The elucidation of signalling and survival mechanisms of fungi to counteract the antifungal protein AFP

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To my parents

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world" Louis Pasteur

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Chapter 1

General introduction

1.1. Introduction

The emergence of fungal resistant pathogens to antifungal agents is becoming more prevalent in the medical and agricultural fields and the need for new safe and effective antifungal agents which are not toxic to mammalian cells and plants is imperative. The past two decades has witnessed a dramatic growth in knowledge as regards natural peptides and approximately 400 peptides have been investigated to date for their antifungal properties (De Luca and Walsh, 2000). These have been obtained from many different sources; most peptides that have been studied are natural, although an increasing number are either semisynthetic or completely synthetic. They have different modes of action with hydrophobic and amphipathic properties. The application of these antifungals agents in both medicine and agriculture is mandatory. However, they need to meet several criteria such as a specific mode of antifungal action, being safe for humans and the environment, high efficacy and inexpensive and sustainable production. Interestingly, filamentous fungi produce antifungal compounds to protect themselves against other fungi which might act as nutrient competitors or other stress responses in the same environment. For example, the genus *Aspergillus* has been described to produce small-size basic antifungal peptides with suppressive effects on fungal growth (Meyer, 2008).

1.2. Antifungal proteins from filamentous fungi

A great abundance of biologically active proteins and peptides with antifungal properties are found in filamentous fungi (Table 1). These are small peptides with diverse structures and diverse modes of action (Galgoczy and Vagvolgyi, 2009). Some antifungal proteins are connected to lipids and form a lipopeptide structure (De Luca and Walsh, 2000). The major targets of antifungal proteins which have been discovered in sensitive organisms are the cell wall, cell membrane and intracellular organelles (Theis and Stahl, 2004; Yeaman and Yount, 2003). Most antifungal proteins which target the cell wall inhibit glucan synthesis; echinocandins are antifungal proteins which display this characteristic. Examples of echinocandins comprise echinocandins, pneumocandins, aculeacins, mulundocandins, anidulafungins and WF11899 (De Lucca, 2000). Unfortunately, native echinocandins are haemolytic and analogues which retain their antifungal properties with reduced haemolysis have been created. Moreover, if the knowledge of the mode of action of the antifungal proteins produced by filamentous fungi is

important for their effective use in medical or agricultural fields, there are still some antifungal proteins with unknown modes of action. Amongst these are leucinostatin, 1901-II, 1907-VIII and trichopolyns which are effective against *C. neoformans*, *C. albicans* and other clinically important fungi (De Lucca, 2000; Fuji et al., 1978; Fujita et al., 1981). However, the overall overview of the antifungal proteins discovered to date indicates that the fungal-produced antifungal proteins are more active than those from bacteria and plants.

1.3. Antifungal proteins from Aspergillus

Scientists working with *Aspergillus* seem to be fascinated by its potential to produce extracellular enzymes, organic acids and secondary metabolites with biotechnological importance. Among them are low molecular weight proteins with suppressive effects on fungal growth. These proteins are also characterized by their basic nature and a high amount of cysteines residues forming disulfide bridges. To date, *A. clavatus*, *A. niger*, *A. giganteus and A. oryzae* are *Aspergillus* species known to produce antifungal proteins called respectively ACAFP, ANAFP, AFP and exAP-AO17 (Gun Lee et al., 1999; Lacadena et al., 1995; Seong-Cheol et al., 2008; Skouri-Gargouri and Gargouri, 2008). These proteins can be active against members of important zoopathogenic and plant-pathogenic fungi (Table 2). However, they have a limited antifungal spectrum with different species specificity (Marx, 2004). Furthermore, AFP and ACAFP have no effect on yeast and bacteria. However, ANAFP and exAP-AO17 exhibit activity against yeast. In addition to inhibiting yeast and filamentous fungi, exAP-AO17 is effective against pathogenic bacteria such as *S. aureus* and *E. coli0157* (Seong-Cheol et al., 2008). The antimicrobial activity of exAP-AO17 is probably due to its larger structure (17kDa) in comparison to other proteins from the *Aspergillus* species. The antifungal proteins AFP, ANAFP and ACAFP have molecular weights of 5.8 kDa, 6.6 kDa and 5.77 KDa respectively and their amino acid sequences exhibit high similarity to each other (Fig 1) (Marx, 2004; Meyer, 2008; Skouri-Gargouri and Gargouri, 2008). Although they are known to be effective against different fungal species, their mode of action is mostly not well understood. Nevertheless, the antifungal protein secreted by *A.giganteus* (AFP) presents an advantage as regards to its mechanism of action in comparison to the other proteins. The mature form of AFP is a 51 amino acid with a high content of cysteine, tyrosine and lysine residues (Meyer, 2008). It binds to the cell wall and/or plasma membrane of sensitive fungi

and disturbs polarized growth. Its growth inhibition in filamentous fungi is shown to be due to the inhibition of cell wall chitin biosynthesis, followed by a permeabilization of the membrane (Hagen et al., 2007; Theis et al., 2003). Plasma membrane and cell wall perturbation is the main inhibiting mechanism for most antifungal proteins produced throughout all kingdoms, ranging from prokaryotes to lower and higher eukaryotes. Thus, it can be hypothesized that there is a putative receptor in the outer layers of the sensitive fungi, allowing the proteins to become active (De Samblanx et al., 1997; Thevissen et al., 1997). Moreover, the amphipathic structure of most of the antifungal proteins probably facilitates the interaction with the membrane or/and cell wall receptor. Using immunofluorescence microscopy, it has been demonstrated that AFP binds to the plasma membrane of *A.niger* and *A.awamori* (two sensitive strains), but it is localized intracellularly in AFP resistant fungi such as *P.chrysogenum* and *A.clavatus* (Theis et al., 2003). The potential relevance of AFP and its related proteins as new antifungal drugs to combat fungal infections requires more investigation as regards their mode of action and clinical evaluation. As the complete clarification of its molecular targets and mode of action are major prerequisites for the future application of the protein, this study aims to bring AFP closer to being an alternative to currently used drugs.

Table 1: Antifungal protein of filamentous fungi

Name	Source	Structure	Target	Typical target organisms	References
Aculeacins	*A. aculeatus*	Lipopeptide	Glucan synthesis	*C. albicans, A. niger, A. fumigatus*	(De Lucca and Walsh, 1999)
Aureobasidin A	*A. pullulans*	Cyclic depsipeptide	Actin assembly	*C. neoformans*	(De Lucca and Walsh, 1999)
Echinocandin	*A. nidulans* *A. rugulosus*	Lipopeptide	Glucan synthesis	*C. albicans*	(De Lucca and Walsh, 1999)
Mulundocandin	*A. syndowi*	Lipopeptide	Glucan synthesis	*C. albicans, A. niger*	(De Lucca and Walsh, 1999)
1901-II	*P. lilacinus*	Lipopeptide	Unknown	*C. tropicalis*	(De Lucca and Walsh, 1999)
1907-VIII	*P. lilacinus*	Peptide	Unknown	*C. tropicalis*	(De Lucca and Walsh, 1999)
Aureobasidin A	*A. pullulans*	Cyclic depsipeptide	Actin assembly	*C. neoformans*	(De Lucca and Walsh, 1999)

Name	Source	Structure	Target	Typical target organisms	References
Leucinostatin A	*P. lilacinum*	Lipopeptide	Unknown	*C. neoformans*	(De Lucca and Walsh, 1999)
Leucinostatin H	*P. marquandii*	Lipopeptide	Unknown	*C. albicans*	(De Lucca and Walsh, 1999)
Leucinostatin K	*P. marquandii*	Lipopeptide	Unknown	*C. albicans*	(De Lucca and Walsh, 1999)
Mulundocandin	*A. syndowi*	Lipopeptide	Glycan synthesis	*C. albicans*, *A. niger*	(De Lucca and Walsh, 1999)
Pneumocandin A_0	*Z. arboricola*	Lipopeptide	Glucan synthesis	*C. albicans* isolates	(De Lucca and Walsh, 1999)
Trichopolyn B	*T. polysporum*	Lipopeptide	Unknown	*C. neoformans*	(De Lucca and Walsh, 1999)
WF11899 A	*Coleophoma empetri*	Lipopeptide	Glucan synthesis	*C. albicans*	(De Lucca and Walsh, 1999)
WF11899 B	*C. empetri*	Lipopeptide	Glucan synthesis	*C. albicans*	(De Lucca and Walsh, 1999)
WF11899 C	*C. empetri*	Lipopeptide	Glucan synthesis	*C. albicans*	(De Lucca and Walsh, 1999)
AFP	*A. giganteus*	Peptide	Plasma membrane, chitin biosynthesis	*A. niger, F. oxysporum*	(Hagen et al., 2007; Meyer, 2008; Theis et al., 2003)
PAF	*P. chrysogenum*	Peptide	Plasma membrane	*A. niger, A. nidulans, A. fumigatus*	(Leiter et al., 2005)
ANAFP	*A. niger*	Peptide	Plasma membrane	*A. flavus, A. fumigatus, F. oxysporum, C. albicans, S.cerevisiae*	(Gun Lee et al., 1999)
NAF	*P. nalgiovense*	Peptide	Unknown	*P. roqueforti, P. italicum,*	(Geisen, 2000)
ACAFP	*A. clavatus*	Peptide	Cell wall	*F. oxysporum, F. solani, A. niger*	(Skouri-Gargouri and Gargouri, 2008)
exAP-AO17	*A. oryzae*	Peptide	Unknown	*F. moniliforme, C. coccoides*	(Seong-Cheol et al., 2008)

Table 2: Sensitivity of microorganisms to the antifungal proteins AFP, ANAFP, ACAFP and exAPAO17

Organism	AFP[a,b]	ANAFP[c]	ACAFP[d]	exAP-AO17[e]
Filamentous fungi				
A. awamorii	++	n.d.	n.d.	n.d.
A. clavatus	-	n.d.	n.d.	n.d.
A. flavus[f]	-	++	n.d.	n.d.
A. fumigatus[f]	+	++	n.d.	n.d.
A. giganteus	+	n.d.	n.d.	n.d.
A. nidulans	+ -	n.d.	-	n.d.
A. niger[f]	++	-	++	n.d.
A. oryzae	-	n.d.	n.d	n.d.
A. terreus[f]	n.d.	n.d.	n.d	n.d.
Alternaria solani[g]	n.d.	n.d.	++	n.d.
Colletotrichum coccoides[g]	n.d.	n.d.	n.d.	+
Botrytis cinerea[g]	n.d.	n.d.	++	n.d.
Fusarium aquaeductuum[g]	+	n.d.	n.d.	n.d.
F. bubigenum[g]	++	n.d.	n.d.	n.d.
F. culmorum[g]	+	n.d.	n.d.	n.d.
F. equiseti[g]	++	n.d.	n.d.	n.d.
F. lactis[g]	++	n.d.	n.d.	n.d.
F. lini[g]	++	n.d.	n.d.	n.d.
F. moniliforme[f,g]	++	n.d.	n.d.	+
F. oxysporum[f,g]	++	++	++	n.d.
F. poae[g]	+	n.d.	n.d.	n.d.
F. proliferatum[g]	++	n.d.	n.d.	n.d.
F. solani[f,g]	+	++	++	n.d.
F. sporotrichoides[g]	++	n.d.	n.d.	n.d.
F. vasinfectum[g]	++	n.d.	n.d.	n.d.
Magnaporthe grisea[g]	++	n.d.	n.d.	n.d.

++ Highly sensitive, + sensitive, - resistant, n.d. not determined
a(Lacadena et al., 1995) b(Theis et al., 2003) c(Gun Lee et al., 1999) d(Skouri-Gargouri and Gargouri, 2008) e(Seong-Cheol et al., 2008)

[f] Opportunistic zoo-pathogenic organism [g] Potentially plant-pathogenic organism

Organism	AFP[a,b]	ANAFP[c]	ACAFP[d]	exAP-AO17[e]
Penicillium chrysogenum	-	n.d.	n.d.	n.d.
P. frequentas	-	n.d.	n.d.	n.d.
P. occitanis	n.d.	n.d.	-	n.d.
P. purpurogenum	++	n.d.	n.d.	n.d.
Stacchybotris microbiospora	n.d.	n.d.	++	n.d.
Trichoderma harzianum	+	n.d.	n.d.	n.d.
T. koningii	++	n.d.	n.d.	n.d.
T. reesei	n.d.	n.d.	+++	n.d.
Yeasts				
Candida albicans[f]	-	++	-	+
Pichia pastoris	n.d.	n.d.	-	n.d.
P. membranaefaciens	-	n.d.	n.d.	n.d.
Rhodotorula mucilaginosa[f]	-	n.d.	n.d.	n.d.
Saccharomyces cerevisiae	-	++	-	+
S. exiguous	-	n.d.	n.d.	n.d.
Trichosporon beigelii[f]	n.d.	++	n.d.	n.d.
Bacteria				
Bacillus megaterium	-	n.d.	-	n.d.
B. subtilis	-	-	-	n.d.
Escherichia coli[f]	-	-	-	+
Micrococcus luteus	-	n.d.	-	n.d.
Pseudomonas aeruginosa[f]	-	n.d.	-	n.d.
P. fluorescens	-	n.d.	-	n.d.
Salmonella enteritidis[f]	-	n.d.	-	n.d.

++ Highly sensitive, + sensitive, - resistant, n.d. not determined
a(Lacadena et al., 1995) b(Theis et al., 2003) c(Gun Lee et al., 1999) d(Skouri-Gargouri and Gargouri, 2008) e(Seong-Cheol et al., 2008)

[f] Opportunistic zoo-pathogenic organism [g] Potentially plant-pathogenic organism

Organism	AFP[a,b]	ANAFP[c]	ACAFP[d]	exAP-AO17[e]
Serratia marcescens[f]	-	n.d.	-	n.d.
Staphylococcus aureus[f]	-	n.d.	-	+

++ Highly sensitive, + sensitive, - resistant, n.d. not determined
a(Lacadena et al., 1995) b(Theis et al., 2003) c(Gun Lee et al., 1999) d(Skouri-Gargouri and Gargouri, 2008) e(Seong-Cheol et al., 2008)

[f] Opportunistic zoo-pathogenic organism [g] Potentially plant-pathogenic organism

```
AFP      MKFVSLASLGFALVAALGAVATPVEADSLTAGGLDARDESAVLATYNGKCYKKDNICKYK 60
ACAFP    MKVVSLASLGFALVAALGVAASPVDADSLAAGGLDARDESAVQATYDGKCYKKDNICKYK 60
ANAFP    ---MQLTSIAIILFAAMGAIANPIAAE---ADNLVAR--EAELSKYGGECSVEHNTCTYL 52
            :.*:*:.: *.**:*. *.*: *:   *..* **  .*  :.*.*:*  :.* *.*

AFP      -QSGKTAICKCYVKK---CPRDGAKCEFDSYKGKCYC---- 93
ACAFP    AQSGKTAICKCYVKV---CPRDGAKCEFDSYKGKCYC---- 94
ANAFP    -KGGKDHIVSCPSAANLRCKTERHHCEYDEHHKTVDCQTPV 92
          :.**  * .*       *  :  :**:*.:: .  *
```

Figure 1: **AFP (GenBank accession no.:X60771.1), ACAFP (GenBank accession no.: GU390689.1), and ANAFP (GenBank accession no.:XP_001391221) amino acid alignment**. "*" means that the residues in that column are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, "." means that semi-conserved substitutions are observed. Dashes in the alignment indicate gaps in individual sequences.

1.4. The antifungal protein from Aspergillus giganteus

AFP is secreted by *A. giganteus* as a 94 amino acid long preproprotein. After hydrolysis of the presequence (aa 1-26) and the prosequence (aa 27-43), the mature AFP consists of 51 amino acids, resulting in a molecular weight of 5.8 kDa. This mature form can be isolated from a culture supernatant of *A. giganteus*. It has a pI of 8.8 due to the high amount of lysine and tyrosine residues, thus it is positively charged under physiological conditions (Nakaya et al., 1990). The conformational structure of the protein is well resolved and consists of five highly twisted antiparallel β-strands (Fig 2). This structure is stabilized by four disulfide bridges which grant remarkable resistance to heat and protease degradation (Lacadena et al., 1995; Theis et al., 2005). Several environmental conditions have been shown to regulate the transcriptional expression of the *afp* gene. These conditions include carbon starvation, high concentrations of NaCl, heat shock and ethanol (Meyer and Stahl, 2002; Meyer et al., 2002). In addition, it was found that co-cultivation modulates *afp* expression on the transcription level (Meyer and Stahl, 2003; Meyer et al., 2002). Thus, an increase in AFP expression can be found in co-culture with *F. oxysporum*, whereas a co-culture with *A. niger* leads to the suppression of *afp* expression. AFP shows

antifungal activity against several filamentous fungi in particular, whereas it does not affect the growth of yeast or bacterial cells. The effect of AFP can be fungistical or fungicidal, according to the concentration applied. Theis et al have reported that AFP provokes severe plasma membrane alteration followed by a permeabilization in sensitive fungi (Theis et al., 2005). Although the targets of AFP have not yet been identified, it has been demonstrated that AFP is predominantly localized to the cell wall-attached "outer layer" in AFP-sensitive fungi (Theis et al., 2005). Most recently, it has been found that Δ3-desaturated glucosylceramides are involved in AFP activity as their cellular depletion leads to the reduction of AFP susceptibility in *A. niger* and *A. fumigatus* (Hagen, 2006). Glucosylceramides belong to the group of sphingolipids, localised to lipid rafts, mostly found in the plasma membrane (Rittenour et al., 2011; van Meer et al., 2003).

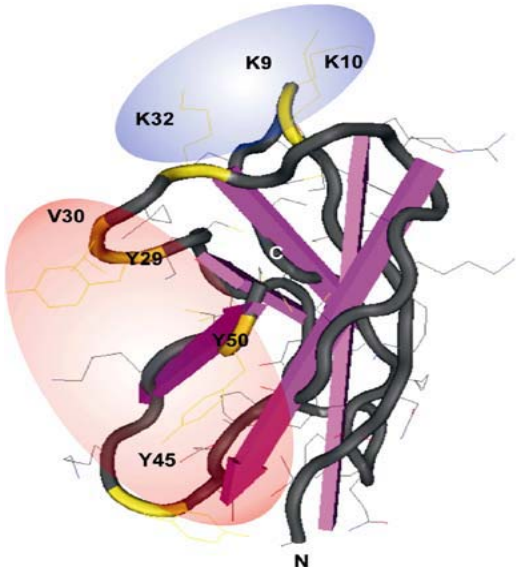

Figure 2: Conformational structure of the AFP from *A. giganteus*. β-sheet structures are shown as arrows. The cationic site is depicted in blue and created by Lys9, Lys10 and Lys32. The hydrophobic stretch formed by Tyr29, Val30, Tyr45 and Tyr50 is marked in red. The amino- and carboxyterminal ends are marked N and C, respectively. The image was calculated with CD3D 4.0 on the basis of the AFP structure reported by (Campos-Olivas et al., 1995).

1.5. ***Aim of the thesis***

AFP was first purified from the culture supernatant of the imperfect ascomycete *A. giganteus* and shown to inhibit the growth of many filamentous fungi; in particular strains belonging to the genera *Fusarium* and *Aspergillus* (Table 2). There is more knowledge about the mode of action of AFP as opposed to other peptides with antifungal activity. Early experiments indicate that AFP induces the proteins kinase C (PKC)-dependant cell wall integrity pathways in sensitive fungi (Hagen et al., 2007). It has also been reported that AFP binds to the cell wall and/or plasma membrane of AFP sensitive fungi, but is localized intracellularly in AFP-resistant fungi such as *P. chrysogenum* and *A. clavatus.* In addition to permeabilizing the membrane in sensitive fungi, AFP inhibits in vivo chitin synthases activity in *A. niger*, *A. oryzae* and *F. oxysporum* probably inhibiting chitin synthases target of class III and V (Hagen et al., 2007). The identification of these AFP targets was made possible by analyzing AFP susceptibility of class V chitin synthase mutant strains of *F. oxysporum* and *A. oryzae*, as well as the class III chitin synthase mutant of *A. oryzae.* These mutants were less susceptible to AFP in comparison to the corresponding wild type (Hagen et al., 2007). Hagen (2006) thus postulated a mechanism of action of AFP in sensitive and resistant filamentous fungi (Fig 3).

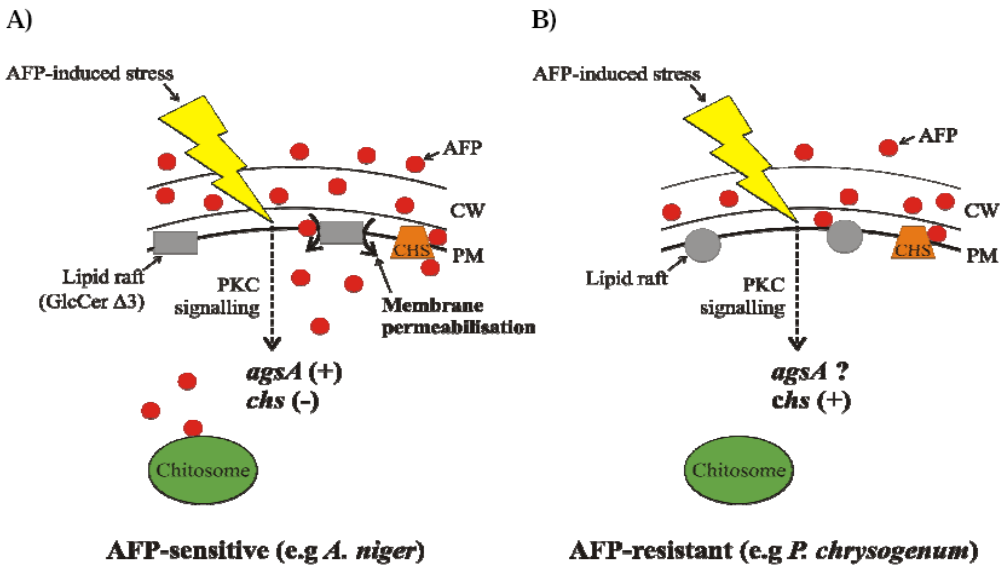

Figure 3: Postulated mechanism of action of AFP in sensitive and resistant fungi (Hagen, 2006). AFP initially binds to cell wall chitin. A) In sensitive fungi, AFP may then interact with lipid rafts containing Δ3-deasturated glycosphingolipids, which could result in plasma membrane destabilization and subsequent membrane permeabilization. AFP-induced stress triggers protein kinase C (PKC) signaling, which leads to the transcriptional activation of the α-1,3-glucan synthase gene *agsA (ags+)*. Intra –and/or extracellularly localized AFP may prevent PKC-signaling-induced chitin biosynthesis *(chs-),* possibly by directly and/or indirectly (e.g. involving chitosomes) targeting class III and V chitin synthases. In consequence, PKC signaling cannot (sufficiently) counteract for AFP-induced cell damage. Finally growth stalls and the fungi will eventually die. B) In AFP-resistant fungi, lipid rafts are assumed to lack Δ3-desaturated glycosphingolipids. Here, AFP is suggested to be incapable of destabilizing plasma membranes, thus no plasma membrane permeabilization takes place. AFP triggers PKC signaling, which results in increased chitin synthase activities (*chs+*), counteracting for AFP-induced cell damage. Whether *agsA* is also transcriptionally activated is to date unknown. Abbreviations: CW = cell wall; PM = plasma membrane; CHS = chitin synthase; GlcCer Δ3 = Δ3-deasturated glycosphingolipids (Hagen, 2006).

