die.

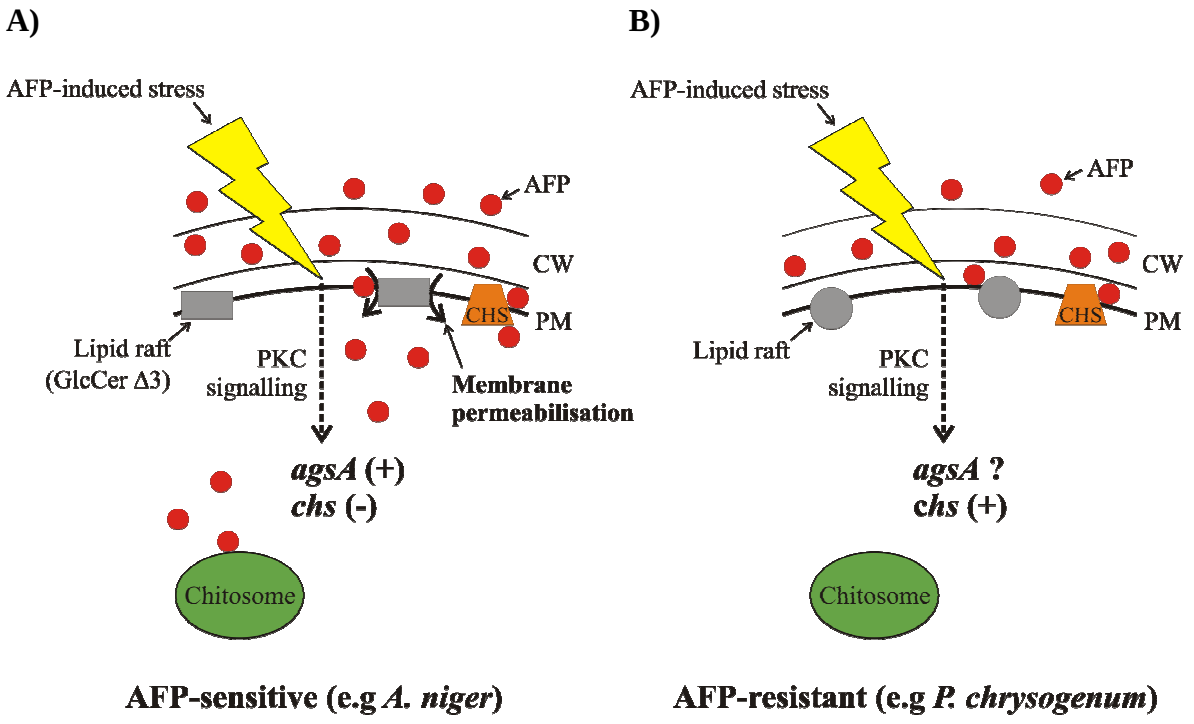

Figure 22. Postulated mechanism of action of AFP in sensitive and resistant fungi. AFP initially binds to cell wall chitin. A) In sensitive fungi, AFP may then interact with lipid rafts containing Δ3-deasturated glycosphingolipids, which could result in plasma membrane destabilisation and subsequent membrane permeabilisation. AFP-induced stress triggers protein kinase C (PKC) signalling, which leads to the transcriptional activation of the α-1,3-glucan synthase gene *agsA* (*ags* +). Intra- and/or extracellulary localised AFP may prevent PKC-signalling-induced chitin biosynthesis (*chs* -), possibly by directly and/or indirectly (e.g. involving chitosomes) targeting class III and V chitin synthases (for detailed explanation see text). In consequence, PKC signalling cannot (sufficiently) counteract for AFP-induced cell damage. Finally, growth stalls and the fungus will eventually die. B) In AFP-resistant fungi, lipid rafts are assumed to lack Δ3-desasturated glycosphingolipids. Here, AFP is suggested to be incapable of destabilising plasma membranes, thus no plasma membrane permeabilisation takes place. AFP triggers PKC signalling, which results in increased chitin synthase activities (chs +), counteracting for AFP-induced cell damage. Whether *agsA* is also transcriptionally activated is to date unknown (*agsA* ?). Abbreviations: CW = cell wall; PM = plasma membrane; CHS = chitin synthase; GlcCer Δ3 = Δ3-deasturated glycosphingolipids.