Still more knowledge on the AFP mode of action could be obtained by using strains with mutations in important genes which are supposedly involved in cell protection or integrity signaling. Preliminary screening of deleted mutant strains from the naturally AFP resistant strain *S. cerevisiae* for their susceptibility to AFP has reinforced this hypothesis (Hagen, 2006). The goals of this thesis are thus (i) to elucidate the response mechanism of some fungi to AFP by using mutant strains from the model resistant fungus *S. cerevisiae* and the sensitive filamentous fungus *A. niger*, (ii) to identify which response mechanism is important for resistant fungi to

survive an AFP attack, and (iii) to identify the role of chitin synthase class III and class V of *A. niger* during treatment with AFP.

Chapter 2 provides a literature review on the composition of the plasma membrane and the cell wall of *Aspergillus sp* as well as the maintenance of their cell wall and membrane integrity. As it is known to be the major target of antifungal compounds, the cell wall and cell membrane signaling mechanism are reviewed as regards ensuring the integrity and the survival response of *Aspergillus*.

In chapter 3, the roles of the class III and class V chitin synthase of *A. niger* during growth development, stress conditions, as well as in the response to AFP attack are studied. The role of the chitin synthase class III and V in fungal cell wall integrity and growth has already been well studied in *A. fumigatus* and *A. nidulans*; however their role in *A. niger* has not been studied yet.

In chapter 4, the survival strategies of yeast and filamentous fungi against the antifungal protein AFP are studied. Selected mutant strains from a yeast genomic deletion collection for AFP-sensitive phenotypes were screened. The results demonstrate that a concerted action of different signaling pathways is likely to safeguard fungi against AFP.

In chapter 5, a screening method for compounds exerting antifungal activities has been described. Due to increase resistance of human and plant pathogenic fungi against currently used drugs, it is important to develop methods and screens for the identification of compounds which specifically kill fungi but do not affect men and environment.

Chapter 6 provides a general discussion and concluding remarks of all chapters previously described.

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Chapter 2

Maintaining cell wall and membrane integrity in *Aspergillus*

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Abstract

In recent years, there have been a significantly increasing number of studies of cell wall and membrane integrity signaling networks, particularly for the yeasts *Saccharomyces cerevisiae* and *Candida albicans.* For instance, fungal cell wall integrity plays a key role in the regulation and maintenance of the fungal physiology and in the defense against stress conditions through the modulation of different pathways and signaling factors. Cell wall/membrane biogenesis and integrity signaling in *S. cerevisiae* have been well studied. However, studies of cell wall and cell membrane biogenesis and their regulation via signaling in filamentous fungi are limited. In molds, such as *Aspergillus sp*., the cell wall and membrane integrity maintaining is important to assure cellular processes such as cell growth, cell shape and to prevent cell lysis against adverse environment such as antifungal compounds or harsh conditions in bioreactors. In this review, we give an overview of the genus *Aspergillus* cell wall and membrane composition and thereby the role of these cell compartments in fungal cell protection.

Keywords: *Aspergillus*. Cell wall and membrane integrity. Antifungal compounds. Survival response.

2.1. Introduction

Aspergillus is a taxonomic group which is found in various climates worldwide and includes organisms whose characteristics have high pathological, agricultural, industrial, pharmaceutical and biotechnological importance. *Aspergillus fumigatus*, *A.nidulans*, *A. oryzae* and *A. niger* are the four predominant *Aspergillus* strains studied nowadays because of their multiple importance cited (Galagan et al., 2005; Gupte et al., 2002; Machida et al., 2005; Nierman et al., 2005; Pel et al., 2007). It is well known that the genus *Aspergillus* can survive in a wide range of environments; moreover the genus can also inhabit extreme environments which present challenges that must be overcome for survival, including oxidative stress, osmotic changes, heat shock, nutrients limitation, pH changes and chemical stresses. When exposed to stress conditions, the fungal cell responds by modifying several genes expression to be able to endure the adversities. The major key defense of fungi to withstand environmental aggressions is their cell wall and cell membrane. The membrane and the wall are intimately bound. The cell wall represents a dynamically forming exoskeleton which protects the fungal protoplast. Under stress

conditions which cause cell damage, the cell wall is repaired and remodeled through cell wall biosynthesis which requires an intact cell wall integrity pathway. In addition to being an entry barrier for antifungal components, the cell membrane dictates the entry of nutrients and the exit of metabolites and represents a selective barrier for their translocation. The cell membrane in *Aspergillus* is composed of a phospholipid bilayer interspersed with globular proteins and ergosterol as the major sterol instead of cholesterol which is found in the membrane of animals and phytosterols in plants (Pasanen et al., 1999). On the other hand, the cell wall is mainly composed of different polysaccharides (α-1,3-glucan, β-1,3-glucan, β-(1,3)-(1,4)-glucan, chitin, galactomannan and a polymer of galactosaminogalactan) with proteins and glycoproteins (de Groot et al., 2009; Gastebois et al., 2009; Johnston, 1965) (Fig 1). Both the membrane and fungal cell wall components are potential drug targets in fact they harbor most of the fungal antigens (Bernard and Latge, 2001). Knowledge on the mechanisms to maintain cell wall integrity in *Aspergillus* is extremely important for different reasons. Firstly although *Aspergillus* is one of the most abundant and important fungal genus, the function and maintenance of its cell wall is not fully understood. Secondly, this knowledge is the way to find more effective antifungal drugs with the cell wall/membrane integrity as target and will help to understand the pharmacology of the antifungal agents already discovered.

Much work has been carried out on the composition and integrity of the cell wall of ascomycetous fungi such as *Saccharomyces cerevisiae, Candida spp., and Schizosaccharomyces pombe*. (Bernard and Latge, 2001; de Groot et al., 2007; Klis et al., 2006; Latge et al., 2005; Lesage and Bussey, 2006). In *S. cerevisiae*, maintaining a proper cell wall architecture requires cell wall components such as β-1,3-glucan, β-1,6-glucan, chitin, and mannoproteins (Kapteyn et al., 1999; Klis, 1994). Its integrity signaling involves the mitogen-activated protein kinase (MAKP) cascade (Philip and Levin, 2001).

The aim of this review is to provide an overview of the composition of the membrane and the cell wall of the genus *Aspergillus*, and to elucidate the signal transduction cascade regulating their synthesis and the survival mechanisms to respond to the major antifungal compounds.

2.2. Cell membrane and wall constituents in Aspergillus: an overview of the composition

2.2.1. Cell membrane composition

The cell membrane in the genus *Aspergillus* and in most of the filamentous fungi comprises a phospholipid bilayer, sphingolipids and sterols. Phospholipids or phosphoglycerides constitute the major structural lipids in the membrane and are derivatives of glycerol 3-phosphate esterified to fatty acyl chains. Modifications of the phosphate group with alcohols further classify the phospholipids into phosphatidyl-choline, phosphatidyl-ethanolamine, phosphatidyl-inositol or phosphatidyl-serine. In addition, the phospholipid bilayer contains globular proteins. Sphingolipids are long-chain of fatty acids attached in an amide linkage to the amino group of sphingosine and amino alcohol. The various phospholipids and sphingolipids differ not only in the modification of the phosphate group, but also in the number of carbon atoms. Their composition is known to change during cell growth, cell differentiation and upon exposure to stresses (Sakai and Kajiwara, 2004). Previous studies have implicated the role of sphingolipids, particularly sphingosine and ceramide in fungal cell signaling and cytoskeletal organization (Futerman and Hannun, 2004). The major sterol in filamentous fungi is ergosterol instead of cholesterol in mammalian cells and phytosterols in plants (Pasanen et al., 1999). The amount of ergosterol varies between *Aspergillus* species and depends on the age of the culture, the developmental stage and the growth environment (Gessner and Chauvet, 1993; Newell, 1994; Schnurer, 1993). As a result of this variation, ergosterol is used as an estimate of fungal biomass under different growth conditions due to its correlation with the fungal dry mass (Matcham et al., 1985; Newell, 1994; Schnurer, 1993). The sterols and fatty acids of membrane of industrially important *Aspergillus* species (*A. niger*, *A. fumigatus*, *A. oryzae*, *A. flavus, A. nidulans* and *A. terreus*) have been studied in some detail (Birch et al., 1998; Nemec et al., 1997). The typically major fatty acids that occur in the cell membrane phospholipids and storage triacylglycerols of these *Aspergillus* species are palmitic and stearic acids with their unsaturated derivatives palmitoleic, oleic, linoleic and linolenic acids (Suutari, 1995). With its bilayer structure which prevents the free passage of most molecules into and out of the cell, the amount of cell membrane lipids also varies between species and differs according to the developmental processes and in response to environmental conditions (Suutari, 1995). For example, fatty acid unsaturation in *A. niger* increases when the temperature is lower than 26-20°C, particularly in the linolenic acid content (Suutari, 1995).

The amount of membrane proteins differs among species. Localized integrally or peripherally to the membrane, some proteins have been attached to carbohydrates in glycoproteins. The glycoproteins and the proteins transporters have important functions in the fungal cell physiology. The protein transporters are known to ensure transmembrane solute transport (Gustavo and Stephen, 2008). Depending on their energetic requirements, protein transporters are classified as primary active transporters, secondary active transporters and facilitators (channels) (Gustavo and Stephen, 2008). The first type of transporter catalyzes the transport of metabolites up to an electrochemical gradient using ATP hydrolysis. The secondary actives transporters catalyze the transport of substrates and most commonly an ion (Na^+, K^+, H^+) down an electrochemical gradient. Depending on the substrate and direction of the ions transport, the secondary actives transporters are called symporters (transport of the substrate and ions in the same direction) or antiporters (transport of the substrate and ions in the opposite direction) (Gustavo and Stephen, 2008). While active transporters need energy to be functional, protein facilitators mediate the transport of a solute across the cell membrane along its concentration gradient. It is estimated that in the genus *Aspergillus,* there are more than 600 genes which encode proteins catalyzing the transport of solutes and ions across the cell membrane (http://www.membranetransport.org). As a result, the genus *Aspergillus* is placed as the second on the list of eukaryotes which have more proteins transporters after *Cryptococcus neoformans* (Gustavo and Stephen, 2008). A comparative genomic of transporters of *A. oryzae*, *A. fumigatus* and *A. nidulans* revealed that the secondary transporters are the most required transporters in the membrane function (Table 1). Further data concerning comparative genomics of transporters can be obtained at http://www.membranetransport.org.

Table 1: Comparative genomic of transporters of three *Aspergillus* species (*A. oryzae, A. fumigatus*, *A. nidulans*) (http://www.membranetransport.org/)

	A.oryzae	*A. fumigatus Af293*	*A. nidulans FGSC A4*
Genome size (Mb)	32	32	31
Total transporters	934	600	683
No. of transporters per Mb genome	29.19	18.75	22.03
Primary active transporters (ATP-dependent)	108 (11.6%)	81 (13.5%)	81 (11.9%)
Secondary active transporters	794 (85%)	491 (81.8%)	570 (83.5%)
Facilitators (channels)	28 (3%)	23 (3.8%)	28 (4.1%)
Unclassified	3 (0.3%)	4 (0.7%)	4 (0.6%)

Glycoproteins in particular are a large and heterogeneous group of macromolecules and have a variety of functions in the cell membrane and cell wall. The cell membrane/cell wall proteins of filamentous fungi represent approximately 20-30% of the cell wall mass and are generally covalently bound to carbohydrates with N-linked and O-linked (Bowman and Free, 2006). Most glycosylation of *Aspergillus* cell membrane proteins are N-glycosylation and O-mannosylation, in which oligosaccharides are attached either to the β-amide group of an asparagine residue or to β-hydroxyl group of mainly serine and threonine residues (Deshpande et al., 2008; Esser and Bennett, 2004). The consequence of protein glycosylation, in particular on the membrane and cell wall is that glycosylation regions in the glycoproteins are relatively resistant to proteinases and may help to protect the peptide core. Such proteins function as enzymes, antibodies, hormones and structural or carrier receptors. They are able to mediate a cell's communication with the

outside word (Deshpande et al., 2008). As a consequence, they are considered to be a target for many antifungal compounds. The carbohydrates attached to the glycoproteins are species specific. The major carbohydrates in *Aspergillus* are galactomannans composed of galactose and mannose residues (Latge et al., 1994). In addition, some glycoproteins are modified through a phosphatidylinositol linkage, forming glycosylphosphatidylinositol (GPI) anchor (Bowman and Free, 2006). The GPI-anchored proteins contain a hydrophobic C-terminus sequence which acts as a GPI-anchored signal and a hydrophobic N-terminus sequence which is important for import into the ER (Damveld et al., 2005b). Many genes encoding for GPI-anchored proteins have been characterized in various ascomycetes such as *S. cerevisiae*, *Schizosaccharomyces pombe*, *C. albicans*, *A. nidulans* and *N. crassa* with 59, 28, 169, 74 and 87 genes respectively. Disruption of these genes profoundly affects the structure and function of the fungal cell membrane and cell wall (Bowman and Free, 2006; De Groot et al., 2005). At least seven genes encoding for a GPI-anchored proteins have been found in *A. niger* and disruption of *cwpA* (cell wall protein gene A) increased sensitivity to CFW (Damveld et al., 2005b).

2.2.2. Cell wall composition

The cell wall of *Aspergillus* and of most fungi is an essential extracellular organelle whose structural composition varies with the fungal morphotype and culture condition (Latge, 2007). The cell wall of *Aspergillus* differs significantly from the cellulose-base plant cell wall but is more similar to the cell wall of yeast-like fungi. In fact, it accounts for 20 to 40 % of the cellular dry weight and is comprised of glycoproteins and polysaccharides that can be divided into two groups discriminated by their solubility in hot alkali (Fontaine et al., 2000). The first group is composed of the alkali soluble fraction which is mainly α-1,3-glucan and some galactomannan. The second group which is represented by the alkali insoluble fraction is the group of polysaccharides responsible for cell wall rigidity and is composed by β-1,3-glucan and chitin. The glycoproteins, glucan and chitin constitute the structural basis of the cell wall and are cross-linked together, forming a complex network (Fig 1).

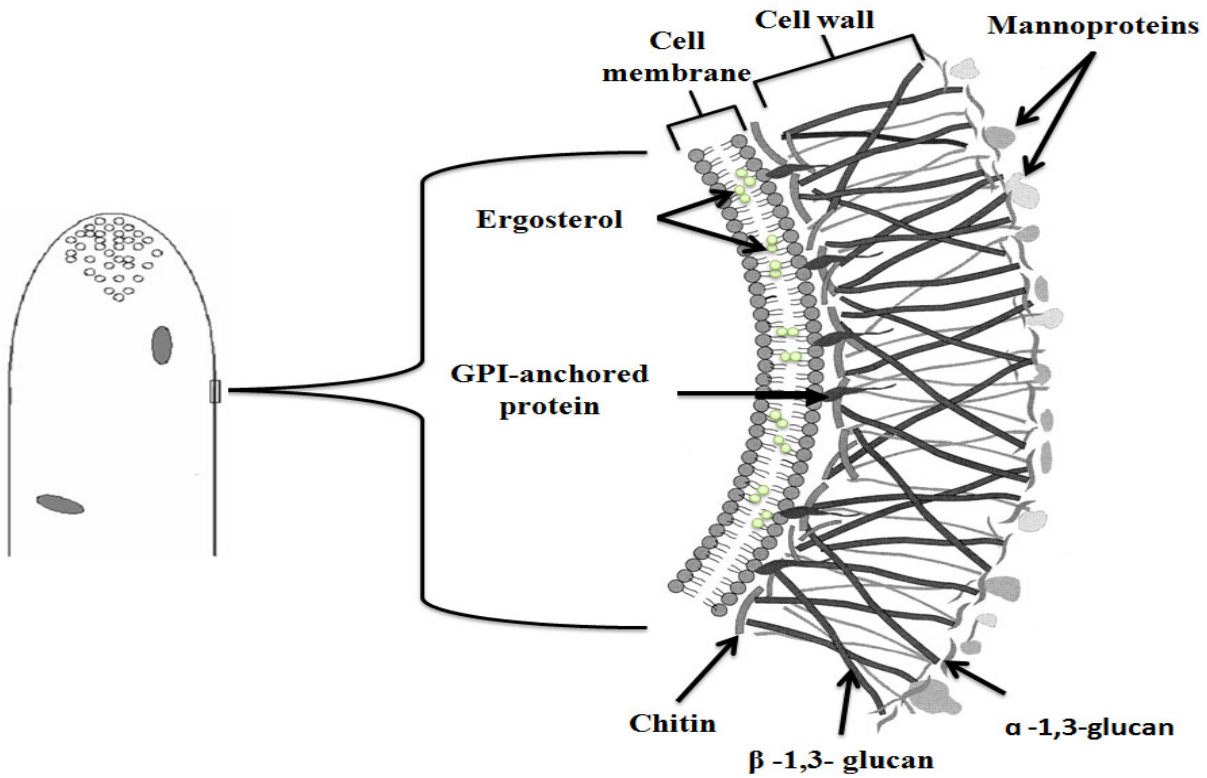

Figure 1: Proposed cell membrane and cell wall structure in *Aspergillus sp*. after (Selitrennikoff, 2001).

2.3. β-1,3-glucan

β-1,3-glucan forms the central core and inner bilayer of the *Aspergillus* cell wall architecture (see Fig 1). Moreover, it represents 25 to 35 % of the total polysaccharide contained in the cell wall (Gastebois et al., 2009). The synthesis of β-1,3-glucan in *Aspergillus* is catalyzed by the β-1,3-glucan synthase like in the yeast *S. cerevisiae* through transcription of the gene FKS. However, in contrast to *S. cerevisiae* in which three FKS genes have been found (FKS1, FKS2 and FKS3), *Aspergillus* species contain one FKS orthologue which is essential (Mouyna et al., 2004). FKSA is a plasma membrane-bound protein (> 200 kDa) and uses UDP-glucose on the membrane intracellular side as a substrate to form the β-1,3-glucan chains which is transferred into the cell wall space (Latge, 2007). The β-1,3-glucan synthesis is regulated by the GTPase Rho1 protein whose mechanism is well studied in *A. fumigatus* (Beauvais et al., 2001; Lesage and Bussey, 2006). The FKSA protein in *Aspergillus* is highly conserved (*A. fumigatus* FKSA amino-acid identity and conservation of the intron positions are 98 % , 92 % and 91 % respectively to *A. lentulus*, *A. clavatus* and *A. terreus*; 89 % to *A. flavus* and *A. oryzae*; 88 % to *A.*

niger and *A. nidulans*) (Bernard and Latge, 2001; Mount, 2007). The β-1,3-glucan polymer is branched to a linear β-1,4-glucan, conferring more robustness to the cell wall. In addition to the β-1,4-glucan links, the cell wall of *A. fumigatus* and *A. niger* are known to have β-1,6-glucan linked to β-1,3-glucan in a similar way as described for the ascomycetous yeasts *C. albicans* and *S. cerevisiae* (Brul et al., 1997; De Groot et al., 2005; Gastebois et al., 2009). This link has not yet identified in other *Aspergillus* species. However, the update of *A. nidulans* genome annotation reveals the presence of gene (AN8444, designated celA) whose product is similar to plant cellulose synthases and is hypothesized to be responsible for the production of β-1,3/β-1,4-glucan in this species (Wortman et al., 2009). A link between β-1,3-glucan and other cell wall polysaccharides such as chitin has also been proven in some *Aspergillus* (Fontaine et al., 2000). These covalent linkages occurring between the β-1,3-glucans and between the other cell wall components are catalysed by glucanosyltransferases (Goldman et al., 1995). A family of at least seven related 1,3-β-glucanosyltransferases (*GEL1-7*) has been found in *A. fumigatus* (Gastebois et al., 2010).