5.5 Biotechnological application of AFP

AFP exhibits a restricted range of activity, inhibiting exclusively the growth of filamentous fungi mainly belonging to the genera *Aspergillus* and *Fusarium*. The fact that bacteria and yeast are not affected by AFP (Lacadena et al., 1995; Theis, 2003) makes this polypeptide particularly attractive for putative applications in the food industry, crop control or medicine. In addition, it was demonstrated that AFP has no detrimental effects on mammalian cell types (Szappanos et al., 2006), which further underlines its excellent potential as a safe antifungal agent. With the aim to expand the potential applicability of AFP to the building industry, AFP susceptibilities of major wood decaying fungi were investigated. In fact, it was shown that several important strains (*A. penicilloides*, *Cladosporium herbarum*, *C. cladosporioides*, *Chaetomium globosum*, *Stachybotrys chartarum*, *Ceratocystis moniliformis* and *Aureobasidium pullulans*), all implicated in the destruction of cellulose-containing materials, exhibited AFP-induced growth restrictions. These results indicate that AFP may indeed be suitable to prevent fungal infections occurring in the building industry as well as with fields associated therewith.

In analyses aimed at identifying biodegradability, AFP was demonstrated to neither become degraded in media simulating stomach nor small intestine environments in man. At the same time, it was shown that the potency of AFP is also not affected by prolonged acid or bile treatments. This is certainly advantageous if it comes to treating endemic mycoses, such as gastrointestinal aspergilloma in humans (Prescott et al., 1994). However, due to the fact that the biodegradability assay applied here simulates human gastrointestinal environments *in vitro*, it cannot be excluded that AFP at one point becomes degraded within the digestive system nonetheless. And even if AFP should leave the digestive system of man unaffected, it is very likely to be degraded afterwards, e.g. inside activated sludge during wastewater treatment. In summary, these data indicate that AFP is a promising and safe candidate to be used as antifungal agent in multiple fields of biotechnology and industry.

5.5 Outlook

The results in this thesis strongly indicate that one major AFP target is represented by chitin synthesis. In particular class III and V chitin synthases seem to play significant roles in determining AFP susceptibility; however, the mechanism by which AFP interferes with chitin synthase activity has remained speculative. Further in-depth analyses will have to be performed to elucidate the interaction of AFP with chitin synthases in more detail.

Site-directed mutagenesis of the putative CBD is assumed to reveal whether this specific site is indeed required for efficient binding to chitin.

It was demonstrated that glucosylceramides may constitute putative AFP targets. Analyses of filamentous fungi deficient in the synthesis of Δ3-desaturated glucosylceramides may provide valuable information regarding the involvement of these sphingolipids in determining AFP-susceptibility. In addition, it should be clarified whether the interaction of AFP with distinct sphingolipids can result in the inhibition of vesicle transport and polarity establishment.

The screening of *S. cerevisiae* mutants affected in cell wall or lipid composition has given an insight into the molecular complexity by which fungi may respond towards AFP. However, it would be advisable to screen the complete EUROSCARF gene bank, aiming at the identification of additional mutants that exhibit AFP-sensitivity. This may contribute to a more wholistic understanding of how fungi cope with AFP-induced cell damage. Gene complementation in yeast mutants exhibiting significantly deviating growth effects in the presence of AFP will ensure that the observed effects are due to the deletion of specific genes. In order to analyse whether homologues of conspicuous genes also result in altered phenotypes in filamentous ascomycetes, respective genes should be overexpressed and/or deleted accordingly.

6 Summary

The antifungal protein (AFP) of *Aspergillus giganteus* is a small, amphipathic and basic polypeptide with antifungal activity against many important plant- and human pathogenic fungi. Interestingly, it exerts no effects on yeast, bacteria or mammalian cell types, making it an attractive agent to combat fungal infection. However, in order to bring AFP to application, its molecular mode of action has to be elucidated first. In the course of this work, it was shown that AFP binds readily to chitin *in vitro*. The identification of a putative chitin binding domain supports the finding that AFP is a chitin-binding protein. Significantly reduced susceptibilities of class III and class V chitin synthase mutants of AFP-sensitive fungi suggest that these may represent putative AFP targets. In *in situ* chitin synthase assays it was demonstrated that AFP-sensitive strains respond to AFP with a reduced chitin synthase activity, while chitin synthesis in AFP-resistant strains is increased. From these results is concluded that AFP targets chitin biosynthesis and may trigger the fungal cell wall integrity pathway. Further putative AFP targets may be represented by glucosylceramides, as depletion of these rendered AFP-sensitive strains significantly less susceptible towards AFP. A model is proposed in which present knowledge about the mode of action of AFP is summarised.