2.4. Chitin

Chitin is a long linear homopolymer of β-1,4 linked N-acetylglucosamine. Chitin synthases are responsible for chitin synthesis and use UDP-N-acetylglucosamine as a substrate. The enzyme is integrally located in the membrane. Chitin accounts for only 1-2% of the dry weight of the yeast cell wall, however the cell wall of *Aspergillus sp*. contains 10-20% chitin (Bartnicki-Garcia, 1968; de Nobel et al., 2000). It contributes enormously to the overall integrity of the cell wall because when its synthesis is disrupted, the wall becomes malformed and unstable (Shaun and Stephen, 2006; Specht et al., 1996). Multiple chitin synthases reported to be involved in septum synthesis, lateral wall biosynthesis and spore formation were identified in *Aspergillus sp.* (Bulawa, 1993; Munro and Gow, 2001; Roncero, 2002). This multiplicity is certainly due to the different roles that the enzymes play in the physiology of the organism. The chitin synthases in *Aspergillus* species are generally classified into seven classes according to the similarities in the peptides sequences (Choquer et al., 2004) (Table 2). The high amount of chitin content in *Aspergillus sp.* when compared to *S. cerevisiae* is probably due to the presence of more chitin synthase genes in the first organisms (only 3 classes found in *S. cerevisiae*) (Table 2). In addition,

the chitin synthases belonging to classes III, V, VI and VII are only present in filamentous fungi but not found in yeast, hinting at their possible role in hyphal tip growth (Horiuchi, 2009).

Table 2: The different chitin synthase classes homologous in *Aspergillus* species (Bulawa and Osmond, 1990; Choquer et al., 2004; Wang et al., 1999)

Class	*A .nidulans*	*A. fumigatus*	*A. niger*	*A .terreus*	*A .oryzae*	*A .clavatus*	*A. flavus*	*S. cerevisiae*
I	ChsC	ChsA	An07g05570	Chs2	AO090011000449	ChsA	ChsA	Chs1
II	ChsA	ChsB	An14g00660	ChsA	AO090206000079	ChsB	ChsB	Chs2
III	ChsB	ChsG	An09g04010	ChsG	AO090701000589	ChsG	ChsG	-
IV	ChsD/ChsE	ChsF	An09g02290	ChsD	AO090005000579	ChsF	ChsF	Chs3
V	CsmA	ChsE	An02g02360	Chs 6	AO090026000323	ChsE	ChsE	-
VI	CsmB	Afu2g13430	An02g02340	Chs6	AO090026000321	ACLA 072240	AFL2G 06937	-
VII	ChsG	ChsD	An08g05290	ATEG 00451	AO090113000128	ChsD	ChsD	-

2.5. α- 1,3-glucan

α-1,3-glucan is the main component of the alkali-soluble fraction of *Aspergillus* cell wall and in most of filamentous fungi. It is present at level of 9 to 46 % of the cell wall of Ascomycetes and Basidiomycetes but absent in *S. cerevisiae* and *C. albicans.* In contrast to β-1,3-glucan and chitin which are located on the inner layer of the cell wall, α -1,3-glucan is mainly localized on the outer layer of the cell wall (Beauvais et al., 2007). The chemical structure of α-1,3-glucan in *Aspergillus sp*. contains a small percentage of (1,4)-linked residue in particular in the cell wall of *A. niger* (Bobbitt et al., 1977). However, in contrary to β-1,3-glucan, which is known to play an essential role in the morphology and integrity of the cell, little is known about the function and biosynthesis of α-1,3-glucan in the majority of *Aspergillus* species. Some studies have been performed on the structure and biosynthesis of *A. fumigatus* α-1,3-glucan, hypothesising that it is probably implicated in the virulence of this pathogenic *Aspergillus* (Beauvais et al., 2005). In fact, due to the peripherical localization of the protein, it has been postulated to be a potential cell wall antigen (Beauvais et al., 2005).

Two high homologous genes, *ags1* and *ags2* which are responsible for α-1,3-glucan synthesis have been found in *A. fumigatus* (Beauvais et al., 2005). However, a family of five α-1,3-glucan synthase-encoding genes (*agsA-E*) has been identified in *A. niger* (Damveld et al., 2005c). Deletion of *ags1* and *ags2* in *A. fumigatus* demonstrated their role in cell polarity organization and conidia formation (Beauvais et al., 2005). The role of α-1,3-glucan in the maintenance of cell wall integrity has been verified for *A. nidulans* in which α-1,3-glucan mutant strains show an increased level of β-1,3-glucan in the cell wall (Polacheck and Rosenberger, 1977). This role has been proved in *A. niger* in which an induction of the α-1,3 glucan synthesis has been observed during cell wall stress (Damveld et al., 2005c; Hagen et al., 2007).

2.6. Galactomannan (mannoproteins)

First isolated from the culture medium and in serum of patients with aspergillosis infection, galactomannan is frequently found in alkali-soluble fractions of the mycelial wall (Hearn and Sietsma, 1994). Although galactomannan is used as a major antigen of *Aspergillus sp*. during aspergillosis diagnosis, little is known about its biosynthesis. It has only been proved that a coordinated action of mannosyl-and galactosyltransferases is required to synthesize the linear α-mannan backbone and the galactofuranose residues (Gastebois et al., 2009). Galactomannan is found to be linked to β-1,3-glucan in *A.fumigatus*, indicating its involvement in the cell wall organization. Additional binding has been found between galactomannan and glycosylphosphatidylinositol (GPI) in the cell wall and it could be involved in the plasma membrane-cell wall interactions as well (Fontaine et al., 2000).

General overviews of hyphae cell wall composition show some differences in comparison to the cell wall of spores. The outer layer of *Aspergillus* conidia is in addition mainly characterized by the presence of hydrophobic proteins (hydrophobins), conferring hydrophobic propriety to *Aspergillus* conidia (Bernard and Latge, 2001). Glycoprotein and dense pigmented components called melanin have additionally been characterized (Latge, 1999). Melanin plays an important role in the survival and longevity of fungal conidia. In addition, it is an important virulence factor for animal- and plant-pathogenic fungi (Tsai et al., 1999). At least six genes encoding for melanin have been identified in the pathogenic species *A. fumigatus* (Bernard and Latge, 2001).

2.7. Signaling mechanisms to ensure membrane and cell wall integrity

All living cells are challenged by environmental changes and have to respond to threatening conditions to survive. Maintaining membrane and cell wall requires action of cell wall integrity (CWI) signaling pathways. First, membrane receptors, sense cell surface stress and transfer the signal to effectors which collectively regulate diverse processes that lead to the expression of genes related to the membrane and the cell wall biogenesis (Levin, 2005). *S. cerevisiae* is the fungus where CWI pathways have mostly been studied. In this organism, CWI signal is a *mitogen activated protein kinase* (*MAPK*) signaling cascade resulting in the activation of the Rlm1p transcription factor which regulates the transcription of at least 25 genes implicated in cell wall biogenesis and functions (Jung and Levin, 1999). The transduction cascade from the external signal to catalyze cell membrane and wall biogenesis is conserved between all eukaryotes via a conserved module composed of these protein kinases (Levin, 2005). In *Aspergillus* where the membrane and cell wall composition is close to *S. cerevisiae*, MAPK are also the central elements for signal transduction which act sequentially, using cascade-like mechanisms which maintain the membrane and cell wall integrity. Another signaling system whose importance should be noted for cell membrane integrity in *Aspergillus* is the c-AMP-mediated PKA which is involved in the regulation of membrane lipid biosynthesis. An interaction system between c-AMP/PKA, MAP kinase pathway and G protein signaling has been demonstrated (Kronstad et al., 1998; Levin, 2005). Therefore, the MAPK kinase pathways and c-AMP/PKA system, together control cell wall/cell membrane integrity through a signal cascade in *Aspergillus*. This cascade mechanism starts on the surface of the cell through the cell-surface receptors which are composed of three main families: The ions-channel-linked receptors, the G-protein-linked receptors and the enzyme-linked receptors (Li et al., 2007). The ion channels are proteins that form macromolecular pores in the cell membranes. They open and close to control the passage of ions across the cell membrane and they sense and respond to signals. The G-proteins are the most studied receptors in fungi and are localized on the inner membrane surface and act as transducer receptor-activated signals into intracellular responses that underlie membrane and cell wall genes responses. In *A. nidulans*, nine putative G-protein receptors encoding genes, designated GprA to GprI were identified and GprD was found to be involved in fungal asexual development (Han et al., 2004). In silico analysis revealed the presence of 15 G-protein receptors encoding genes in *A. fumigatus*. In this species, deletion of GprC and GprD causes growth and germination defect

(Gehrke et al., 2010; Lafon et al., 2006). This demonstrated the importance of the receptors G-protein in the maintaining of *Aspergillus* cell integrity. The enzyme linked receptors manifest a slow and more complex molecular mechanism but can achieve a great amplifying signal effect. The initiation of the membrane and cell wall integrity signaling events relies primarily on interactions between the three different effectors and different types of molecules that can serve as extracellular signals.

As the MAPK kinase cascade is the main signal route to maintain the membrane and cell wall integrity, it should mediate cell wall biogenesis and remodeling, necessary to maintain the cell membrane/cell wall integrity in *Aspergillus*. Four genes have been found to encode MAPKs in *Aspergillus* species. They are known as *mpkA*, *mpkB*, *mpkC* and *sakA/hogA* in *A. fumigatus* (May et al., 2005; Reyes et al., 2006; Valiante et al., 2008). Blastp analysis shows a closer relationship in comparison to the amino acid sequence of MAPKs between *A. fumigatus* and some other *Aspergillus species* (*A. nidulans*, *A. niger*, *A. terreus*, *A. oryzae*, *A. clavatus*, *A. flavus*). The MAPK family of kinases connects extracellular stimuli through the cell surface receptors with diverse cellular responses including the membrane and cell wall maintainance (Widmann et al., 1999). Among the four MAPK genes, *mpkA* which is a counterpart of *S. cerevisiae mpk1/slt2*, is the central module of the MAPKs family in which the signal is made via Pkcp (Beauvais et al., 2001; Fujioka et al., 2007; Ichinomiya et al., 2007; Teepe et al., 2007) (Fig 2). This pathway functions in a cascade of kinases, sending signals from one molecule to another, thereby amplifying the signals. Briefly, an intracellular signal, created from upstream activators responding to a receptor-ligand interaction activates the central module which phosphorylates a serine and threonine residue in a conserved amino-terminal domain of the MAP kinase, which in turn adjusts membrane and cell wall genes expression (Bussink and Osmani, 1999; May et al., 2005; Valiante et al., 2008). Only few transcription factors and target genes of CWI pathways are known in filamentous fungi (Fujioka et al., 2007). In *A. nidulans* in which the role of MpkA in cell wall integrity signaling has been investigated, it has been shown that the transcription of *mpkA* is autoregulated by CWI signaling via MpkA but not RlmA or by AnSwi4-AnSwi6 (Fujioka et al., 2007). However, in *A. niger,* RlmA is required for cell wall reinforcement in response to cell wall stress (Damveld et al., 2005a). Furthermore, the activation of the CWI signaling pathway through the small G protein RhoA via PkcA with the MAPK as a downstream target is also demonstrated in *A. nidulans*. Mutations of *rhoA* tend to affect cell wall integrity, and

its hyper activation result in a thickened cell wall and an increased chitin levels in the cell wall (Guest et al., 2004). *RhoA* plays a central role in fungal polarity and viability in *A. niger* (Kwon et al., 2011). Additionally, (Valiante et al., 2008) have shown that the *A. fumigatus* MpkA controls the cell wall integrity in an oxidative stress response. These findings indicate the important role of MpkA signaling to ensure the membrane and cell wall integrity in *Aspergillus.* To further reinforce this argument, (Mizutani et al., 2004) have analyzed the CWI pathway in *A. oryzae by* using a morphological mutant in which the *kexB* gene, which encodes a subtilisin-like processing enzyme similar to *S. cerevisiae* Kex2p, was disrupted. The gene expression profiles of the Δ*kexB* shows a significant increase of MpkA and cell wall related genes encoding β-1,3-glucanosyltransferases and chitin synthases, which is presumably due to CWI through the high level of MpkA expression (Mizutani et al., 2004). Apart from its involvement in membrane and cell wall integrity, MpkA also controls the germination of conidial spores and polarized growth in *A nidulans* (Bussink and Osmani, 1999). However, studies have shown the existence additional of the CWI signaling mechanisms parallel to MpKA and RlmA (Fujioka et al., 2007).

In addition, evidence for crosstalk between the MAPK and the calcium-calcineurin pathways has previously been proved (Muller et al., 2003), showing the eventual role of the calcium-calcineurin pathway in *Aspergillus* cell wall and the membrane integrity signaling. In *A. nidulans*, the deletion of *CrzA*, the *CRZ1* orthologue in *S. cerevisiae* leads to alteration of the fungal cell, which has for result an extreme sensitivity to alkaline pH and Ca^{2+} (Spielvogel et al., 2008). Recently, involvement of *CrzA* in cell wall gene synthesis has been proved in *A. niger,* reinforcing the role of calcium-calcineurin pathway in cell wall integrity signaling (Ouedraogo et al., 2011).

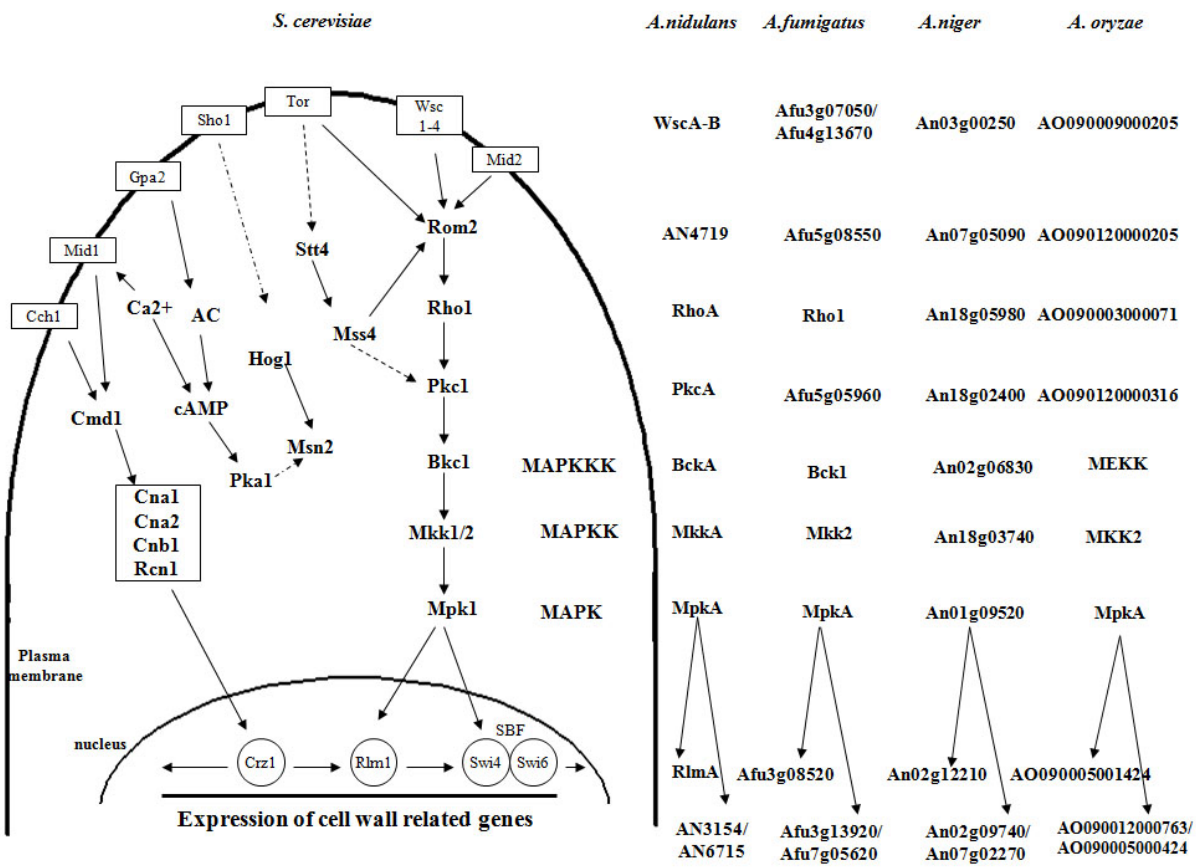

Figure 2: *Aspergillus* CWI pathway based on *S. cerevisiae* and *Aspergillus* genome information (after (Fujioka et al., 2007)). The putative orthologous proteins involved in CWI signaling including the MAPK signaling pathway in *Aspergillus* and *S. cerevisiae* are shown. Through upstream activators, the MAP kinase kinase kinase (MAPKKK) is activated. This activation will phosphorylate a MAP kinase kinase (MAPKK) through signal cascade which in turn phosphorylates the MAPK. The downstream target of the signal cascade is a transcription factor which will adjust gene expression to respond to cell wall and membrane integrity maintaining requirement. Other signaling pathways in *S. cerevisiae* in interaction with the CWI and also found in *Aspergillus* to safeguard cell integrity are represented (Ouedraogo et al., 2011).

2.8. Survival response of Aspergillus against cell-surface acting compounds

Survival response of *Aspergillus* against cell wall/cell membrane active antifungals requires four main mechanisms: efflux of the antifungal compounds through the membrane, an strengthening of the cell membrane and cell wall through synthesis of structural components (Lesage and Bussey, 2006; Levin, 2005), overexpression of the target enzyme to stop the antifungal action and remodeling of the cell wall and membrane constituents through mutation of the target component

gene in order to prevent the binding with the antifungal compound (Eleusa et al., 2007; Garcia-Rodriguez et al., 2000).

Current antifungal compounds directed against the major components of the cell wall include echinocandin and nikkomycin (Christine et al., 2000). Echinocandins are antifungal compounds which are currently evaluated in clinical trials. The first echinocandin approved for clinical use was caspofungin, followed by anidulafungin and micafungin (Louise et al., 2010). They interfere noncompetitively with the cell wall synthesis by inhibition of the β-1,3-glucan synthase. The human pathogens *A. fumigatus*, *A. terreus* and *A. flavus* have comparable susceptibilities to caspofungin, anidulafungin and micafungin which might be a consequence of similarity in their cell wall composition (Charalampos et al., 2008). Echinocandin acts particularly by lysing the hyphal tip, blocking the nascent cell wall synthesis and provoking inhibition of the cell growth (Louise et al., 2010; Meyer et al., 2007). A study of the survival response to caspofungin has been done for *A. niger* and shows that the group of genes involved in cell wall biogenesis and maintenance such as *MKKA* was up-regulated in the presence of the compound (Meyer et al., 2007). Furthermore, it has become evident that overexpression of genes encoding cell wall components in response to echinocandin is a general survival response of *Aspergillus* as this mechanism is not only found in *A. niger* but also in *A. fumigatus*. In this species, mutants where the cell wall was affected indicated increased expression of 28 genes involved in cell wall biogenesis enzyme and signal transduction (Gardiner et al., 2005). Another survival response mechanism of *Aspergillus* against echinocandin is a mutation of the gene *fksA*. Fks1 mutations have been shown to confer also resistance in yeast against echinocandins, which may suggest that this mechanism is pervasive in the fungal kingdom (Douglas et al., 1997; Kurtz et al., 1996).

The nikkomycin antifungal drug is produced by *Streptomycetes* and competitively inhibits fungal chitin synthase enzymes. It acts as an analog of the chitin synthase substrate UDP-GlcNAc. The in vivo activity of nikkomycin has been proved against *Candida* species.

However, *Aspergillus spp* are usually resistant to this agent and clinically usefulness of nikkomycin against *Aspergillus* is only possible in combination with other antifungal drugs such as fluconazole or itriconazole (Damveld et al., 2005c; Li and Rinaldi, 1999; Meyer et al., 2007; Vincent, 1999). The mechanism of this natural resistance is not clear yet. One speculation is that

Aspergillus species are able to synthesize more chitin in the cell wall in response to the inhibitor of chitin (Hector et al., 1991).

It has been demonstrated that the fluorochrome dyes calcofluorwhite (CFW) and congo red (CR) exert affinity to chitin and β-(1,3) glucan (Wood, 1980). Both CFW and CR act in the cell wall by binding to nascent chitin chains, thereby inhibiting the enzymes which connect chitin to β-(1,3) glucan and β-(1,6) glucan (Ram et al., 2004). The binding of CFW and CR to the cell wall in *A.niger* results in swelling and lysis of the hyphal, activating cell wall integrity (Damveld et al., 2005a; Damveld et al., 2005c). Whereas, echinocandins and nikkomycin are used to treat fungal infection, the fluorochrome dyes are rather used for characterization of cell wall defective mutants (Ram and Klis, 2006).

The cell wall glucan of *Aspergillus* includes α-1,3-glucan which is not found in the yeast *S. cerevisiae* and *C. albicans* (Beauvais et al., 2005). The *agsA* gene encoding for α-1,3-glucan has been found to be strongly induced in the presence of CFW, suggesting the involvement of α-1,3-glucan synthesis in securing or reinforcing cell wall integrity (Damveld et al., 2005c). There is evidence that a reinforced cell wall is necessary to counteract CFW or CR, in *A. niger*, *A. nidulans*, *and A. awamori* (Damveld et al., 2005a; Damveld et al., 2005c; Oka et al., 2005; Shaw and Momany, 2002). In addition, an increased susceptibility of CFW and CR in cell wall defect strains was also demonstrated in other fungi such as *S. cerevisiae*, *C. albicans* and *Yarrowia lipolytica* (Popolo and Vai, 1998; Roncero et al., 1988; Ruiz-Herrera et al., 2003), highlighting the importance of an intact cell wall and membrane integrity to sufficiently counteract the antifungal compounds.

Many antifungal compounds targeting the cell membrane have been developed based on their activity against ergosterol, the principal constituent of *Aspergillus'* cell membrane. Two major classes of compounds (azole and polyene) are known to target ergosterol.

Azole antifungal agents contain two or three nitrogens in the azole ring and are classified as imidazoles (keteconazole and miconazole) or triazoles (itraconazole, ravuconazole, voriconazole and posconazole), respectively (Christine et al., 2000). They interact with the membrane by inhibiting the synthesis of ergosterol. In particular, cytochrome P-450 C14 α-demethylase (cyp51A) which promotes the conversion of lanosterol to ergosterol is the molecular target of azoles. They exhibit a large spectrum of activity against important fungal pathogens such as

Aspergillus and *Candida* (Clancy and Nguyen, 1998; Macro et al., 1998). Polyenes are clinically useful antifungal drugs with a broad spectrum activity against filamentous fungi, including *Aspergillus*. The most important polyenes are amphotericin B, nystatin and natamycin. They bind through hydrogen bonds or van der waals forces to the cell membrane ergosterol and lead to the formation of aqueous channels, increasing the membrane permeability to univalent and divalent cations (Beauvais and Latge, 2001; Christine et al., 2000). Recently, their oxidative damage has also been demonstrated which may also contribute to their fungicidal effect (Brajtburg et al., 1990).