6 Zusammenfassung

Das Antifungalprotein (AFP) aus *Aspergillus giganteus* ist ein kleines, amphipathisches und basische Polypeptid, das antifungale Wirkung gegen eine Vielzahl an pflanzen- und human-pathogenen Pilzen aufweist. Interessanterweise übt es keine Effekte auf Hefen, Bakterien oder Säugetierzellen aus, was es zu einem attraktiven Wirkstoff zur Bekämpfung von Pilz-infektionen macht. Bevor das AFP jedoch zur Anwendung gebracht werden kann, muss zunächst sein Wirkmechanismus aufgeklärt werden. Im Rahmen dieser Arbeit wurde in *in vitro* Experimenten gezeigt, dass das AFP an Chitin bindet. Die Identifizierung einer mutmaßlichen Chitinbindedomäne unterstützt die Vermutung, dass das AFP ein Chitinbinde-protein ist. Deutlich reduzierte Suszeptibilitäten von Klasse III und Klasse V Chitinsynthase-mutanten von AFP-sensitiven Pilzen deuten darauf hin, dass diese ein putatives AFP Target darstellen. Die Durchführung von *in situ* Chitinsynthase-Assays hat gezeigt, dass AFP-sensitive Stämme mit einer reduzierten Chitinsynthaseaktivität auf AFP reagieren, während die Chitinsynthese in AFP-resistenten Stämmen erhöht vorliegt. Daraus kann geschlossen werden, dass das AFP die Chitinbiosynthese beeinflusst und möglicherweise den pilzlichen Zellwandintegritäts-Signalweg auslöst. Ein weiteres putatives AFP Target stellen die Glucosylceramide dar, da bei deren Reduktion eine deutlich verminderte AFP Sensitivität vorliegt. Ein Modell wird postuliert, in dem das gegenwärtige Wissen bezüglich der AFP Wirkweise zusammengefasst ist.

7 References

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Appendix

Table A: AFP susceptibility of different fungi and bacteria. Abbreviations: MIC = minimal inhibitory concentration; NE = no effect; > 400* = no complete growth inhibition was detected at concentrations of 400 µg/ml.

Organism	MIC (µg/ml)	Reference	Remark
Fungi:			
Aspergillus awamorii ATCC 22342	2	Theis et al., 2003	
A. clavatus ATCC 1007	NE	Theis et al., 2003	
A. flavus JB Collections 1147	NE	Lacadena et al., 1995	
A. flavus JB Collections 1057	NE	Lacadena et al., 1995	
A. fumigatus 15/0809	10	Theis, 2003	
A. giganteus IfGB 15/0903	> 400*	Theis et al., 2003	
A. giganteus MDH 18894	> 400*	Theis et al., 2003	
A. giganteus MDH 18894	NE	Lacadena et al., 1995	
A. nidulans DSM 969	200	Theis et al., 2003	
A. nidulans G191	200	Theis et al., 2003	
A. niger A365 (mcC)	2.5	This work	Mutant derived from *A. niger* A395.
A. niger A395	1	This work	
A. niger A493 (DC)	3	This work	Mutant derived from *A. niger* A395.
A. niger ATCC 9029	1	Theis et al., 2003	
A. niger IfGB 15/1803	1	Theis et al., 2003	
A. niger IJFM A483	NE	Lacadena et al., 1995	
A. niger NRRL 372	1	Theis et al., 2003	
A. oryzae A1560	1	This work	
A. oryzae ATCC 11488	NE	Theis et al., 2003	

Table A: Continued.

Organism	**MIC (µg/ml)**	**Reference**	**Remark**
A. oryzae C100 (*chsB*)	> 400*	This work	Mutant derived from *A. oryzae* A1560.
A. oryzae C101 (*csmA*)	> 400*	This work	Mutant derived from *A. oryzae* A1560.
A. penicilloides IfGB 15/1301	< 400	This work	
Aureobasidium pullulans IfGB 0112	< 400	This work	
Candida albicans	NE	S. Hagen, unpublished data	AFP resistant in yeast and filamentous state.
C. albicans CYC 1146	NE	Lacadena et al., 1995	
Ceratocystis moniliformis IfGB 15/0101	< 400	This work	
Chaetomium globosum IfGB 26/0102	< 400	This work	
Cladosporium cladosporioides IfGB 28/0101	< 400	This work	
C. herbarum IfGB 0203	< 400	This work	
Colletotrichum gloeosporioides	1	S. Hagen, unpublished data	
Fusarium aquaeductuum IfGB 39/0101	> 400*	Theis et al., 2003	
F. bubigenum IfGB 39/0301	10	Theis et al., 2003	
F. cerealis DSM 8704	1	S. Hagen, unpublished data	
F. culmorum IfGB 39/0403	> 400*	Theis et al., 2003	
F. equiseti IfGB 39/0701	1	Theis et al., 2003	
F. lactis IfGB 39/0701	1	Theis et al., 2003	
F. lini IfGB 39/0801	8	Theis et al., 2003	
F. moniliforme IfGB 39/1402	0.1	Theis et al., 2003	
F. oxysporum 4287	1	This work	

Table A: Continued.

Organism	MIC (µg/ml)	Reference	Remark
F. oxysporum chsV	NE	This work	Mutant dervived from *F. oxysporum* 4287.
F. oxysporum IfGB 29/1201	1	Theis et al., 2003	
F. oxysporum strains Fot 1a, Fot 1b, Folr2, Fosrl 13, Fol 39-87	7 – 25	Lacadena et al., 1995	
F. poae IfGB 39/0901	180	Theis et al., 2003	
F. proliferatum IfGB 39/1501	1	Theis et al., 2003	
F. sambucinum DSM 62396	1	S. Hagen, unpublished data	
Fusarium sp. strain IfGB 39/1101	1	Theis et al., 2003	
F. solani IfGB 39/1001	120	Theis et al., 2003	
F. sporotrichoides IfGB 39/1601	0.1	Theis et al., 2003	
F. vasinfectum IfGB 39/1301	10	Theis et al., 2003	
Penicillium chrysogenum ATCC 10002	NE	Theis et al., 2003	
P. chrysogenum IJFM A487	NE	Lacadena et al., 1995	
P. chrysogenum CECT 2306	NE	Lacadena et al., 1995	
P. frequentans IJFM A569	NE	Lacadena et al., 1995	
P. purpurogenum IJFM A575	10	Lacadena et al., 1995	
Pichia membranaefaciens CYC 1057	NE	Lacadena et al., 1995	
P. pastoris GS115	< 400	S. Hagen, unpublished data	
Rhodotorula mucilaginosa CYC 1024	NE	Lacadena et al., 1995	
Saccharomyces cerevisiae CYC 1058	NE	Lacadena et al., 1995	
S. cerevisiae AH22	NE	Theis et al., 2003	
S. cerevisiae ALG5	< 400	This work	
S. cerevisiae CHO2	< 400	This work	
S. cerevisiae DIE2	< 400	This work	
S. cerevisiae GSY1	< 400	This work	

Table A: Continued.

Organism	**MIC (µg/ml)**	**Reference**	**Remark**
S. exiguus CYC1057	NE	Lacadena et al., 1995	
Stachybotrys chartarum IfGB 87/0101	< 400	This work	
Trichoderma harzianum IJFM A217	127	Lacadena et al., 1995	
T. koningii IJGM A219	6	Lacadena et al., 1995	
Ustilago maydis FB1	< 400	S. Hagen, unpublished data	
U. maydis FB2	< 400	S. Hagen, unpublished data	
Bacteria:			
Bacillus megaterium ATCC 10778	NE	Theis et al., 2003	
B. subtilis	NE	Lacadena et al., 1995	
B. subtilis ATCC 6051	NE	Theis et al., 2003	
B. subtilis L170	NE	This work	Exhibits no cell wall.
Escherichia coli	NE	Lacadena et al., 1995	
E. coli ATCC 11775	NE	Theis et al., 2003	
E. coli K12	NE	This work	
E. coli LWF^{+}	NE	This work	Exhibits no cell wall.
Micrococcus luteus	NE	Lacadena et al., 1995	
Proteus mirabilis	NE	This work	Exhibits no cell wall.
Pseudomonas aeruginosa	NE	Lacadena et al., 1995	
P. fluorescence IfGB 0301	NE	Theis et al., 2003(Theis et al., 2003)	
Salmonella enteriditis	NE	Lacadena et al., 1995	
Serratia marcescens	NE	Lacadena et al., 1995	
Staphylococcus aureus	NE	Lacadena et al., 1995	