Gene mutation as a response mechanism in *Aspergillus* has been shown for the antifungal compound azole, which, very often consists of point mutations in the cyp51A, known as an antifungal azole target (Verweij et al., 2009). Cyp51A encodes for the enzyme responsible for the biosynthesis of ergosterol and mutation in the amino acid composition of Cyp51Ap leads to modification of the azole target site, thereby an unrecognized target by the antifungal. Although there are two genes, Cyp51A and Cyp51B, which code for ergosterol in *Aspergillus sp*, almost all of those mutations were restricted to Cyp51A (Krishnan-Natesan et al., 2008). However, the mechanisms of the origin of these mutations are not well known yet. The hypothetic reasons come from the spontaneous mutations or from the repeated exposure of the strains to the antifungal compounds (Qiao et al., 2008). Azole antifungal resistances have been studied in several other species of fungi and include changes in the cellular content of azole. In *C. albicans* for example, a survival response to azole is described by a reduction of azole accumulation through active efflux of drug or overexpression of the target that lead to the accumulation of less toxic sterols (Lamb et al., 1999; Sanglard et al., 1998; White et al., 1998). In this organism, at least five different genes (CDR1 to CDR5) have been described to be involved in drug efflux and two genes CDR1 and CDR2 are overexpressed when *C. albicans* is exposed to the antifungal (Sanglard et al., 1998). Increased efflux as evidence of reduced azole intracellular accumulation has been described in *A. fumigatus* clinical and laboratory strains, followed by an overexpression of the genes AfuMDR3 and AfuMDR4 (a class of transporter) (Slaven et al., 2002). However, the understanding of the role of these transporters in a survival response of *Aspergillus* against azole require further study as their overexpression usually co-occurs with mutations in the gene encoding for the target of the antifungal (da Silva Ferreira et al., 2004).

As regards to the survival response of *Aspergillus* to polyene little is known about the mechanism. Most studies on survival response to polyene come from the non pathogenic yeast *S. cerevisiae* and in the pathogenic yeasts *Cryptococcus neoformans* and *C. albicans* and they are related to mutations in the ergosterol synthesis pathway (Kelly et al., 1994; Kontoyiannis and Lewis, 2002; Sterling and Merz, 1998; Warnock et al., 1999). However, it is known that polyene also acts in *Aspergillus* by interfering with ergosterol; the survival response against this antifungal could also due to mutations in ergosterols biosynthesis pathway, resulting by a diminution in the binding of the compound to the target. On the other hand, a survival response of *Aspergillus* to polyene could also be to reinforcement of the cell wall and membrane by cell wall proteins to prevent the entry of the antifungal at the cell membrane/cell wall level. *A niger* exposed to fenpropimorph, an antifungal compound which inhibits ergosterol synthesis shows increased expression of genes responsible for membrane reconstruction, lipid signaling, oxidative stress and cell wall remodeling (Meyer et al., 2007).

Nowadays, fungal infections are becoming more prevalent and antifungal agents of fungal origin such as the antifungal protein (AFP) secreted by *Aspergillus giganteus* and the antifungal protein (PAF) from *Penicillium chrysogenum* bear great potential for future use as they are not toxic to mammalian cells (Marx et al., 2008; Meyer, 2008). AFP and PAF have been shown to disturb membrane integrity in filamentous fungi but show different specific mode of action. PAF provokes a rapid hyperpolarization of the cell membrane at the hyphal tips, increases the efflux of ions as K+ (Binder et al., 2010a). In contrast, AFP provokes membrane permeabilization, inhibits chitin synthase and induces cell wall integrity pathway in *A. niger* (Hagen et al., 2007).

More knowledge is available concerning the survival response of *Aspergillus* and other filamentous fungi against AFP in comparison to PAF (Binder et al., 2010a; Hagen et al., 2007). A survival response against AFP has been studied in *A. niger* and resulted in increased expression of the *agsA* gene, encoding an α-1,3-glucan synthase, thereby reinforcing the fungal cell wall (Hagen et al., 2007). Nevertheless, an induction of the cell wall integrity pathway in the sensitive filamentous fungi such as *Aspergillus* seems to be inadequate to fully counteract AFP. It has recently been demonstrated that mutants yeast strains which are moderately sensitive to AFP respond by a concerted action of several signaling pathways (Ouedraogo et al., 2011). Calcium signaling, TOR signaling, cAMP-PKA signaling and CWI signaling are together involved in the protection of *S. cerevisiae* against AFP. A concerted survival mechanism also seems to be used

by the resistant filamentous fungi *P. chrysogenum* to counteract AFP as has been shown by increased chitin level similar to the moderately sensitive mutant yeast in the presence of AFP (Ouedraogo et al., 2011). The survival response against PAF has recently been investigated and it has been shown that PAF activates the cAMP/PKA signaling cascade but has no significant effect on the transcription levels of cell wall remodeling enzymes (Binder et al., 2010b), suggesting that both proteins trigger different responses most probably because they target different cellular processes.

2.9. Conclusions

The survival response of *Aspergillus* species against antifungal compounds can then be summarized in four important mechanisms (Fig 3): (i) the reinforcement and remodeling of the cell membrane/cell wall level through signaling pathways to prevent or stop the binding and entry of the antifungal compound; (ii) the overexpression of the target enzyme so that the antifungal does not inhibit the biochemical reaction anymore; (iii) the alteration of the antifungal target by mutation to avoid the binding with the antifungal compound; (iv) the antifungal compound is pumped out by an efflux pump. All these mechanisms should be adequately performed to effectively protect the fungal cell. A too weak response results in a susceptibility of the fungus towards the antifungal, and a too strong response can disturb the fungal metabolism and lead to self cell damage and death (Casadevall and Pirofski, 1999; Ouedraogo et al., 2011) (Fig 4 and chapter 4).

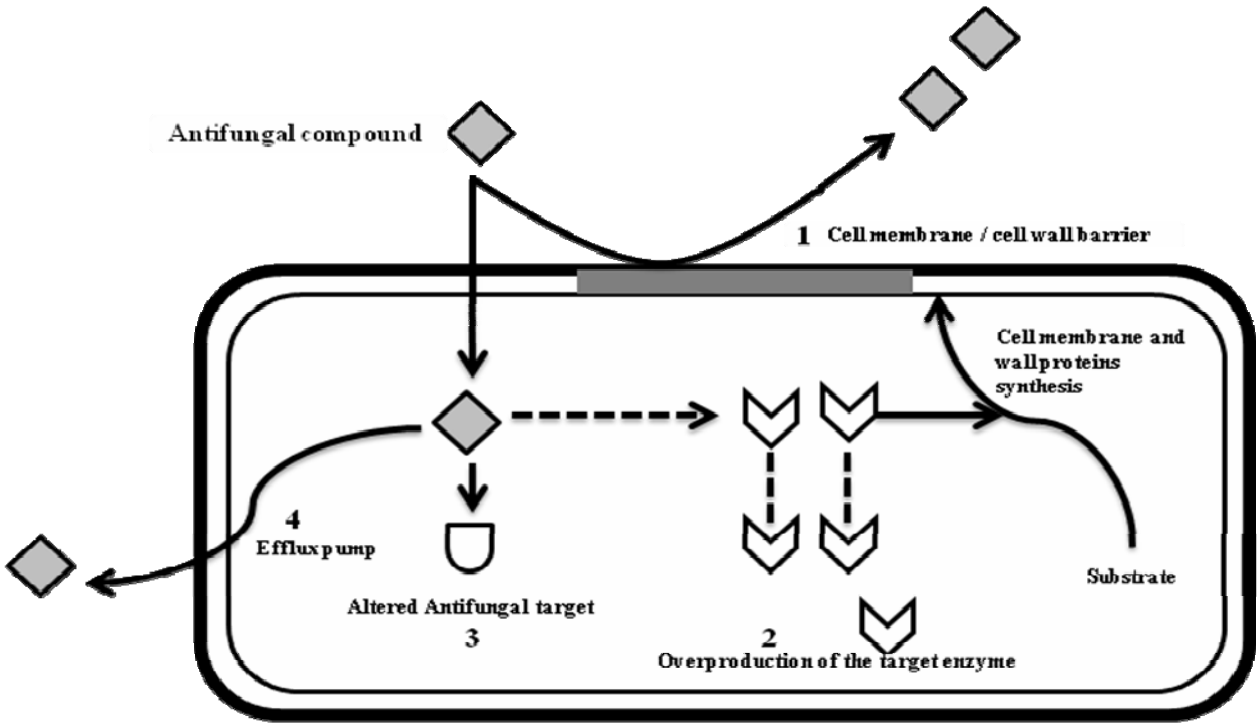

Figure 3: Different survival strategies by which *Aspergillus* can respond to antifungal compounds. **1**. The reinforcement and remodeling of the cell membrane/cell wall level through the cell membrane and cell wall integrity signaling to prevent or stop the binding and entry of the antifungal compound. **2**. Overexpression of the target enzyme so that the antifungal does not inhibit the biochemical reaction completely. **3**. Alteration of the antifungal target by mutation to avoid the binding with the antifungal compound. **4**. Antifungal compound is pumped out by an efflux pump.

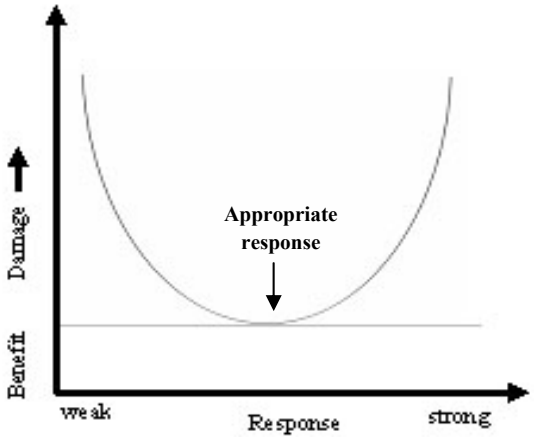

Figure 4: Damage-response framework of fungal-antifungal interaction (after (Casadevall and Pirofski, 1999; Ouedraogo et al., 2011)). The survival of the fungal cell is dependent on the response mechanism to an antifungal compound. This response can be weak or too strong. In both cases the fungal cell is damaged or dies. Only an appropriate response allows the fungal cell to survive in the presence of the antifungal compounds.

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Chapter 3

Functional characterization of *A. niger* class III and class V chitin synthases and their role in cell wall integrity

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Abstract

Class III and V chitin synthases play an important role in morphogenesis and cell wall integrity in many filamentous fungi. However, their function in the filamentous fungus, *A. niger* has not yet been elucidated. To address this, deletion mutants of class III and V chitin synthase-encoding genes of *A.niger*, *chsB* and *csmB*, and their role in cell wall integrity have been studied. Deficiency in conidiation and abnormal swollen conidiophores have been observed in *chsB* and *csmB* deletion mutants. Using cell wall inhibitor reagents, it was shown that the mutants are hypersensitive towards cell wall stress. However, there are differences between them as regards susceptibility to the antifungal protein AFP. These results suggest that ChsB and CsmB play an important role during asexual development and in ensuring cell wall integrity of *A. niger*. Interestingly, the data indicate that only chitin synthase *csmB* is important to counteract AFP inhibitory effects.

3.1. Introduction

Chitin is a β-1,4 linked linear polymer of N-acetylglucosamine present in the cell wall of fungi. The homopolymer is one of the major structural components of the fungal cell wall and contributes to structural rigidity and osmotic integrity of the wall (Borgia et al., 1996; Ichinomiya et al., 2002). Chitin synthases encoded by *chs* genes vary from yeast to filamentous fungi. Studies have shown that chitin synthesis in the yeast *Saccharomyces cerevisiae* requires three related genes which encode chitin synthase isozymes (Bulawa, 1993). The three isozymes are temporally and spatially regulated and play an important role in morphogenesis during fungal growth and development. Like *Saccharomyces cerevisiae*, *Candida albicans* harbors three classes of chitin synthases: class I (CaChs2p and CaChs8p), class II (CaChs1p) and class IV (CaChs3p) (Bulawa et al., 1995; Gow et al., 1994; Mio et al., 1996; Munro et al., 2003). In contrast to yeasts, filamentous fungi contain more chitin synthase genes which indicate that they are more complex in function (Bartnicki-Garcia, 1968). In recent years, more information about fungal chitin synthase gene has emerged, resulting in their subdivision into two families. The first family is subdivided into classes: I, II and III. The second family includes classes IV and V (Roncero, 2002). Class I, II and IV chitin synthase genes are present in both yeast and filamentous fungi. However classes III and V are only found exclusively in filamentous fungi (Roncero, 2002). Several other class V chitin synthase genes have been isolated and found to encode proteins

containing a myosin motor-like domain in their amino-terminal part. Some authors therefore propose the classification of class V chitin synthases into two groups. One group, named class V subgroup A, comprises chitin synthase with a larger version of the myosin motor-like domain containing an ATP-binding site motif (Amnuaykanjanasin and Epstein, 2003; Martin-Urdiroz et al., 2008; Roncero, 2002). The second group named class V subgroup B, or class VI or class VII, depending on which authors, includes the chitin synthase with a shorter myosin motor-like domain lacking ATP binding site motifs (Amnuaykanjanasin and Epstein, 2003; Chigira et al., 2002; Choquer et al., 2004; Nino-Vega et al., 2004). The myosin motor like-domains are exclusive to filamentous fungi and are not only important for the maintenance of cell wall integrity, but are also involved, directly or indirectly, in pathogenicity (Garcera-Teruel et al., 2004; Liu et al., 2004; Madrid et al., 2003; Martin-Urdiroz et al., 2008; Weber et al., 2006). They are also able to bind to actin (Takeshita et al., 2005; Takeshita et al., 2006). *Aspergillus fumigatus* contains seven chitin synthase genes (Mellado et al., 1995). At least five chitin synthase genes have been isolated in *A. nidulans* and in *Neurospora crassa* (Motoyama et al., 1994; Takeshita et al., 2006; Yarden and Yanofsky, 1991). The role of the different chitin synthase families in fungal wall integrity and fungal growth has been studied in detail in *A. fumigatus* and *A. nidulans*. Disruption of chitin synthase D (encoding a class V chitin synthase) and chitin synthase E (encoding a class IV chitin synthase) in *A. nidulans* results in chitin deficiency and cell wall integrity disruption (Specht et al., 1996). Moreover, the deletion of *chsB* (which encodes a class III chitin synthase) in *A. nidulans* results in mutants characterized by very slow growth, small colonies and highly branched hyphae (Borgia et al., 1996; Yanai et al., 1994) (Table 1). A similar phenotype has been found in *N. crassa* after inactivation of the gene encoding *chs1*; a class III chitin synthase highly similar to *chsB* in *A. nidulans* (Yarden and Yanofsky, 1991). *A. fumigatus* has two chitin synthases belonging to class III, *chsC* and *chsG*. Although deletion of *chsC* does not cause any phenotypic change, the deletion mutant *chsG* results in slow growth, defects in conidiation and highly branched hyphae (Mellado et al., 1996). Recently, Fukuda et al have investigated the localization of ChsB in *A. nidulans* and demonstrated its functions in normal cell wall formation as well as in conidiophores and conidia development (Fukuda et al., 2009). Deletion of *csmA* (a class V chitin synthase) in *A. nidulans* leads to abnormal hyphal morphology such as intrahyphal hyphae and the formation of balloons like hyphae (Horiuchi et al., 1999). The phenotype of the deleted mutant *csmB* (a class VI chitin synthase) in *A. nidulans* has been further characterized and shown to be similar to the *csmA* null mutant phenotype. Furthermore, it has

also been shown that *csmA csmB* double mutants are not viable, demonstrating that both *csmA* and *csmB* play an essential role in fungal growth and survival (Takeshita et al., 2006).

The functional role of the different chitin synthase genes in *A. niger* has not yet been elucidated. In this study, the different roles of *chsB* (class III chitin synthase) and *csmB* (class V chitin synthase) for *A. niger* in asexual development and cell wall formation is demonstrated. Moreover, the function of these chitin synthase genes in the response to the antifungal protein AFP has been studied. In the course of the past few years *A. niger* has been characterized by its high sensitivity to the antifungal protein AFP with minimal inhibitory concentration (MIC) at 1µg/ml. The mode of action of AFP has further been elucidated using the filamentous fungi *A. niger* as well as mutants of the yeast *S. cerevisiae* (Hagen et al., 2007; Ouedraogo et al., 2011). AFP inhibits chitin synthesis in *A. niger*, causes cell wall stress and triggers cell wall integrity (CWI) pathway. Furthermore, chitin synthase inhibition is a general AFP mechanism of action in all sensitive filamentous fungi; this has also been demonstrated in the wild type of *Fusarium oxysporum* and *A. oryzae* (Hagen et al., 2007). Chitin synthase mutants of class III (*chsB* of *A. oryzae*) and class V (*csmA* of *A. oryzae* and *chsV* of *F.oxysporum*) became less sensitive to AFP in comparison to the respective wild type strains, thus supporting the hypothesis that the chitin synthases of class III and V are targets of AFP (Hagen et al., 2007). Hence, in this study the response of the null mutant *chsB* (class III chitin synthase) and *csmB* (class V chitin synthase) of *A. niger* to AFP and to other cell wall stress conditions has been elucidated.

Table 1: Phenotype of deleted specific chitin synthase in some filamentous fungi

Species	Gene name	Class	Deletion phenotype	References
A. nidulans	*chsB*	III	slow growth and small colonies highly branched hyphae no formation of conidiophores and conidia	(Borgia et al., 1996; Fukuda et al., 2009)
	**csmA*	V	slow growth formation of few conidiophores formation of balloons and intrahyphal hyphae lysis of hyphae	(Horiuchi et al., 1999)
	csmB	VI	slow growth formation of balloons lysis of hyphae	(Takeshita et al., 2006)
A. fumigatus	*chsG*	III	slow growth defect in conidiation highly branched hyphae	(Mellado et al., 1996)
	chsE	V	reduction of conidiation reduction of chitin	(Aufauvre-Brown et al., 1997)
A. oryzae	*chsB*	III	reduction of conidiation sensitivity to CFW hyperbranched hyphae	(Muller et al., 2002)
	csmA	V	slow growth formation of intrahyphal hyphae	(Muller et al., 2002)
F. oxysporum	*chsV*	V	slow growth swollen balloon like hyphae sensitive to CFW	(Madrid et al., 2003)
	chsVb	VII	slow growth swollen balloon like hyphae sensitive to CFW	(Martin-Urdiroz et al., 2008)
N. crassa	*chs1*	III	slow growth aberrant hyphal morphology decrease in chitin synthase activity	(Yarden and Yanofsky, 1991)

*Nomenclature conflict: *csmA* has been referred to as *chsD* by some authors

3.2. Materials and methods

3.2.1 Strains, media and molecular techniques

A.niger strains used in this study are listed in Table 2. Strains were cultivated in minimal medium (MM) (Bennett and Lasure, 1991a) containing 55 mM glucose, 7 mM KCl, 11mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO^3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6,4 nM $CuSO_4$, 6.2 nM Na_2MoO_4, 174 nM EDTA; or in complete medium (CM) containing, in addition to MM, 0.1 % (w/v) casamino acids and 0.5 % (w/v) yeast extract. When required, 10 mM uridine or/and 100 µg/ml of hygromycin was added. YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L dextrose) was used to perform sensitivity tests towards AFP. Basic molecular techniques were performed according to standard procedures (Sambrook and Russel, 2001b). *Escherichia coli XL 10-Gold*® was used for transformations. Transformation of *A. niger*, genomic DNA extraction, screening procedures, diagnostic PCR, Northern and Southern analysis were performed as previously described (Meyer et al., 2010).

Table 2: *A. niger* strains used in this study

Name	Genotype	Reference
N402	*csp*, *amdS*$^-$	(Bos et al., 1988)
MA70.15	Δ*kusA, pyrG*$^-$*, amdS*$^+$	(Meyer et al., 2007)
MA169.4	*kusA::DR-amdS-DR, pyrG*	(Carvalho et al., 2010)
JP1	Δ*kusA, pyrG*$^+$*, amdS*$^+$,Δ*csmB*	This study
JP2	Δ*kusA, pyrG*$^+$*, amdS*$^+$,Δ*chsB*	This study
JP3	Δ*kusA,pyrG*$^+$*, amdS*$^+$*,* Δ*chsB,* pAMA-chsB	This study

3.2.2. Construction of A. niger strains

CsmB and *chsB* deletion mutants were constructed as follows. The *A. niger csmB* gene (*An02g02340*) was deleted in MA70.15 by replacing its open reading frame (ORF) with a DNA fragment containing the *pyrG* marker from *A. fumigatus* (Yang et al., 2004). The cassette used for *csmB* deletion was obtained by fusion-PCR in two steps. First, independent amplification of the *csmB* promoter and terminator regions and the *pyrGAf* gene, respectively, using the primers summarized in Table 3. Genomic DNA of the wild type strain N402 served as template DNA. Second, fusion-PCR using the three fragments as template DNAs with primers PP1 and GSP4 (Table 3). Deletion of the *A. niger chsB* ORF (*An09g04010*) followed the same approach as described for *csmB*. The respective primers are listed in Table 3. The different deletion cassettes

were transformed into strain MA70.15 and uridine prototrophic transformants were selected and analyzed by PCR and Southern hybridization.

Table 3: Primers used in this study

Primer name	Sequence (5'→3')	Targeted sequence
csmB PP1	TAACCGGAGCGTAAGCTAATACCTTGACAT	*csmB* promoter
csmB PP2	GATAGAGCGGAGCATAATCAGCCAACTAAC	*csmB* promoter
csmB GSP3	TCCTGCAGCACGCATACATAGATAACATAC	*csmB* terminator
csmB GSP4	AGCGTCTCGAGTTGATACATATTACGAGTC	*csmB* terminator
chsB PP1	CCGACTAAGGAAGGTCTTACATGGTACCTG	*chsB* promoter
chsB PP2	GAATGCAAGGATGGCGCAAGAAGCAATGAG	*chsB* promoter
chsB GSP3	GTCCGGCTATCACCTGTCTCTGATAATCTC	*chsB* terminator
chsB GSP4	AATCTATCCAGTCGCTAATTAATCCAAGTG	*chsB* terminator
csmB SMP1	*GTTAGTTGGCTGATTATGCTCCGCTCTATC*ACCGGTCGCCTCA AACAATGCTCT	*PyrGAf*
csmB GFP2	*GTATGTTATCTATGTATGCGTGCTGCAGGA*GTCTGAGAGGAG GCACTGATGCG	*PyrGAf*
chsB SMP1	*CTCATTGCTTCTTGCGCCATCCTTGCATTC*ACCGGTCGCCTCA AACAATGCTCT	*PyrGAf*
chsB GFP2	*GAGATTATCAGAGACAGGTGATAGCCGGAC*GTCTGAGAGGAG GCACTGATGCG	*PyrGAf*
FW ChsB not1	AAGGAAAAAA<u>GCGGCCGC</u>AAGGAAAAAAATCCCGCCCAG TCTCACTTC	*chsB* ORF
Rev ChsB not1	AAGGAAAAAA<u>GCGGCCGC</u>AAGGAAAAAATCAGAGCCGTC GTAGTGTTG	*chsB* ORF
Fw CsmB not1	AAGGAAAAAA<u>GCGGCCGC</u>AAGGAAAAAAATCCGCATCAC CCGAGTAAC	*csmB* ORF
Rev CsmB not1	AAGGAAAAAA<u>GCGGCCGC</u>AAGGAAAAAAAGCCCTCATCG CATTTCTCC	*csmB* ORF

Complementary overhangs used in the fusion PCR are indicated in italics and the underlined sequence corresponds to the indicated restriction site

3.2.3 Complementation of Δchsb and Δcsmb strains using AMA1-based complementation vector

The complementation of Δ*chsB* and Δ*csmB* was performed using the plasmid pMA171 described previously (Carvalho et al., 2010). The ORF of *chsB* including 1000 bps of the promoter and terminator regions, was amplified by PCR using N402 genomic DNA as template and respective primers containing *NotI* overhangs (Table 3). The fragment was cloned into *NotI*-linearised pMA171. The plasmid pMA171-chsB was then transformed into the *chsB* deletion mutant. Transformants containing the complementation plasmid were isolated on MM containing 100 µg/mL of hygromycin and further analyzed by PCR and Southern blot. The same approach was used for Δ*csmB* complementation.

3.2.4. Determination of conidiation efficiency

Conidiation efficiency was determined according to the plating method previously described, but with some modifications (Ichinomiya et al., 2005). 10^3 conidia of each strain were point-inoculated on MM and CM plates and incubated at 37°C for 72h. Conidia were then harvested and suspended in 1.0 ml of distilled water containing 0.005 % Triton X by scraping the surface of the colonies. The number of conidia was counted using a haemocytometer. The diameters of the different colonies were also measured. Data were taken from three colonies for each strain to determine the average and standard deviation.

3.2.5. Microscopy

3 x 10^5 conidia of *A. niger* strains were inoculated in MM supplemented with 0.003 % yeast extract and grown until germ tubes were visible (5h at 37°C) on coverslips, which were placed into petri dishes containing the liquid medium. AFP (10 and 50 µg/ml) or the same volume of AFP puffer (negative control) was added; the petri dishes were incubated for 2h at 30°C. Germlings which adhered to the coverslips were observed using an Axioplan 2 (Zeiss) equipped with a DKC-5000 digital camera (Sony). The phenotype and morphology tests of the wild type and chitin synthase mutants were viewed directly on the surface of the solid media or on coverlips in liquide medium, using an Axioskop (Zeiss) equipped with a digital camera (Olympus).

3.2.6. AFP susceptibility assay

The sensitivity of *A. niger* strains to AFP was determined using a protocol according to (Theis et al., 2003). In brief, 10^3 spores were used to inoculate 150 µl YPD medium in the absence or presence of different AFP amounts (0.1 – 15 µg/ml). Cultivations were carried out in technical triplicates in microtiter plate format (28 °C, 28 h, 120 rpm) and repeated at least twice. Growth was assessed by measuring the optical density at 600 nm.

3.2.7. Sytox Green uptake assay

The assay was carried out in microtiter plate format using the method recently described (Theis et al., 2003). In brief, 100 conidia were cultivated in 150 µL YPG medium for 20 to 40 h at 28°C.

AFP and SYTOX Green were added to final concentrations of 100µg/ml and 0.2 µM, respectively. Fluorescence was quantified immediately after addition of SYTOX Green and AFP. Measurements were obtained for 225 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 for samples incubated in the absence of AFP. Triplicate experiments were performed.

3.2.8. ***Phenotypic tests***

Phenotypic characterization was performed by point-inoculating approximately 10^4 freshly isolated conidia of the different *A. niger* strains for 72 h at 37 °C in CM or/and in MM medium plates with the supplements indicated: $CaCl_2$ 100 mM; Na_2HPO_4 100 mM; NaCl 1 M; SDS 0.005 %; CFW 0.1 mg/ml; caspofungin 50 µg/ml; amphotericin B 1 mg/ml; sorbitol 1.2 M. To test temperature sensitivity, conidia were cultivated at 42 °C.

3.2.9. Chitin and β-1,3- glucan content determination

To determine cell wall polymers chitin and β-1,3- glucan, 10^6 fungal spores were inoculated per ml of MM or CM medium and incubated in the presence (10 µg/ml) or absence of AFP. Cell wall chitin was isolated according to (Ram et al., 2004), measured according to (Popolo et al., 1997; Tracey, 1956) and calculated per dry biomass. β-1,3-glucan levels were determined according to (Jarrod et al., 2009) and calculated per dry biomass. All isolations and measurements were calculated from at least two independent experiments.

3.2.10. Computer analysis

Similarity of the amino acid sequences of chsB and csmB of *A. niger* to other *Aspergillus* species chitin synthases was determined using the BLASTP program at the Joint Genome Institute website (JGI). The chitin synthases homologous to chsB and csmB of *A. niger* was obtained for *A. fumigatus* and *A. nidulans*. Deduced amino acid sequences of the chitin synthases were aligned and a phylogram was constructed using the multiple alignment program CLUSTAL W2 (Gonnet 350) (Thompson et al., 1994).

3.3. Results

3.3.1. *Comparison of predicted CsmB and ChsB of A. niger to their homologous chitin synthases in A. fumigatus and A.nidulans*

Sequences of CsmB and ChsB of *A. niger* were aligned to their respective homologous chitin synthases in *A.fumigatus* and *A.nidulans*. The *csmB*-encoded polypeptide is 80 % identical to the polypeptide encoded by the *A. fumigatus* chitin synthase (*Afu2g13430*) gene and 85 % identical to the polypeptide encoded by the *A. nidulans* chitin synthase *csmB*. As regards to *chsB,* the polypeptide is 91 % identical to the polypeptide encoded by the *A. fumigatus chsG* gene and 89 % identical to *chsB*-encoded polypeptide *in A. nidulans*. Several conserved amino acids have been found in class III and V chitin synthases of *A. niger* and their homologous chitin synthase genes in *A. fumigatus* and *A. nidulans*, suggesting that these chitin synthase genes function identically (Fig 1A and B).

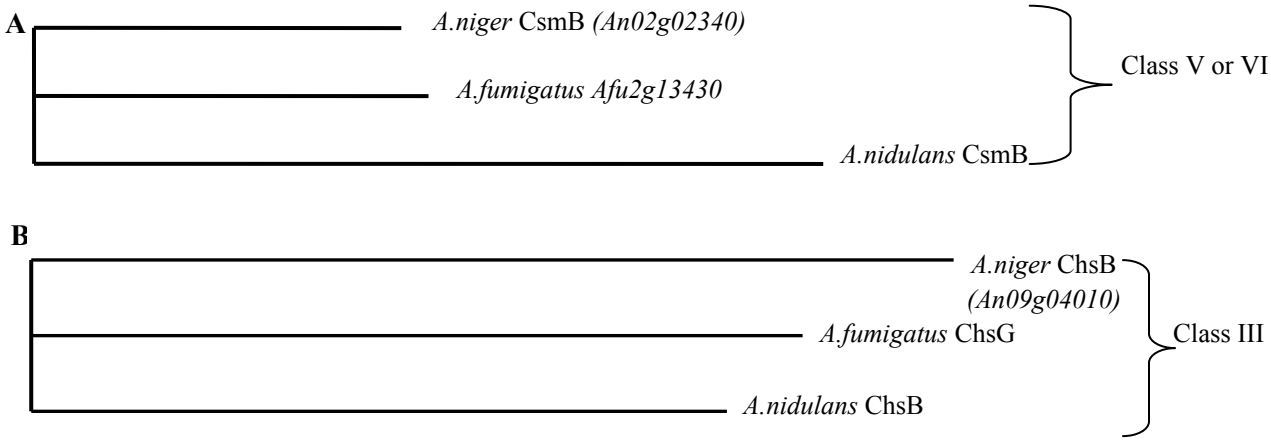


Figure 1: Homologies of CsmB and ChsB proteins of *A.niger* in *A .fumigatus* and *A. nidulans*, using multiple sequence alignment software. (A) Sequence distances for each chitin synthase of the class V/VI (CsmB in *A. niger CBS513.88*). (B) Sequence distances for each chitin synthase of the class III (ChsB in *A. niger CBS513.88*).

3.3.2. Construction of the chsB and csmB null mutants and complementation

CsmB and *chsB* null mutants were constructed by homologous recombination in the strain MA70.15 (Δ*kusA, pyrG*$^{-}$) by replacing the *csmB* and *chsB* ORF with PCR constructs containing the *A. fumigatus pyrG*. The MA70.15 strain is characterized by its high efficiency for generating gene knockouts (Meyer et al., 2007). Genomic DNA from selected *pyrG*$^{+}$ transformants was selected and analyzed by Southern hybridization using the 1kb promoter from *csmB* and *chsB*

gene as respective probes (Fig.2 A-D). Following digestion with EcoRV, the expected fragments of 2.1kb and 3.3 kb were detected in the wild type and in the *csmB* null mutant, respectively. For *chsB* null mutant transformant analysis, a BglII 3.4kb fragment was detected instead of 5.1kb in the wild type, proving successful fine unockout. The correct transformants were designated as JP1 (Δ*csmB*) and JP2 (Δ*chsB*). The AMA1 based vector pMA171 conferring hygromycin resistance was used for complementation experiments. The vector pMA171-chsB was constructed as described in "Materials and Methods "and transformed into Δ*chsB* (JP2) giving strain JP3. Transformants were selected and purified on MM without uridine but containing hygromycin. Complementing strains were analysed by Southern hybridisation which confirmed the presence of the complementing plasmid containing the gene *chsB* (data not shown).

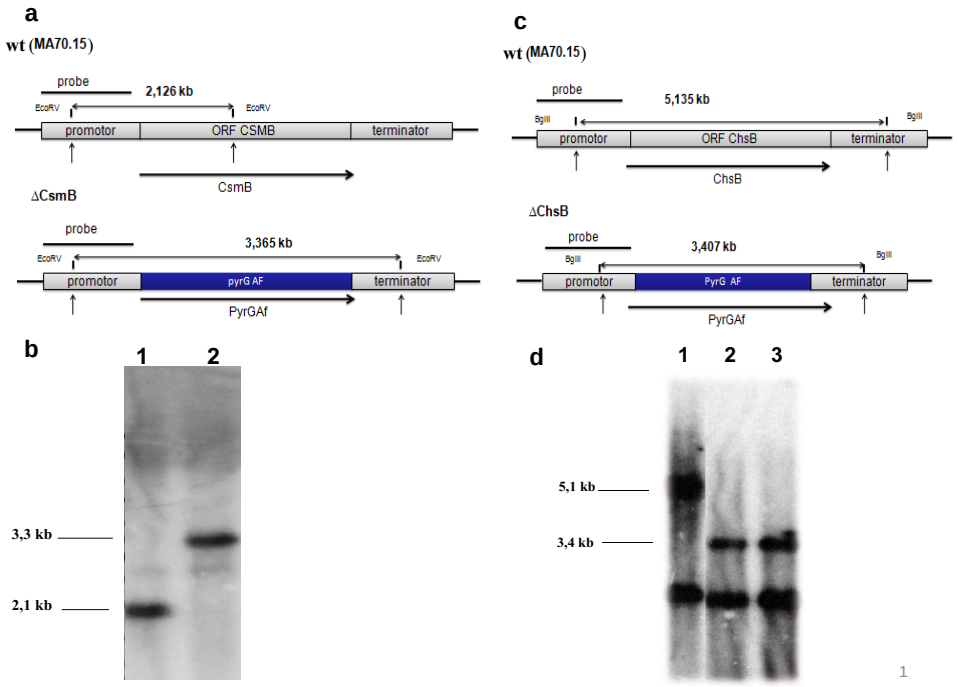


Figure 2: Construction of *csmB* and *chsB* null mutants. A) Scheme for the construction of *csmB* null mutant. A 1.1 kb of the promoter *csmB* was used as a probe. Restriction sites and the predicted sizes of genomic fragments are indicated. B) Southern analysis of the genomic DNA of MA70.15 (lane 1) and Δ*csmB* (lane 2) digested with *EcoRV*. A 2.1 kb band was detected in lane 1. In lane 2, a 3.3 kb band was detected instead. The size of marker DNA fragments is indicated on the left (kilobases). C) Scheme for the detection of the *chsB* gene replacement. A 1.3 kb of the promoter *chsB* was used as probe. Restriction sites and the predicted sizes of genomic fragments are indicated. D) Southern analysis of genomic DNA of MA70.15 (lane 1), ΔchsB (lane 2 and 3) digested with *BglII*. The probe indicated 5.1 kb band in lane 1 and 3.4 kb band in lane 2 and 3.

3.3.3. Effects of csmB or chsB deletion on growth and conidia formation

The effects of *csmB* or *chsB* deletion on growth and conidiation efficiency on CM and/or MM plate were examined. As shown in Fig 3, *csmB* and *chsB* null mutants grow slower than the wild type strain on CM and MM plates. Although no significant differences in the size of the different colonies grown on CM and MM plates could be observed, both chitin synthase mutants formed less conidia in comparison to the wild type. On CM medium, the efficiency of *csmB* and *chsB* null mutants were 8 % and 1 % respectively, in comparison to the wt conidiation efficiency in CM (considered as 100%) (see Table 4). Hence, CsmB and ChsB proteins could thus play an important role in *A. niger* during sporulation.

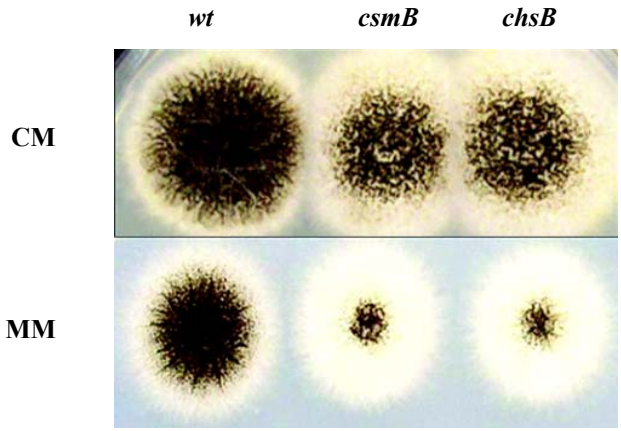


Figure 3: Colonies of the wild type strain (MA77.1) and chs mutants. Conidia from each strain were point-inoculated on solid CM and MM media and incubated for 72h at 37°C.

Table 4: Growth of *A. niger* wild type and chs mutant strains. Strains were growth for 72 h at 37 °C on CM and MM plates. Means ± SD were calculated from three independent experiments. Conidiation efficiency has given in % in comparison to the wt conidiation efficiency in CM.

Strain	Relevant genotype	Colony diameter (mm)		Conidiation efficiency (%)	
		CM	MM	CM	MM
MA77.1	Wild type	23 ± 1.2	14 ± 1.3	100	16.92
JP1	Δ*csmB*	22 ± 1.3	13 ± 1.1	8.23	0.012
JP2	Δ*chsB*	23 ± 1.3	13 ± 0.7	0.92	0.01

3.3.4. Involvement of CsmB *and* ChsB *in fungal wall assembly and morphology*

In order to study the role of CsmB or ChsB gene in fungal wall assembly, the effect of various stress agents on fungal growth were tested including salts (NaCl [1M], Na_2HPO4 [0.1 M], $CaCl_2$ [100 mM]), the detergent SDS (0,005 %), the chitin binding reagent calcofluor white (CFW) (0.1 mg/ml), the cell wall components inhibitors (caspofungin 50 µg/ml, amphotericin B 1 mg/ml). In addition, the sensitivity of the strains toward elevated temperature was examined. Both chitin synthase mutants displayed higher sensitivity to CFW in CM and MM. In addition, the growth of the Δ*chsB* mutant is inhibited in the presence of caspofungin and SDS (Fig.4). CFW sensitivity of the deleted chitin synthase *csmB* and *chsB* mutants correlated to higher sensitivity to elevated temperature; which is indicative for a defective cell wall in both mutants. However, sorbitol added to the medium partially rescued the growth of the chitin synthase mutant strains. Here, the growth of the Δ*csmB* mutant in MM was effectively improved in the presence of sorbitol, indicating an osmotic remediable phenotype. Congruently, the presence of salts such as NaCl improved growth of the chitin synthase mutants.

Interestingly, chitin synthase mutants showed frequently balloon-like swollen conidia hyperswollen vesicles on conidiophores forming less or no conidia (Fig 5 and data not shown).

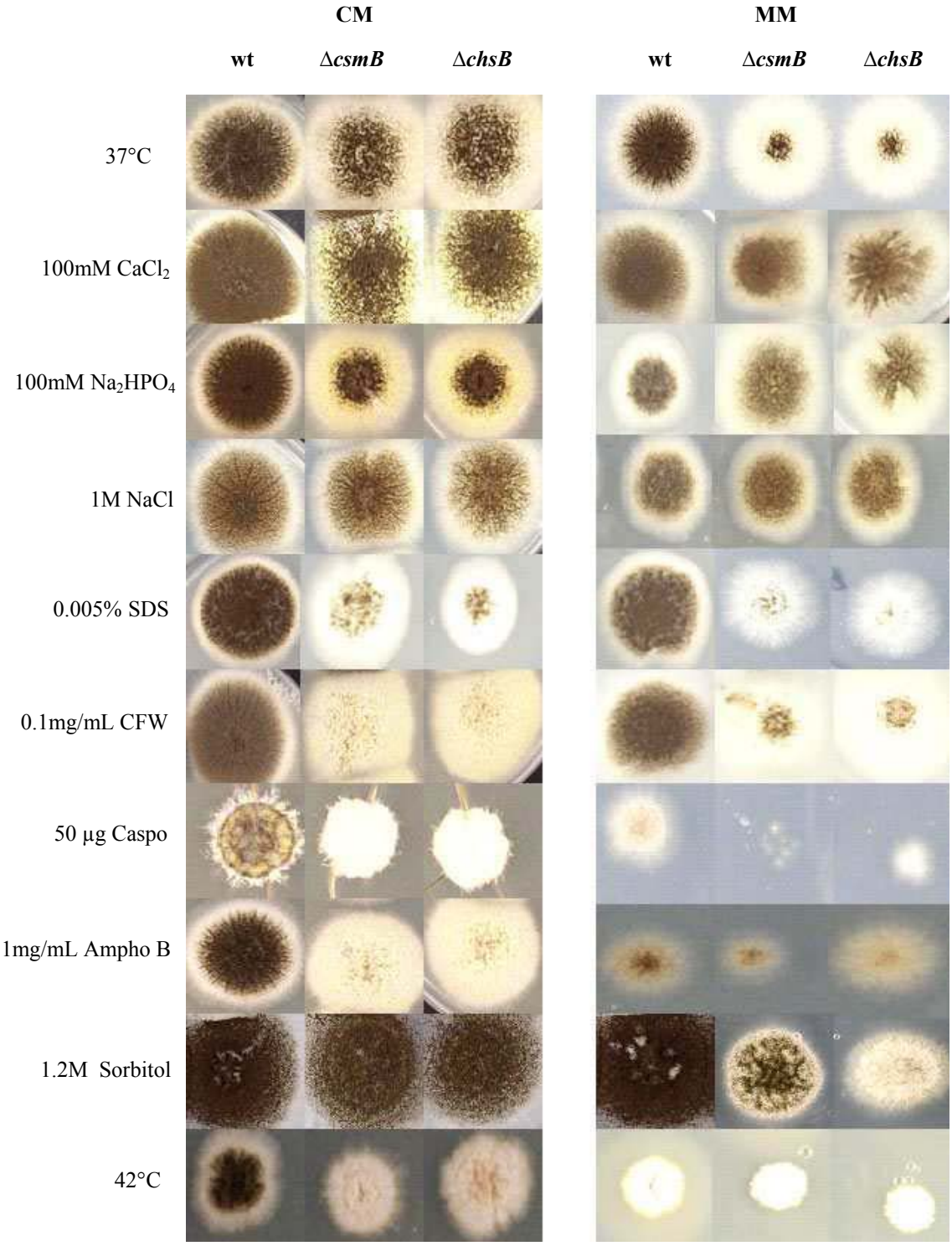


Figure 4: **Phenotype of *A. niger* chitin synthase mutants under different conditions**. Relevant strain names are indicated at the top. 10^4 conidia from each strain were plated on CM and MM containing different reagents and cultivated at 37 °C or 42 °C for 3 days.