Table B: Summary of *S. cerevisiae* mutants tested in AFP susceptibility screening experiments. Mutants are listed in alphabetical order. Description of mutants is given (www. yeastgenome.org).

Mutation	Description
ALG5	UDP-glucose:dolichyl-phosphate glucosyltransferase, involved in asparagine-linked glycosylation in the endoplasmic reticulum.
ALG6	Glucosyltransferase, involved in transfer of oligosaccharides from dolichyl pyrophosphate to asparagine residues of proteins during *N*-linked protein glycosylation; mutations in human orthologue are associated with disease.
ATG26	UDP-glucose:sterol glucosyltransferase, conserved enzyme involved in synthesis of sterol glucoside membrane lipids, involved in autophagy.
BCK1	Mitogen-activated protein (MAP) kinase kinase kinase acting in the protein kinase C signalling pathway, which controls cell integrity; upon activation by Pkc1p phosphorylates downstream kinases Mkk1p and Mkk2p.
CHO2	Phosphatidylethanolamine methyltransferase (PEMT), catalses the first step in the conversion of phosphatidylethanolamine to phosphatidylcholine during the methylation pathway of phosphatidylcholine biosynthesis.
CHS1	Chitin synthase I, requires activation from zymogenic form in order to catalyse the transfer of *N*-acetylglucosamine to chitin; required for repairing the chitin septum during cytokinesis; transcription activated by mating factor.
CHS3	Chitin synthase III, catalyses the transfer of *N*-acetylglucosamine to chitin; required for synthesis of the majority of cell wall chitin, the chitin ring during bud emergence and spore wall chitosan.
CHS4	Activator of Chs3p, recruits Chs3p to the bud neck via interaction with Bni4p; has similarity to Shc1p, which activates Chs3p during sporulation.
CHS5	Protein of unknown function, involved in chitin biosynthesis by regulating Chs3p localisation, also involved in cell fusion during mating.
CHS6	Protein of unknown function, involved in chitin biosynthesis by regulating Chs3p localisation.
CHS7	Protein of unknown function, involved in chitin biosynthesis by regulating Chs3p export from ER.
CRZ1	Transcription factor that activates transcription of genes involved in stress response; nuclear localisation is positively regulated by calcineurin-mediated dephosphorylation.
CWP1	Cell wall mannoprotein, linked to a beta-1,3- and beta-1,6-glucan heteropolymer through a phosphodiester bond; involved in cell wall organisation.
CWP2	Covalently linked cell wall mannoprotein, major constituent of the cell wall; plays a role in stabilising the cell wall; involved in low pH resistance; precursor is GPI-anchored.
DIE2	Dolichyl-phosphoglucose-dependent glucosyltransferase of the ER, functions in the dolichol pathway that synthesises the dolichol-linked oligosaccharide precursor for *N*-linked protein glycosylation, has a role in regulation of ITR1 and INO1.
EKI1	Ethanolamine kinase, primarily responsible for phosphatidylethanolamine synthesis via the CDP-ethanolamine pathway; also exhibits choline kinase activity.
EPT1	sn-1,2-diacylglycerol ethanolamine- and cholinephosphotranferase.
FKS1	Catalytic subunit of 1,3-beta-D-glucan synthase, functionally redundant with alternate catalytic subunit Gsc2p; binds to regulatory subunit Rho1p; involved in cell wall synthesis and maintenance; localises to sites of cell wall remodelling.
FKS2	Catalytic subunit of 1,3-beta-glucan synthase, has similarity to an alternate catalytic subunit, Fks1p (Gsc1p); Rho1p encodes the regulatory subunit; involved in cell wall synthesis and maintenance.
FPS1	Plasma membrane glycerol channel, member of the major intrinsic protein (MIP) family of channel proteins; involved in efflux of glycerol and in uptake of the trivalent metalloids arsenite and antimonite.
FRT1	Tail-anchored endoplasmic reticulum membrane protein that is a substrate of the phosphatase calcineurin, interacts with homolog Frt2p, promotes cell growth in conditions of high Na^+, alkaline pH and cell wall stress.