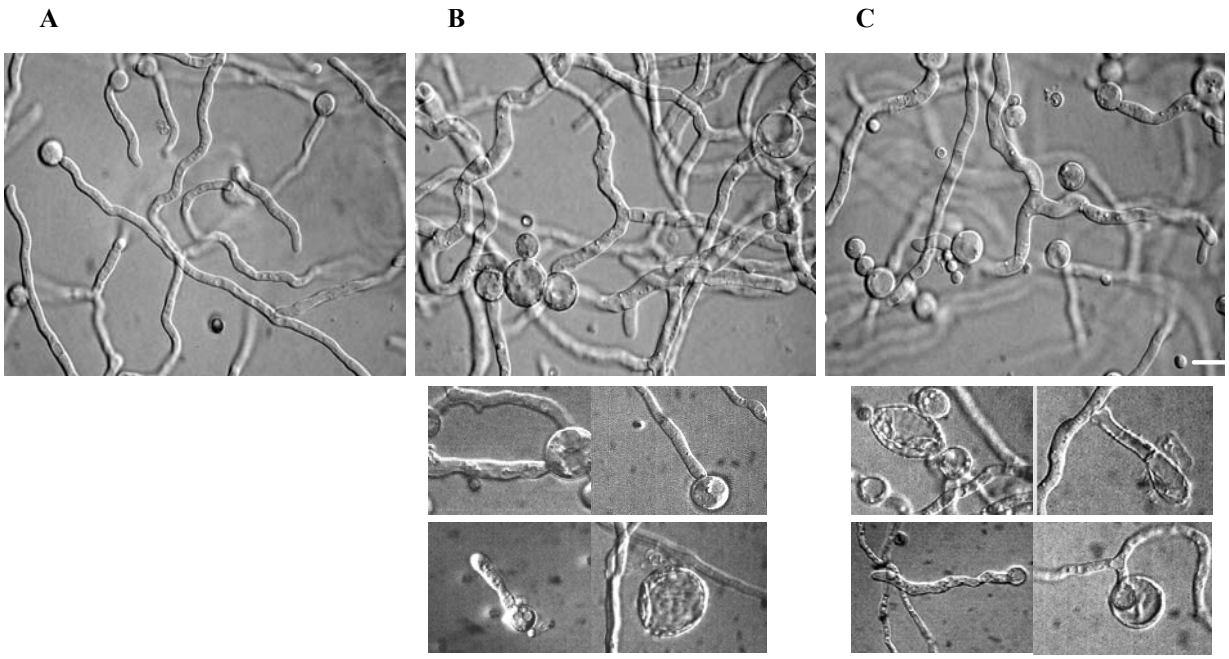


Figure 5: Morphology of *A. niger* wt and chs mutants strains with balloon-like swollen conidia. (A) Wild type *A. niger*. (B) Δ*csmB* strain. (C) Δ*chsB* strain. Bars = 10 µm

3.5. *Complementation of chsB and csmB null mutant*

The AMA1-based method pMA171 conferring hygromycin resistance described in "Materials and Methods" was used to complement the deletion mutants. The transformants were selected and purified on MM containing hygromycin and lacking uridine. The complementing strains were verified by Southern analysis (data not shown). Although the complementation of Δ*chsB* was successful, the complementation of Δ*csmB* was not, due to cloning problems of the ORF (7.5kb) which rendered the ligation with the plasmid inefficient (11.2kb). The phenotype of the successful complemented strain *chsB/AMA-chsB* was further tested in different reagents in comparison to the Δ*chsB* strain. The results show that complementation partially restored resistance of the mutant strain to CFW, confirming the role of *chsB* in cell wall formation (Fig 6). Moreover, the result clearly shows the capacity of the complement strain to sporulate. However, the wild type phenotype was not completely restored in the AMA-chsB strain, suggesting that the cellular amount of ChsB is highly controlled. A higher copy number caused by multiple *pAMA* plasmid copies might have induced an overexpression phenotype.

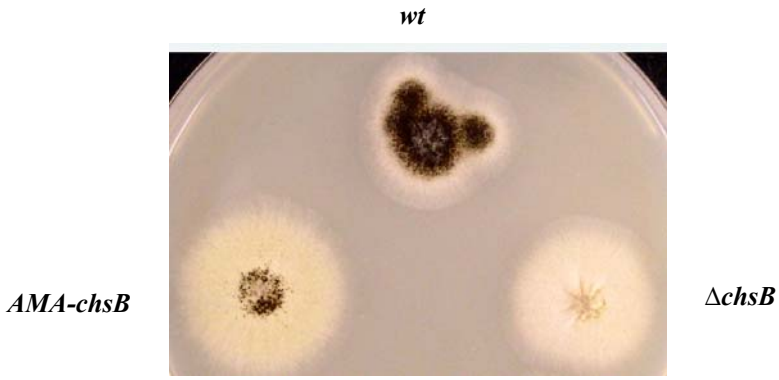
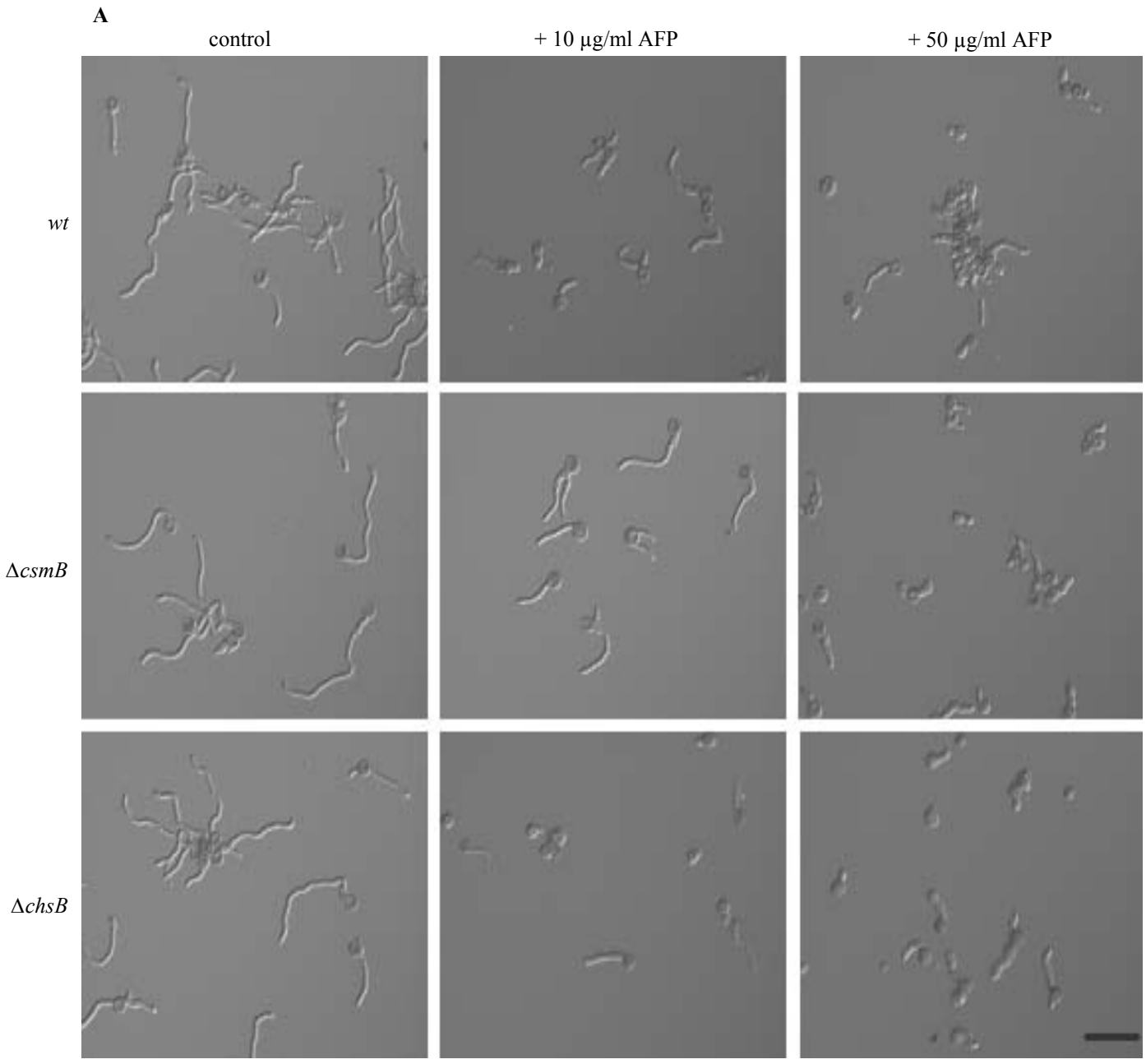


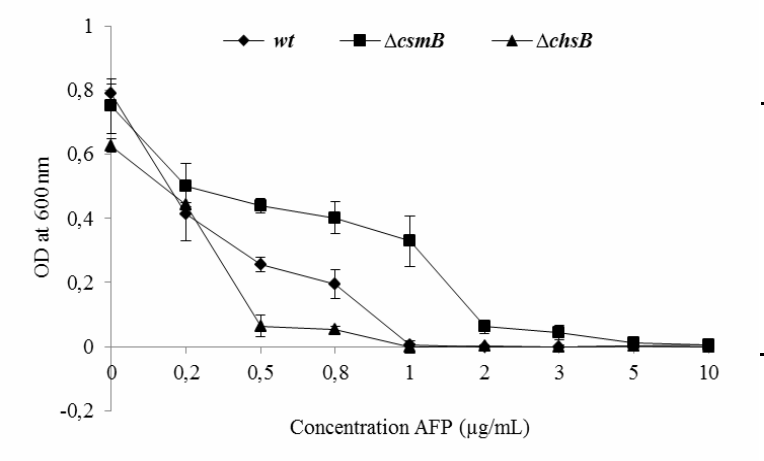
Figure 6: The growth of AMA-chsB strain and deleted mutant chsB in CM+CFW. 10^3 conidia from each strain were plated on CM containing 0.1 mg/ml CFW cultivated at 37°C for 3 days.

3.6. Susceptibility comparison of csmB and chsB null mutants to AFP

To check whether the *csmB* and *chsB* null mutants respond differently to AFP attacks in comparison to the wild type, the minimal inhibitory concentration (MIC) of AFP was determined. In addition, conidia germination in the presence of AFP as well as membrane stability was tested. As can be seen in Figure 7, AFP has an effect on the growth of the wild type and the chitin synthase mutants. The wt and the Δ*chsB* strains exhibited high sensitivity to AFP in comparison to Δ*csmB* with the MIC successively determined at 1µg/ml, 0.5µg/ml and 10µg/ml (Fig 7B). AFP was also able to halt germination of the different strains. However, no significant difference in AFP effect on the germination could be detected between the three different strains using 10µg/ml and 50µg/ml of AFP, confirming that AFP stops germination in all AFP sensitive fungi (Fig 7A). The membrane permeabilization assay performed based on the uptake of the fluorogenic dye SYTOX Green (which can only penetrate cells that have compromised plasma membranes), revealed that AFP acts on all mutants by permeabilizing their plasma membrane. However, the plasma membrane of the chitin synthase mutant *chsB* is more readily permeabilized in comparison to the mutant *csmB* (Fig 7C), which is in agreement with a more susceptible phenotype compared to wt and Δ*csmB*.


A
control
+ 10 µg/ml AFP
+ 50 µg/ml AFP
wt
ΔcsmB
ΔchsB

B



Strains	Genotype	MIC (µg/mL)
MA77.1	wt	1
JP2	Δ*chsB*	0.5
JP1	Δ*csmB*	10

C

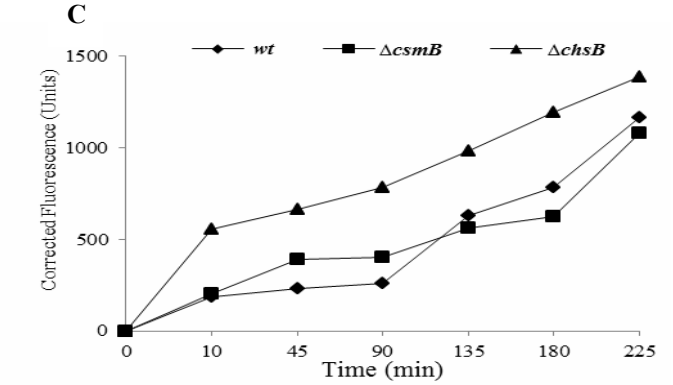


Figure 7: Effect of AFP on *A. niger* chitin synthase mutants. A) Inhibition of conidia germination in *A. niger* wt and chitin synthase mutants by AFP. Conidia were incubated in YPG medium at 37°C. After 5h, AFP was added at 10µg/ml and 50µg/ml. Micrograph was taken after 3h of further incubation in absence and presence of different concentration of AFP. Bars = 10 µm B) Analysis of growth inhibition of the wild type and chitin synthase mutants of *A. niger*. Susceptibility assays were carried out in microtiter plate with the wild type (MA77.1), Δ*csmB* and Δ*chsB* mutants. Fungi were cultivated in the presence of different AFP concentrations and growth was measured at 600 nm after 28h of incubation at 28°C. C) Detection of AFP-induced SYTOX Green uptake. *A.niger* wt and chitin synthase mutants were incubated with 100 µg/ml AFP in the presence of 0.2 µM SYTOX Green. Fluorescence was measured up to 225 min.

3.3.7. Chitin synthase mutants differ in their cell wall remodeling response in the presence of AFP

Next, the content of two major fungal cell wall polysaccharides, chitin and β-1,3-glucan was examined in all strains. In general, the amount of chitin and β-1,3-glucan was reduced in both chitin synthase mutants in comparison to the wild type strain, suggesting that chitin synthases

csmB and *chsB* are involved in cell wall organization and remodeling (Fig 8). In the presence of AFP, the chitin content of the wild type and the mutant *chsB* changed to 0.94 and 0.83 fold respectively. However, the chitin content of the mutant *csmB* is increased in the presence of AFP (1.34 fold changing) (Fig 8). This finding reveals that *A. niger* responds to an AFP attack by remodeling its cell wall chitin and that the chitin synthase mutants respond differently to AFP. However, the β-1,3-glucan content of the mutant Δ*chsB* slightly increased in the presence of AFP in comparison to the absence of AFP, whereas, the wt and the Δ*csmB* have their β-1,3-glucan reduced in the presence of AFP. This allows the hypothesis that AFP provokes different responses in these strains.

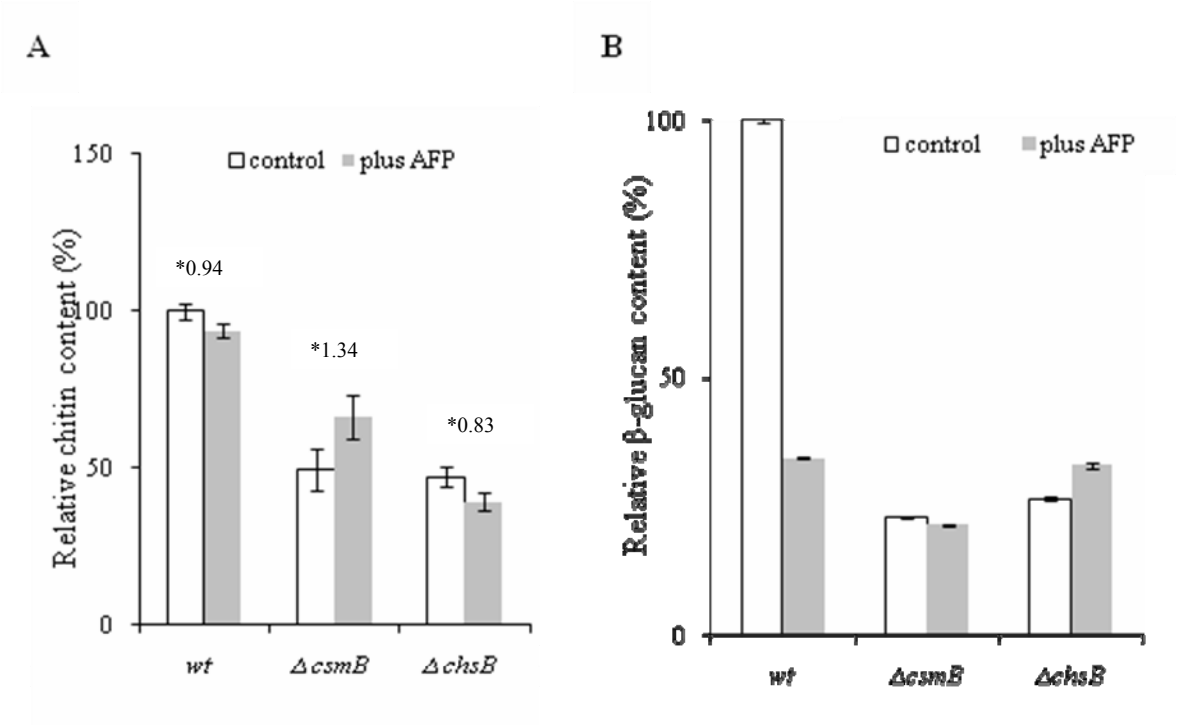


Figure 8: Cell wall remodeling of *A.niger* chitin synthase mutant in response to AFP. A) Amount of chitin in the wild type and mutants chitin synthase strains in the absence and presence of 50 µg/ml AFP. B) β-1,3-glucan content in the absence and presence of AFP**.** The data are given in % of dry weight in comparison with the wt polysaccharide content in the absence of AFP. The error bars indicate standard deviations for two different experiments. *: Fold change of chitin amount after adding AFP. Wt: strain MA169.4.

3.4. Discussion

Class III and V chitin synthases are only found in filamentous and some dimorphic fungi, suggesting an important key role for hyphal morphogenesis and cell wall structure. Previous studies have demonstrated that inactivation of chitin synthases belonging to these classes leads to mutant strains with phenotypic defects (Borgia et al., 1996; Fukuda et al., 2009; Horiuchi et al., 1999; Takeshita et al., 2006). The data presented in this work demonstrate that the deletion of chitin synthase of class III and class V in *A. niger* produced strains defective in asexual development and hyphal wall integrity. By comparing sensitivity and the survival response of the deleted chitin synthase strains to the antifungal protein AFP, it was found that they respond differently to AFP attack and that this response includes a remodeling of their cell wall.

3.4.1. ChsB and csmB, the class III and V chitin synthase are important for the maintenance of cell wall integrity and asexual development in A. niger

The role of the non-essential chitin synthase *chsB* and *csmB* of *A. niger* in fungal development and integrity was analyzed by characterizing their null mutants. The mutant Δ*chsB* and Δ*csmB* showed deficiency in conidiation and in hyphal response to cell wall inhibitors. The formation of low numbers of conidia in CM or in MM medium indicates that the chitin synthase *chsB* and *csmB* are involved in the asexual development of *A. niger.* It has been previously reported that ChsB is required for conidial development and the formation of conidiophores in *A. nidulans* (Fukuda et al., 2009; Ichinomiya et al., 2002). It may be that ChsB plays a similar role in morphogenesis in both *Aspergillus* species. In addition, the role of the class V chitin synthase in conidiation has been further demonstrated in *A. nidulans,* showing that the *csmB* transcript was more abundant under low osmotic conditions, thereby revealing its function in maintaining cell wall integrity (Takeshita et al., 2002; Takeshita et al., 2006). The comparison of ChsB and CsmB protein sequences in *A. niger* with their homologous chitin synthases in *A. nidulans* has revealed high homology (Fig 1), which additionally may hint at their similar functional role in conidiation and hyphal morphogenesis. These observations are consistent with the finding that the inactivation of ChsG in *A. fumigatus* also results in a defect in conidiation (Mellado et al., 1996). Both chitin synthases (ChsB and CsmB) are involved in asexual development in *A. niger*, whereas ChsB seems to be more implicated in the conidia formation than CsmB. This is supported by the findings that the Δ*chsB* strain has an about 8-fold lower conidiation efficiency in

comparison to Δ*csmB* and about 100-fold lower conidiation efficiency in comparison to the wt (Table 4). As regards phenotypic characterization, the hyphal growth of the chitin synthase mutants was inhibited on media containing various cell wall-compromising reagents, indicating modification in their cell wall architecture. The sensitivity to SDS demonstrates that the cell wall has became weak, as shown in the yeast *S. cerevisiae* (Ram et al., 1994; Shimizu et al., 1994), indicating that the chitin synthase ChsB and CsmB has a functional role in the maintenance of the fungal cell wall. Disruption of the cell wall in the chitin synthase mutants has additionally been proved by their hypersensitivity to caspofungin, amphotericin B and at elevated temperature. It has been also demonstrated that chitin synthase mutants are highly sensitive to CFW. Elevated sensitivity to SDS and CFW with reduced sporulation has been shown in the *A. nidulans* strain lacking chitin synthases *chsA* and *chsC* genes, supporting the contention that a chitin inhibitor such as CFW can inhibit strains which are deficient in the expression of some chitin synthases (Fujiwara et al., 2000). Moreover, triggering cell wall integrity in *A. niger* by SDS, CFW and caspofungin has been well demonstrated by Damveld et al (Damveld et al., 2005). In fact, the expression of *agsA*, a gene encoding for 1,3-α-D-glucan synthase, is induced in response to the cell wall stress reagents mentioned. There are up to 7 chitin synthase genes in *A. niger* which together contribute to the overall integrity of the fungal cell wall and could differently interact with chitin synthases inhibitors (Bowman and Free, 2006; Roncero, 2002). The chitin content in the chitin synthase mutants was lower than that of the wild type strain. This suggests that the deleted chitin synthase genes *chsB* and *csmB* are involved in the overall chitin synthesis in *A.niger* and that the chitin content of the mutants does not directly reflect their sensitivity to chitin inhibitors such as CFW. In other words, the chitin level in the cell wall is not the only factor determining CFW sensitivity and not all mutants with the CFW-hypersensitivity phenotype display increased chitin levels as shown for *S. cerevisiae* (Imai et al., 2005; Ram et al., 1994). For example, deletion of chs1 in the yeast *S. cerevisiae* resulted in a 90 % decrease in in vitro chitin synthase activity, but led to high sensitivity of the strain to CFW (Bulawa et al., 1986; Roncero et al., 1988). However, one cannot exclude limitations in the sensitivity of the chitin content assays or it might be concevable that chitin may be present at normal levels but is less crystalline, thereby more accessible to the chitin inhibitor (Elorza et al., 1983).

3.4.2. Different responses of A. niger csmB and chsB deletion mutants to AFP

The antifungal protein AFP is highly effective in restricting the growth of the filamentous fungus *A. niger*. With the knowledge that chitin synthesis is the target of AFP in sensitive filamentous fungi, chitin synthase class III and V in *A. niger* were deleted to further understand the mode of action of the antifungal protein and to elucidate possible targets. We selected these chitin synthase genes as they are not found in the AFP resistant yeast *S. cerevisiae* and as these chitin synthase genes play an important role in polar growth of *A.nidulans* (Takeshita et al., 2006). The sensitivity of the class III chitin synthase deletion mutant of *A. oryzae* has been characterized and it was determined that class III chitin synthase is a target of AFP (Hagen et al. 2007). The generated data here demonstrate that AFP differently affects class III (ChsB) and V (CsmB) mutants of *A. niger*. Sensitivity as regards to MIC, the survival response and cell wall remodeling are different between both chitin synthase mutants. Δ*chsB* becomes more susceptible to AFP in comparison to the wt strain. In contrast, Δ*csmB* is less susceptible to AFP than both strains. This finding could lead to the hypothesis that the class V chitin synthase in *A. niger* is a target candidate of AFP and that class III seems to be less important in binding with AFP. Previous studies have shown that *A.oryzae* class III and V and *F.oxysporum* class V, chitin synthases were targets for AFP and that the respective mutants were less susceptible in comparison to the wild type (Hagen et al., 2007). However, these findings suggested that AFP might bind more to the class III chitin synthase in *A. oryzae*. Hense, it might be possible that AFP has different specificities in chitin binding in different species. It could also be possible that chitin synthases belonging to other classes are AFP targets, as they have not yet been tested. Alternatively, the cell walls of the mutants become differently remodeled in response to the deletion of the chitin synthase genes and therefore the unknown direct targets of AFP (eg. Δ3-desaturated glycosylceramides) become less accessible.

To further explain different susceptibility of chitin synthase mutants to AFP, the cell walls have been analyzed by determining the components chitin and β-1,3-glucan in the absence and presence of AFP. Interestingly, the chitin content in class V (Δ*csmB*) is increased in the presence of AFP. However, the β-1,3-glucan in Δ*chsB* is increased instead of chitin. Enhancing the chitin level in Δ*csmB* is paralleled by a reduced susceptibility to AFP, letting us to suppose that increasing the chitin content might be a mechanism of better protection against AFP. High susceptibility in the wt and in the class III mutant (Δ*chsB*), is probably due to the incapacity of

the strains to synthesize chitin in the wall during the antifungal protein attack. Instead of increasing chitin levels, the mutant Δ*chsB* increases its cell wall content with β-1,3-glucan when challenged with AFP. This is a compensation mechanism, probably due to a dual effect. One effect is the presence of less chitin in the cell wall due to deletion of the chitin synthase gene and the second is due to the effect of AFP, as the wt does not present any increasing of β-1,3-glucan content in the cell wall in presence of AFP. However, it could be possible that other cell wall components such as α-1,3-D-glucan or/and mannoproteins have been increased. Studies have revealed that the deletion of some chitin synthase genes can be associated with a reduction in cell wall chitin content without modification of the chitin synthase activity or *vice versa* or with an increase in α-glucan or mannoprotein content (Mellado et al., 2003; Ueno et al., 2011). If increasing the glucan content compensates for the lack of chitin in the cell wall in order to stop AFP inhibition, this compensatory mechanism does not seems to be adequate for efficiently counteracting an AFP attack.

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Chapter 4

Survival strategies of yeast and filamentous fungi against the antifungal protein AFP

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Abstract

The activities of signaling pathways are critical for fungi to survive antifungal attack and to maintain cell integrity. However, little is known about how fungi respond to antifungals, particularly if these interact with multiple cellular targets. The antifungal protein AFP is a very potent inhibitor of chitin synthesis and membrane integrity in filamentous fungi and has so far not been reported to interfere with the viability of yeast strains. With the hypothesis that the susceptibility of fungi towards AFP is not merely dependent on the presence of an AFP-specific target at the cell surface, but relies also on the cell's capacity to counteract AFP, we used a genetic approach to decipher defense strategies of the naturally AFP-resistant strain *Saccharomyces cerevisiae*. The screening of selected strains from the yeast genomic deletion collection for AFP-sensitive phenotypes revealed that a concerted action of calcium signaling, TOR signaling, cAMP-PKA signaling and CWI signaling is likely to safeguard *S. cerevisiae* against AFP. Our studies uncovered that the yeast cell wall gets fortified with chitin to defend against AFP and that this response is largely dependent on calcium/Crz1p signaling. Most importantly, we observed that stimulation of chitin synthesis is characteristic for AFP-resistant fungi but not for AFP-sensitive fungi, suggesting that this response is a successful strategy to protect against AFP. We finally propose the adoption of the damage-response framework of microbial pathogenesis to the interactions of antimicrobial proteins and microorganisms in order to comprehensively understand the outcome of an antifungal attack.

4.1. Introduction

The emergence and spread of pathogenic microorganisms being resistant to virtually all available antimicrobials represents a serious challenge for medicine and agriculture and has stepped up efforts to develop new antimicrobials. The use of 'smarter' antibiotics, also called 'dirty drugs' affecting multiple cellular targets is one discussed strategy to prevent the development of resistance mechanisms (Frantz, 2005; Peschel and Sahl, 2006). Of special interest is the exploitation of antimicrobial peptides (AMPs), which are natural products of pro- and eukaryotic organisms and function as defense molecules to combat nutrient competitors, colonizers or invaders (Epand and Vogel, 1999; Zasloff, 2002). The currently known and studied AMPs have been catalogued in the antimicrobial peptide database (Wang et al., 2009; Wang and Wang, 2004)

which contains by 2010 more than 1,600 members. Although AMPs are different in sequence and secondary structure, they share common attributes. Most of them are derived from precursors, are small in size (< 100 amino acids with frequent use of glycine, alanine, lysine and cysteine), display cationic and amphipathic properties and resist proteolytic degradation due to stabilizing disulfide bonds (Peschel and Sahl, 2006; Wang et al., 2009; Zasloff, 2002). Their positive net charge attracts them electrostatically to negatively charged cell surfaces of microorganisms, where hydrophobic and/or receptor-based interactions allow them to bind, traverse or permeabilize biological membranes (Aerts et al., 2008; Yeaman and Yount, 2003; Zasloff, 2002). Thereby provoked membrane alterations cause dysfunctions such as loss of ion homeostasis and/or cell wall biopolymer synthesis, which in turn represents a potentially lethal stress situation. Additional intracellular interactions of translocated AMPs with molecules displaying negative net charges, e.g. proteins, nucleic acids and oligosaccharides, can eventually lead to cell death (Yeaman and Yount, 2003).

One very promising AMP for the employment in antifungal treatments is the antifungal protein AFP produced by the filamentous fungus *Aspergillus giganteus*. AFP is a 5.8 kDa small, cystein-rich, amphipathic protein with a positive net charge and secreted by *A. giganteus* especially under non-favorable growth conditions (Campos-Olivas et al., 1995; Meyer et al., 2002). The protein is active against filamentous fungi, including serious human and plant pathogens, but inactive against bacteria, yeast, plants or mammalian cells and successfully protects plants from colonization or invasion of filamentous fungi (Barakat et al., 2010; Meyer, 2008). AFP heavily localizes to the cell wall and plasma membrane of sensitive filamentous fungi where it provokes membrane stretching and permeabilization (Hagen et al., 2007; Theis et al., 2005; Theis et al., 2003). It comprises a chitin-binding domain and inhibits chitin synthesis in sensitive filamentous fungi (Hagen et al., 2007). In collapsed and dead cells, AFP can also be found intracellularily (Theis et al., 2005; Theis et al., 2003) where it might bind via its oligonucleotide/oligosaccharide-binding (OB) fold to anionic molecules such as nucleic acids (Martinez Del Pozo et al., 2002). A shorter version of AFP exhibiting the chitin-binding domain but lacking the hydrophobic domain (sAFP) does not disturb the cell wall / cell membrane integrity of AFP-sensitive filamentous fungi although it is able to bind to chitin and nucleic acids under *in vitro* conditions (Hagen et al., 2007). This loss of bioactivity implies that the primary inhibitory effect of AFP is exerted at the cell surface where potential target(s) of AFP reside(s). Most-importantly, not all filamentous

fungi are equally susceptible towards AFP, some are highly sensitive (minimal inhibitory concentration, MIC, 0.1-10 µg/ml; *Aspergillus niger*, *Fusarium oxysporum*) or moderate-sensitive (100 < MIC < 400 µg/ml; *A. giganteus*), others are resistant (*Penicillium chrysogenum*).

Microorganisms confronted with multi-functional AMPs such as AFP have to resist these protein activities to ensure cell survival. Some microorganisms may be inherently resistant as their cell surfaces lack electrostatic affinity or receptors for AMPs. Others comprising these targets can potentially counteract AMP's attack e.g. by shielding their surface via remodeling of their cell wall / cell membranes, by extracellular trapping of AMPs at specific cell surface areas, by expressing specific proteases or by modification of intracellular targets (Peschel and Sahl, 2006; Yeaman and Yount, 2003). Prerequisite for these adaptive survival responses are the activities of signaling cascades which sense and transduce stress signals to activate and coordinate the defense reaction in a timely fashion (Levin, 2005).

Using *A. niger* as model system, we could recently show that one defense mechanism to counteract AFP inhibitory effects is induction of the cell wall integrity (CWI) pathway, a highly conserved signaling cascade which secures cell surface protection in yeast and filamentous fungi (Damveld et al., 2005; Fujioka et al., 2007; Levin, 2005; Meyer et al., 2007) and results in *A. niger* in increased expression of the *agsA* gene, encoding an α-1,3-glucan synthase (Hagen et al., 2007). However, when induction of the CWI pathway is meant to protect *A. niger*, why gets the fungus killed by AFP? One compelling explanation is that up-regulation of the CWI pathway might not be the most adequate response to counteract AFP. To pursue that hypothesis, we studied in this work the counteractive potential of the AFP-resistant yeast *Saccharomyces cerevisiae*. We choose *S. cerevisiae* as a model system because the architecture of its cell wall shares many similarities with the cell wall of filamentous fungi (Lesage and Bussey, 2006) and because a genome-wide deletion mutant collection is available. We speculated that mutations affecting processes which normally guarantee cell wall and plasma membrane integrity could render an AFP-resistant organism, such as *S. cerevisiae*, AFP-sensitive. Thus, by means of screening mutants of an AFP-resistant fungus for AFP-sensitive phenotypes, it may be possible to spot and analyze defense mechanisms which are essential to survive an AFP attack.

4.2. Experimental procedures

4.2.1. Strains, growth conditions and molecular techniques

All strains used in this study are given in Table 1 and Supplemental Table S1. *S. cerevisiae* strains are from the EUROSCARF collection (Frankfurt, Germany) containing gene deletions in the MATa background strain BY4741 (Brachmann et al., 1998). Yeast strains were cultivated in YPD medium (2 % peptone, 1 % yeast extract, 2 % dextrose; pH 6,5), whereas *A. niger* and *P. chrysogenum* were grown in YPD medium at pH 4.5. Alternatively, *A. niger* strains were cultivated in minimal medium (MM) (Bennett and Lasure, 1991) containing 1 % glucose as a carbon source (if not otherwise stated) or in complete medium (CM), consisting of MM supplemented with 1 % yeast extract and 0.5 % casamino acids. 10 mM uridine was added when required. Transformation of *A. niger*, selection procedures, genomic DNA extraction and diagnostic PCR were performed using recently described protocols (Meyer et al., 2010). Standard PCR, general cloning procedures in *E. coli* and Southern and Northern analyses were done according to (Sambrook and Russel, 2001).

4.2.2. Sensitivity tests towards AFP and calcofluor white

Sensitivity of yeast and filamentous fungal strains against AFP was determined using a protocol according to (Theis et al., 2003). In brief, 10^3 cells or spores were used to inoculate 150 µl YPD medium in the absence or presence of different AFP amounts (0.1 - 400 µg/ml). Cultivations were carried out in technical triplicates in microtiter plate format (28 °C, 28 h, 120 rpm) and repeated at least twice. Growth was assessed by measuring the optical density at 600 nm. CFW sensitivity was determined using the protocol according to (Ram and Klis, 2006) by spotting serial dilutions of exponentially growing cultures on YPG agar containing 50 µg/ml CFW. Growth was assessed after 48 h incubation at 28 °C.

4.2.3. SYTOX Green uptake assay

The assay was carried out in microtiter plate format using a slightly modified method described recently (Theis et al., 2003). 10^5 yeast cells were cultivated at 28 °C in 150 µl YPG medium for 12 - 16 h till they reached the mid-logarithmic growth phase. 1 µM SYTOX-Green and AFP (up to final concentrations of 400 µg/ml) were added. Fluorescence values were measured over time using a CytoFluor 2350 fluorescence measurement system (excitation 480 nm, emission 530 nm)

and corrected by subtracting values from AFP-untreated samples. All measurements were carried out in triplicates.

4.2.4. Cell wall polymer quantification

10^6 yeast cells or fungal spores were inoculated per ml of YPD medium (pH 6.5) and incubated in the presence (150 µg/ml) or absence of AFP. For some experiments, 50 nM BAPTA or 10 nM FK506 were added as well. After cultivations for 28 h at 28 °C, biomass was harvested and the amount of chitin and β-1,3-glucan determined. Cell wall chitin was isolated after (Ram et al., 2004), measured according to (Popolo et al., 1997; Tracey, 1956) and calculated per dry biomass. β-1,3-glucan levels were determined after (Jarrod R. et al., 2009) and calculated per dry biomass. All isolations and measurements were done from at least two independent experiments.

4.2.5. Microscopy

Cells or conidia (3×10^5) of *S. cerevisiae* or *A. niger* strains were inoculated in 3 ml liquid medium (YPD or MM supplemented with 0.003 % yeast extract) and cultivated on cover slips in the presence or absence of 400 µg/ml AFP for 28 h at 28 °C. For chitin visualizations, cells adherent to the cover slips were incubated for ten minutes in 10 µg/ml CFW and rinsed thereafter with water before subjected to microscopy. Samples were observed with an Axioplan 2 (Zeiss) equipped with a DKC-5000 digital camera (Sony) using differential interference contrast or DAPI settings. Images were captured with a 100x objective and processed using Adobe Photoshop 6.0 (Adobe Systems Inc.).

4.2.6. Construction of chs1Δcrz1Δ deletion strain of S. cerevisiae

The *CRZ1* gene was deleted in the *chs1*Δ knock out strain Y02020 (Euroscarf) by replacing it with the selection marker *URA3* of *Kluyveromyces lactis*. For this purpose, the *URA3* cassette from plasmid pGEMT-URA3 (unpublished, kindly provided by Udo Schmidt, TU Berlin) was PCR amplified which introduced *CRZ1* homologous flanks necessary for homologous recombination (for primers see Supplemental Table S2). *S. cerevisiae* transformants were selected by uracil prototrophy and *CRZ1* deletion was verified using diagnostic PCR.

4.2.7. Construction of an A. niger csmB deletion strain

A fusion PCR approach was used to construct a *csmB* deletion cassette. In brief, the promoter and terminator regions were amplified from genomic DNA of *A. niger* N402 using primer pairs given in Supplemental Table S2. As selection marker, the *A. fumigatus pyrG* gene was used which was amplified from GFP-AfpyrG cassette (Yang et al., 2004) using primers SMP1 and GFP2. The three amplified fragments were purified and subjected to a fusion PCR using primers PP1 and GSP4. The PCR product obtained was used to transform the *pyrG*$^{-}$ strain MA70.15 which allows targeted integration at high frequencies (Meyer et al., 2007a). Uracil-prototroph transformants were selected, purified and subjected to Southern analyses according to (Meyer et al., 2010) to confirm gene deletions.

4.2.8. Construction of an A. niger chsD overexpression strain and chitin assay

The open reading frame of the *chsD* gene (An09g02290) was PCR amplified from genomic DNA of the *A. niger* wild-type strain N402 and cloned downstream of the doxycycline-responsive promoter *TetO7::*P*min* in plasmid pVG2.2 (Meyer et al., 2011). Plasmid pVG2.2 contains three cassettes: P*gpdA::*rtTA2^{S}-M2::T*cgrA, TetO7::*P*min::*T*trpC* and *pyrG**. P*gpdA::*rtTA2^{S}-M2::T*cgrA* ensures constitutive expression of the doxycycline-responsive transcription factor rtTA2^{S}-M2, *TetO7::*P*min::*T*trpC* allows rtTA2^{S}-M2-dependent expression of a gene of interest when cloned downstream of *Pmin*, *pyrG** targets the complete plasmid to the *pyrG* locus of *A. niger* after which uracil-prototrophy is restored (van Gorcom and van den Hondel, 1988). pVG2.2-chsD was transformed into the AFP-sensitive *A. niger* strain MA169.4 (Carvalho et al., 2010) and uracil-prototroph transformants were selected and purified. Southern analyses according to (Meyer et al., 2010) confirmed integration of P*gpdA::*rtTA2^{S}-M2::T*cgrA-TetO7::*P*min::chsD::*T*trpC* at the *pyrG* locus in clone JP3-K4. 10^{6}/ml conidia of JP3-K4 were used to inoculate liquid CM containing 5 µg/ml of doxycycline (Dox) to induce expression of *chsD*. As a control, no Dox was added. After 16 h of cultivation, 10 µg/ml AFP were added to the cultures and after an additional incubation for 3 h in the presence or absence of AFP, chitin levels were determined as described above. The assay was repeated twice.

4.3. Results

We selected from the *S. cerevisiae* deletion strain collection 100 strains, all being deleted in a single non-essential gene (Table 1). This set of strains included mutants affected in cell wall and plasma membrane assembly and mutants disturbed in signaling processes such as cell wall integrity (CWI) signaling, calcium signaling, high osmolarity glycerol (HOG) signaling, protein kinase A (PKA) signaling and TOR signaling known to be important to fortify and preserve the yeast cell wall (Lesage and Bussey, 2006; Lesage et al., 2005; Levin, 2005). As for several yeast systems it has been reported that an elevated cell wall chitin content very often correlates with hypersensitivity to the chitin antagonist calcofluor white (CFW, (Lesage et al., 2005; Plaine et al., 2008; Roncero et al., 1988)), we also wished to determine whether there is a general relationship between chitin levels in *S. cerevisiae* and susceptibility to AFP. Hence, the selected strain collection also contained mutants displaying increased or decreased chitin levels compared to the wild-type *S. cerevisiae*.

Table 1: Grouping of deletion mutants according to their susceptibility against AFP

Group	Growth (%)*	Cell wall synthesis	Membrane synthesis	Endo-cytosis	CWI signaling	Calcium signaling	TOR signaling	PKA signaling	Other processes
A	10-19	*CHS1(19)*[$]							
	20-29				*WSC1*		*TOR1* *VPS34*		
B	30-39					*CCH1* *MID1 CNB1* *(15) CRZ1*			
	40-49					*RCN1* *CNA1* *CNA2*			
C	50-59			*END3*					
	60-69			*SNC1* *LDB19* *YPK1 (12)* *SUR7 PIL1* *LSP1 ART3* *SLA2*			*SLM1* *SLM2*	*GPA2*	
D	70-79				*SWI6*		*BEM2* *SRV2*	*PKA1*	
	80-89	*SMI1 (55)* *SFH5*			*SWI4 (17)* *MPK1(13)*		*SHE4 (63)* *ATG1*	*MSN2*	*BNI1(28)*
E	90-100	*CHS3(2)* *CHS4 (2)* *CHS5(3)* *CHS6 (2)* *CHS7 (2)* *BNI4 (13)* *CWP1* *CWP2* *FKS2*	*YEH2* *PDR16 SCS7* *FRT1 CHO2* *EKI1* *EPT1* *MUQ* *SFK1* *INP51* *ATG26* *ALG6 ALG5* *DIE2* *GSY1* *GSY2*		*WSC2* *WSC3* *WSC4* *MID2* *ROM1* *ROM2* *BCK1(13)* *MKK1* *MKK2* *RLM1*			*PLC1*	*SHO1* *HOG1* *MSN4* *MNN9 (131)* *ANP1 (113)* *MNN10 (97)* *SAC6 (58)* *GUP1 (54)* *BEM4(53)* *ILM1 (53)* *MNN11(47)* *CYK3 (44)* *CLA4 (31)* *FAB1 (23)* *WHI2 (21)* *PHO5(20)* *EDE1 (19)* *TUS1 (19)* *PKR1 (18)* *OPI3 (17)* *AST1 (16)* *MSN5 (15)* *EMP24 (15)* *ELO1 (14)* *HXT8 (14)* *DEP1 (12)*
F	>>100	*FKS (62)* *GAS1(81)*	*FPS1(17)*						

*: Each 10^3 cells were cultivated in YPD medium containing 400µg/mL AFP for 28 h. Growth is expressed in % compared to the control (cells not treated with AFP). The growth of the wild type strain BY4741 is 100% in both the absence and presence of AFP.

§: Chitin levels as determined in (Lesage et al., 2005) are given in brackets. For comparison, the chitin content of BY4741 according to (Lesage et al., 2005) is 16 nmoles GlcNac / mg dry weight.