Table B: Continued.

Mutation	Description
GAS1	Beta-1.3-glucanosyltransferase, required for cell wall assembly; localises to the cell surface *via* a glycosylphosphatidylinositol anchor.
GSY1	Glycogen synthase with similarity to Gsy2p, the more highly expressed yeast homolog; expression induced by glucose limitation, nitrogen starvation, environmental stress and entry into stationary phase.
GSY2	Glycogen synthase, similar to Gsy1p; expression induced by glucose limitation, nitrogen starvation, heat shock and stationary phase; activity regulated by cAMP-dependent, Snf1p and Pho85p kinases as well as by the Gac1p-Glc7p phosphatase.
INP51	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase, synaptojanin-like protein with an N-terminal Sac1 domain, plays a role in phosphatidylinositol 4,5-bisphosphate homeostasis and in endocytosis; null mutation confers cold-tolerant growth.
MID2	*O*-glycosylated plasma membrane protein that acts as a sensor for cell wall integrity signalling and activates the pathway; interacts with Rom2p, a guanine nucleotide exchange factor for Rho1p and with cell integrity pathway protein Zeo1p.
MKK1	Mitogen-activated kinase kinase involved in protein kinase C signalling pathway that controls cell integrity; upon activation by Bck1p phosphorylates downstream target, Slt2p; functionally redundant with Mkk2p.
MKK2	Mitogen-activated kinase kinase involved in protein kinase C signalling pathway that controls cell integrity; upon activation by Bck1p phosphorylates downstream target, Slt2p; functionally redundant with Mkk1p.
MPK1	Serine/threonine MAP kinase involved in regulating the maintenance of cell wall integrity and progression through the cell cycle; regulated by the PKC1-mediated signalling pathway
MUQ1	Choline phosphate cytidylyltransferase, catalyzes the second step of phosphatidylethanolamine biosynthesis; involved in the maintenance of plasma membrane; similar to mammalian CTP: phosphorcholine cytidylyl-transferases.
PDR16	Phosphatidylinositol transfer protein (PITP) controlled by the multiple drug resistance regulator Pdr1p, localises to lipid particles and microsomes, controls levels of various lipids, may regulate lipid synthesis, homologous to Pdr17p.
PLC1	Phosphoinositide-specific phospholipase C, hydrolyses phosphatidylinositol 4,5-biphosphate (PIP2) to generate inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG); involved in kinetochore function and pseudohyphal differentiation.
RLM1	MADS-box transcription factor, component of the protein kinase C-mediated MAP kinase pathway involved in the maintenance of cell integrity; phosphorylated and activated by the MAP-kinase Slt2p.
ROM1	GDP/GTP exchange protein (GEP) for Rho1p; mutations are synthetically lethal with mutations in rom2, which also encodes a GEP.
ROM2	GDP/GTP exchange protein (GEP) for Rho1p and Rho2p; mutations are synthetically lethal with mutations in rom1, which also encodes a GEP.
SCS7	Sphingolipid alpha-hydroxylase, functions in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids, has both cytochrome b5-like and hydroxylase/desaturase domains.
SFH5	Putative phosphatidylinositol transfer protein (PITP), exhibits phosphatidylinositol- but not phosphatidylcholine-transfer activity, mainly localised to cytosol and microsomes, similar to Sec14p; may be PITP regulator rather than actual PITP.
SFK1	Plasma membrane protein that may act together with or upstream of Stt4p to generate normal levels of the essential phospholipid PI4P, at least partially mediates proper localisation of Stt4p to the plasma membrane.
SMI1	Protein involved in the regulation of cell wall synthesis; proposed to be involved in coordinating cell cycle progression with cell wall integrity.
SWI4	DNA binding component of the SBF complex (Swi4p-Swi6p), a transcriptional activator that in concert with MBF (Mbp1-Swi6p) regulates late G1-specific transcription of targets including cyclins and genes required for DNA synthesis and repair.

Table B: Continued.

Mutation	Description
TOR1	PIK-related protein kinase and rapamycin target; subunit of TORC1, a complex that controls growth in response to nutrients by regulating translation, transcription, ribosome biogenesis, nutrient transport and autophagy; involved in meiosis.
VPS34	Phosphatidylinositol 3-kinase responsible for the synthesis of phosphatidylinositol 3-phosphate; forms membrane-associated signal transduction complex with Vps15p to regulate protein sorting; similar to p110 subunit of mammalian PI 3-kinase.
WSC1	Sensor-transducer of the stress-activated PKC1-MPK1 kinase pathway involved in maintenance of cell wall integrity; involved in organisation of the actin cytoskeleton; secretory pathway Wsc1p is required for the arrest of secretion response.
WSC2	Partially redundant sensor-transducer of the stress-activated PKC1-MPK1 signalling pathway involved in maintenance of cell wall integrity and recovery from heat shock; secretory pathway Wsc2p is required for the arrest of secretion response.
WSC3	Partially redundant sensor-transducer of the stress-activated PKC1-MPK1 signalling pathway involved in maintenance of cell wall integrity; involved in the response to heat shock and other stressors; regulates 1,3-beta-glucan synthesis.
WSC4	ER membrane protein involved in the translocation of soluble secretory proteins and insertion of membrane proteins into the ER membrane; may also have a role in the stress response but has only partial functional overlap with WSC1-3.
YEH2	Steryl ester hydrolase, catalyses steryl ester hydrolysis at the plasma membrane; involved in sterol metabolism.

Table C: Summary of *S. cerevisiae* mutants with altered chitin contents tested in AFP susceptibility screening experiments. Mutants are ranked according to chitin content. Description of individual mutants is given (www. yeastgenome.org). Chitin levels are expressed as mmoles GlcNAc per mg dry weight (Lesage et al., 2005). The wild-type (BY4741) is included for reference.

Mutation	Description	Chitin level
MNN9	Subunit of Golgi mannosyltransferase complex also containing Anp1p, Mnn10p, Mnn11p, and Hoc1p that mediates elongation of the polysaccharide mannan backbone; forms a separate complex with Van1p that is also involved in backbone elongation.	130.9
ANP1	Subunit of the alpha-1.6 mannosyltransferase complex; type II membrane protein; has a role in retention of glycosyltransferases in the Golgi; involved in osmotic sensitivity and resistance to aminonitrophenyl propanediol.	113.3
MNN10	Subunit of a Golgi mannosyltransferase complex also containing Anp1p, Mnn9p, Mnn11p, and Hoc1p that mediates elongation of the polysaccharide mannan backbone; membrane protein of the mannosyltransferase family.	97.3
SHE4	Protein containing a UCS (UNC-45/CRO1/SHE4) domain, binds to myosin motor domains to regulate myosin function; involved in endocytosis, polarization of the actin cytoskeleton, and asymmetric mRNA localisation.	63.1
SAC6	Fimbrin, actin-bundling protein; cooperates with Scp1p (calponin/transgelin) in the organisation and maintenance of the actin cytoskeleton.	58.5
GUP1	Plasma membrane protein with a possible role in proton symport of glycerol; member of the MBOAT family of putative membrane-bound *O*-acyltransferases.	54.2
BEM4	Protein involved in establishment of cell polarity and bud emergence; interacts with the Rho1p small GTP-binding protein and with the Rho-type GTPase Cdc42p; involved in maintenance of proper telomere length.	52.7
ILM1	Protein of unknown function; may be involved in mitochondrial DNA maintenance; required for slowed DNA synthesis-induced filamentous growth.	52.7
MNN11	Subunit of a Golgi mannosyltransferase complex that also contains Anp1p, Mnn9p, Mnn10p, and Hoc1p, and mediates elongation of the polysaccharide mannan backbone; has homology to Mnn10p.	47.0
CYK3	SH3-domain protein located in the mother-bud neck and the cytokinetic actin ring; mutant phenotype and genetic interactions suggest a role in cytokinesis.	43.7
CLA4	Cdc42p activated signal transducing kinase of the PAK (p21-activated kinase) family, involved in septin ring assembly and cytokinesis; directly phosphorylates septins Cdc3p and Cdc10p; other yeast PAK family members are Ste20p and Skm1p.	31.5
FAB1	1-phosphatidylinositol-3-phosphate 5-kinase; vacuolar membrane kinase that generates phosphatidylinositol (3,5)P2, which is involved in vacuolar sorting and homeostasis.	23.4
WHI2	Protein required, with binding partner Psr1p, for full activation of the general stress response, possibly through Msn2p dephosphorylation; regulates growth during the diauxic shift; negative regulator of G1 cyclin expression.	21.0

Table C: Continued.

Mutation	Description	Chitin level
PHO5	Repressible acid phosphatase (1 of 3) that also mediates extracellular nucleotide-derived phosphate hydrolysis; secretory pathway derived cell surface glycoprotein; induced by phosphate starvation and coordinately regulated by PHO4 and PHO2.	20.0
EDE1	Key endocytic protein involved in a network of interactions with other endocytic proteins, binds membranes in a ubiquitin-dependent manner, may also bind ubiquitinated membrane-associated proteins.	19.5
TUS1	Guanine nucleotide exchange factor (GEF) that functions to modulate Rho1p activity as part of the cell integrity signalling pathway; multicopy suppressor of tor2 mutation and ypk1 ypk2 double mutation; potential Cdc28p substrate.	19.5
PKR1	Protein of unknown function; overproduction confers resistance to *Pichia farinosa* killer toxin.	18.2
OPI3	Phospholipid methyltransferase (methylene-fatty-acyl-phospholipid synthase), catalyzes the last two steps in phosphatidylcholine biosynthesis.	17.4
AST1	Peripheral membrane protein that interacts with the plasma membrane ATPase Pma1p and has a role in its targeting to the plasma membrane, possibly by influencing its incorporation into lipid rafts.	16.6
wild-type	-	15.8
CNB1	Calcineurin B; the regulatory subunit of calcineurin, a Ca^{++}/calmodulin-regulated protein phosphatase which regulates Crz1p (a stress-response transcription factor), the other calcineurin subunit is encoded by CNA1 and/or CMP1.	15.4
SWI3	Subunit of the SWI/SNF chromatin remodelling complex, which regulates transcription by remodelling chromosomes; required for transcription of many genes, including ADH1, ADH2, GAL1, HO, INO1 and SUC2.	15.2
MSN5	Karyopherin involved in nuclear import and export; shown to be responsible for nuclear import of replication protein A and for export of several proteins including Swi6p, Far1p, and Pho4p; cargo dissociation involves binding to RanGTP.	15.1
EMP24	Integral membrane component of endoplasmic reticulum-derived COPII-coated vesicles, which function in ER to Golgi transport.	14.8
ELO1	Elongase I, medium-chain acyl elongase, catalyzes carboxy-terminal elongation of unsaturated C12-C16 fatty acyl-CoAs to C16-C18 fatty acids.	14.6
HXT8	Protein of unknown function with similarity to hexose transporter family members, expression is induced by low levels of glucose and repressed by high levels of glucose.	13.8
DEP1	Transcriptional modulator involved in regulation of structural phospholipid biosynthesis genes and metabolically unrelated genes, as well as maintenance of telomeres, mating efficiency and sporulation.	12.6

Curriculum vitae

Persönliche Daten:

Name:	Hagen
Vorname:	Silke
Geburtsdatum:	28.06.1971
Geburtsort:	Mainz

Schulische Ausbildung:

1977 – 1982	Rudolf-Steiner-Schule (Hamburg)
1982 – 1991	Gymnasium Ohlstedt (Hamburg)
11.06.1991	Abschluss: Abitur

Wissenschaftlicher Werdegang:

1991 – 1997	Studium der Genetik an der University of Wales (Cardiff, Großbritannien)
14.07.1997	Abschluss: Bachelor of Science
1998 – 2002	Studium der Biologie an der Ernst-Moritz-Arndt Universität Greifswald
19.09.2002	Abschluss: Diplom-Biologin
2002 – 2006	Wissenschaftliche Mitarbeiterin am Fachgebiet Mikrobiologie und Genetik des Institutes für Biotechnologie der TU Berlin