4.3.1. AFP has the capacity to cause damage to S. cerevisiae.

All mutant strains were grown in YPD medium in the presence of 400 µg/ml AFP and cell growth was compared with cultivations in the absence of AFP. Under such high AFP concentrations, growth of the *S. cerevisiae* wt strain BY4741 is unaffected (Fig. 1 and (Theis et al., 2003)). In contrast, growth of 35 out of 100 deletion strains became compromised by AFP (Fig. 1 and Table 1), demonstrating that *S. cerevisiae* can become moderate-sensitive towards AFP. The inhibitory effect of AFP ranged from strong inhibition (residual growth of 10-29%, group A) to weak inhibition (residual growth of 70-89%, group D). For the majority of the strains, growth remained unaffected by AFP (59 strains, group E). Surprisingly, we also observed improved growth of three strains in the presence of AFP (group F), reminiscent of the paradoxical effect of increased survival of fungal strains at high antifungal drug concentrations (Wiederhold, 2009).

From the growth data obtained, three main conclusions were drawn. First, *S. cerevisiae* mutants with higher chitin content do not simply become AFP-sensitive (strains with up to 10 times higher wt chitin levels were tested, Table 1), suggesting that the overall chitin content is not per se important for the susceptibility against AFP. Second, from seven genes tested, known to orchestrate and catalyze chitin synthesis in *S. cerevisiae* (*CHS1, CHS3, CHS4, CHS5, CHS6, CHS7, BNI4*; note that CHS2 is an essential gene; (Lesage and Bussey, 2006)), only deletion of *CHS1* rendered *S. cerevisiae* AFP-sensitive, making Chs1p as the prime chitin synthase responsible for the resistance of *S. cerevisiae* against AFP. Thirdly, and most interestingly, deletions of almost all genes constituting the classical CWI signaling pathway (Levin, 2005), i.e. *WSC2, WSC3, WSC4, MID2, ROM1, ROM2, BCK1, MKK1, MKK2, MPK1, RLM1, SWI4* and *SWI6* did not or only marginally render *S. cerevisiae* sensitive towards AFP, suggesting that CWI signaling is of only minor importance to counteract any detrimental effects provoked by AFP. The only exception was the *wsc1*Δ strain, which like *chs1*Δ, felt into the sensitive group A.

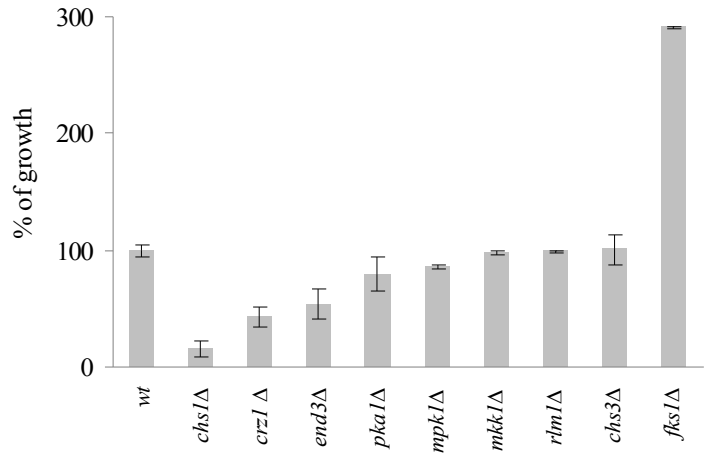

Figure 1: AFP susceptibilities of the *S. cerevisiae* wild-type strain BY4741 and selected deletion mutants when cultivated in YPD medium. 400 µg/ml AFP was used as final concentration. Growth of each strain is expressed in % compared to its growth in the absence of AFP. Growth is expressed as percentages compared with the negative control, which consisted of the strains cultivated in the absence of AFP. Error bars, S.D. for triplicate experiments

4.3.2. S. cerevisiae becomes vulnerable to AFP during cell separation.

Four mutants constituted the group of most susceptible strains – *chs1*Δ, *wsc1*Δ, *vps34*Δ and *tor1*Δ (Table 1). Chs1p acts as a septum repair chitin synthase to replenish chitin lost through chitinase activity during mother-daughter cell separation (Cabib et al., 1992). Wsc1p is, like Wsc2p, Wsc3p and Mid2p, a cell wall- and cell membrane-spanning sensor which signals cell wall stress to the CWI pathway (Lesage and Bussey, 2006; Rodicio and Heinisch, 2010). Some features of Wsc1p, however, distinguishes it from the other sensors, e.g. only Wsc1p localizes to sites of polarized growth, cycles in a cell cycle-dependent manner between the cytoplasm and the plasma membrane and becomes rapidly internalized by endocytosis after completion of cell separation (Grossmann et al., 2008). The gene *VPS34* encodes a phosphatidylinositol 3-kinase (PIK) which is required for phosphatidylinositol metabolism, for endocytic uptake and vacuole partitioning between mother and daughter cells during cell division (Herman and Emr, 1990; Slessareva et al., 2006). Moreover, its activity is also functionally linked to the Tor1p protein in *S. cerevisiae* and higher eukaryotes (Chang et al., 2009; Zurita-Martinez et al., 2007). Tor1p is a membrane localized PIK-related kinase and, as a subunit of the TORC1 complex, implicated in phosphatidylinositol metabolism and myriads of other processes including cell cycle regulation and endocytosis (Inoki et al., 2005).

Common to all four proteins is a function during cell separation, suggesting that *S. cerevisiae* might become vulnerable to AFP in a cell cycle-dependent manner. We thus questioned whether AFP affects cell separation in these mutants and, as shown for all sensitive filamentous fungi so far (Hagen et al., 2007; Theis et al., 2003), is able to permeabilise their plasma membranes. As shown in Figure 2A, the budding pattern of the wt strain was not compromised by AFP and cells separated well after budding. In contrast, *chs1*Δ, *wsc1*Δ, *tor1*Δ and *vps34*Δ cells remained attached to each other and formed aggregates in the presence of AFP, suggesting that AFP interrupts cell division. In addition, the plasma membranes of all four deletion strains became readily permeabilized by AFP, which was not the case for the wt strain (Fig. 2B). These results suggested that *S. cerevisiae* can become attacked by AFP, especially when its plasma membrane is exposed and not sufficiently or timely protected, e.g. by the formation of new cell wall chitin.

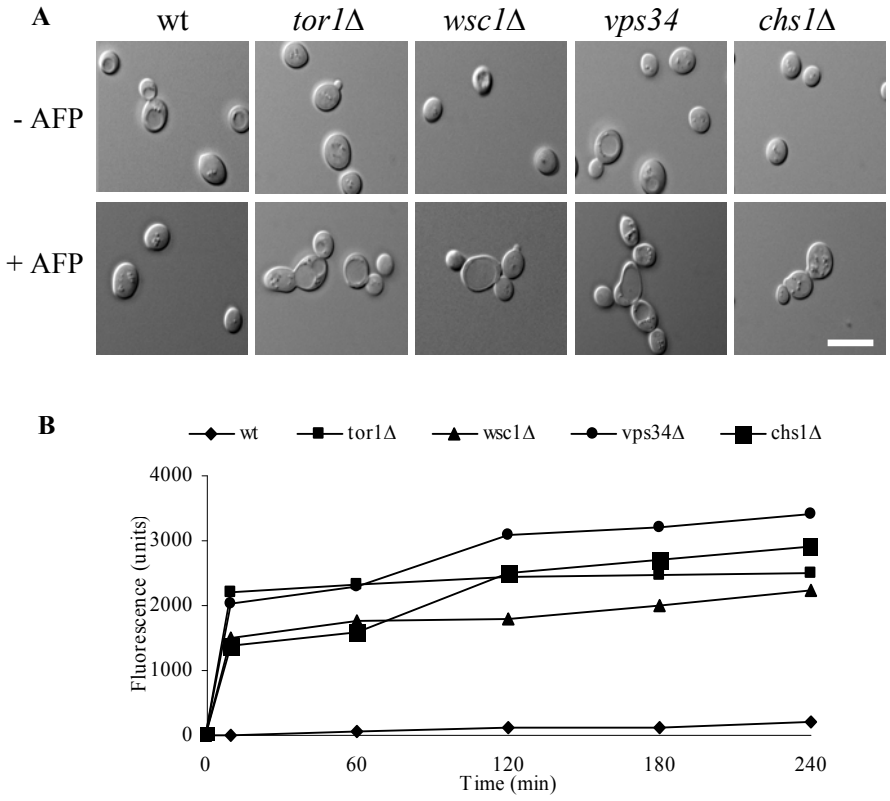

Figure 2: *S. cerevisiae* cell morphology and membrane integrity in the absence and presence of AFP. Cells were treated as described under "Experimental Procedures." A, differential interference contrast micrographs of WT BY4741 and mutant strains. Bars, 10 µm. B, AFP-mediated membrane-permeabilizing effect on mutant and wild-type strains of *S. cerevisiae* using the SYTOX Green uptake assay. This dye can only enter plasma membranes when compromised and previous work has shown that the degree of AFP activity can be correlated with the extent of fluorescence (Hagen et al., 2007; Theis et al., 2003).

4.3.3. S. cerevisiae counteracts AFP with increased chitin synthesis.

Having the moderate-sensitive strains *chs1*Δ, *wsc1*Δ, *tor1*Δ and *vps34*Δ in hand, we sought to identify mechanisms on how these strains responded to AFP and which defense strategies were used to counteract AFP inhibitory effects. For this purpose, we cultivated the wt and mutant strains in the presence and absence of AFP and quantified chitin and β-1,3 glucan levels of their cell walls. All five strains responded with increased chitin synthesis to AFP, whereby strongest response was observed in *chs1*Δ and *vps34*Δ cells (Fig. 3A, B). This data indicated that one counteracting mechanism of *S. cerevisiae* against AFP is fortification of the chitin layer, a defense strategy also used by *S. cerevisiae* to circumvent CFW inhibitory effects (Roncero et al., 1988). Notably, AFP obviously affected more cellular processes than CFW, because only *chs1*Δ and *vps34*Δ cells are vulnerable to CFW but not *wsc1*Δ and *tor1*Δ (Fig. 3C). β-1,3 glucan contents was albeit slight but significantly reduced in all five strains (Fig. 3D), suggesting that the stress-induced increase in chitin levels is paralleled by down-regulated glucan synthesis.

To assess whether the increase in cell wall chitin content in *S. cerevisiae* wt and mutant strains was due to the activities of Chs2p or Chs3p, we measured chitin levels in a *bni4*Δ strain stressed with AFP. Bni4p is a scaffold protein that specifically tethers Chs3p to the bud neck, thus regulating chitin synthesis at the chitin ring (Sanz et al., 2004). If Chs3p is the AFP-responsive chitin synthase, increased chitin synthesis would be abolished in a *bni4*Δ background; however, if Chs2p is the counteractive chitin synthase, increased chitin levels would still be observable in response to AFP treatment. As shown in Figure 3A, the chitin response towards AFP was completely lost in *bni4*Δ cells, strongly suggesting that AFP-stimulated chitin synthesis in *S. cerevisiae* WT and mutant strains is largely dependent on Chs3p.

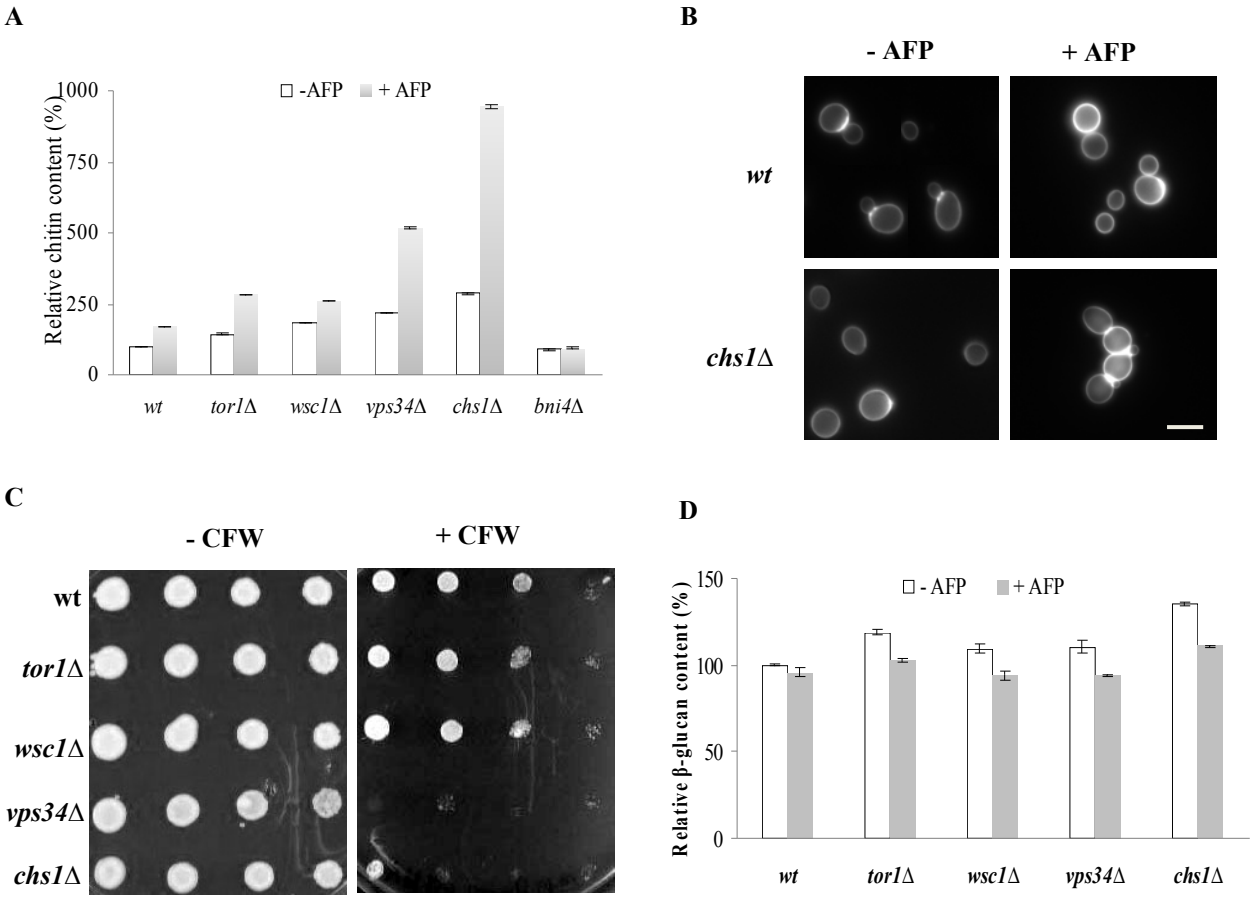

Figure 3: Cell wall remodelling of *S. cerevisiae* in response to AFP. Amount of chitin (*A*) and β-1,3-glucan (*D*) in mutants of *S. cerevisiae* in the absence and presence of 150 µg/ml AFP. The data are given relative to the chitin/glucan amount determined in the WT strain BY4741 in the absence of AFP (set as 100%). *Error bars*, S.D. for quadruple experiments. (*B*) Microscopic images of WT and *chs1*Δ strains stained with CFW. Pictures were taken using fixed exposure time (100 ms). The increase in CFW fluorescence intensity in the presence of AFP (400 µg/ml) reflects enhanced chitin levels at cell walls. *Bar*, 10 µm. (*C*) plate sensitivity assays using 50 µg/ml CFW. Equivalent numbers of cells were serially diluted, and aliquots were spotted on YPD medium containing or lacking CFW. Plates were photographed after 3 days of incubation at 28 °C.

4.3.4. The AFP-induced chitin response is transcriptionally regulated via Crz1p signaling.

Next, we questioned on how *S. cerevisiae* enforced chitin synthesis in response to AFP. Northern experiments revealed that the *S. cerevisiae* WT strain responded with increased transcription of all three chitin synthase genes *CHS1*, *CHS2* and *CHS3*, whereby expression of *CHS3* was stimulated most (Fig. 4A). As our mutant screen revealed that the CWI pathway was not the main pathway that rescued *S. cerevisiae* from AFP, but that deletions in the calcineurin/Crz1p pathway rendered *S. cerevisiae* more susceptible to AFP (Table 1), we suspected that the activity of the

latter might be responsible for these transcriptional up-regulations. Supportive for this assumption was the observation that *CRZ1* expression levels also increased when the wt was treated with AFP (Fig. 4A). The increase in chitin content was in fact absent in strains deleted for components of the calcineurin/Crz1p pathway *CCH1*, *MID1*, *CNA1*, *CNA2*, *CNB1* and *CRZ1* (Fig. 4B; note the calmodulin gene *CMD1* is an essential gene). Furthermore, when the *chs1*Δ strain was co-treated with AFP and the calcium chelator BAPTA, the increase in chitin content was reduced. The chitin response was almost fully abolished when *chs1*Δ was treated with AFP in the presence of the calcineurin inhibitor FK506 (Fig. 4C), strongly suggesting that an intact calcineurin/Crz1p signaling pathway mediates increased chitin synthesis in response to AFP and that the activity of this pathway is sufficient to protect *S. cerevisiae* against AFP. In agreement, we observed a mild sensitization of the wt strain when co-treated with BAPTA or FK506 (Fig. 4D).

To finally prove that increased chitin synthesis is largely controlled by Crz1p, we deleted the *CRZ1* gene in the *chs1*Δ background and determined the growth inhibitory effect of AFP and chitin levels in the double mutant in the presence or absence of AFP. As depicted in Fig. 5A, the growth inhibitory effect of AFP against *chs1*Δ*crz1*Δ is stronger when compared with the *crz1*Δ strain and comparable with the *chs1*Δ strain. Importantly, the increase in chitin amounts in response to AFP became completely lost in the *chs1*Δ*crz1*Δ strain (Fig. 5B), demonstrating that Crz1p is the main regulator responsible for the Chs3p-mediated chitin response.

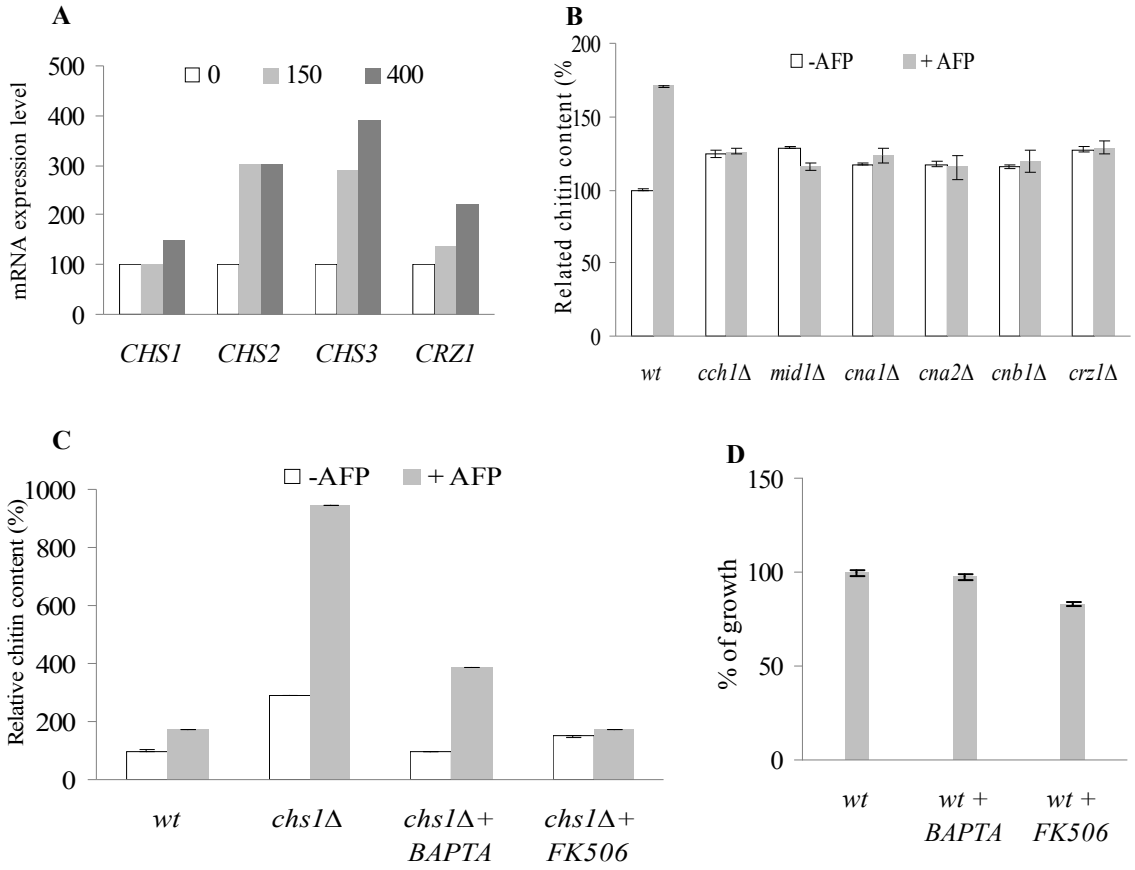

Figure 4: Calcium-dependent cell wall remodelling in *S. cerevisiae*. *A*, BY4741 cells harvested from a logarithmically grown culture were treated with different amounts of AFP (0, 150, and 400 µg/ml) for 1 h, after which total RNA was isolated and analyzed. Transcript levels were quantified by densitometry using the 18 S rRNA signal for calibration. All values are expressed relative to the respective untreated control. Data from a representative experiment are shown. *B* and *C*, amount of chitin in *S. cerevisiae* strains in the absence or presence of 150 µg/ml AFP. The data are given relative to the chitin amount determined in the WT strain BY4741 in the absence of AFP (set as 100%). *Error bars*, S.D. for triplicate experiments. *D*, AFP sensitivity assay of BY4741 (using 150µg/ml AFP) in the presence of 50 nM BAPTA or 10 nM FK506. These concentrations of BAPTA and FK506 were chosen because they are the maximum concentrations that do not inhibit growth of BY4741 (data not shown). The data are given relative to the growth of the WT strain BY4741 in the absence of AFP (set as 100%). *Error bars*, S.D. for quadruple experiments.

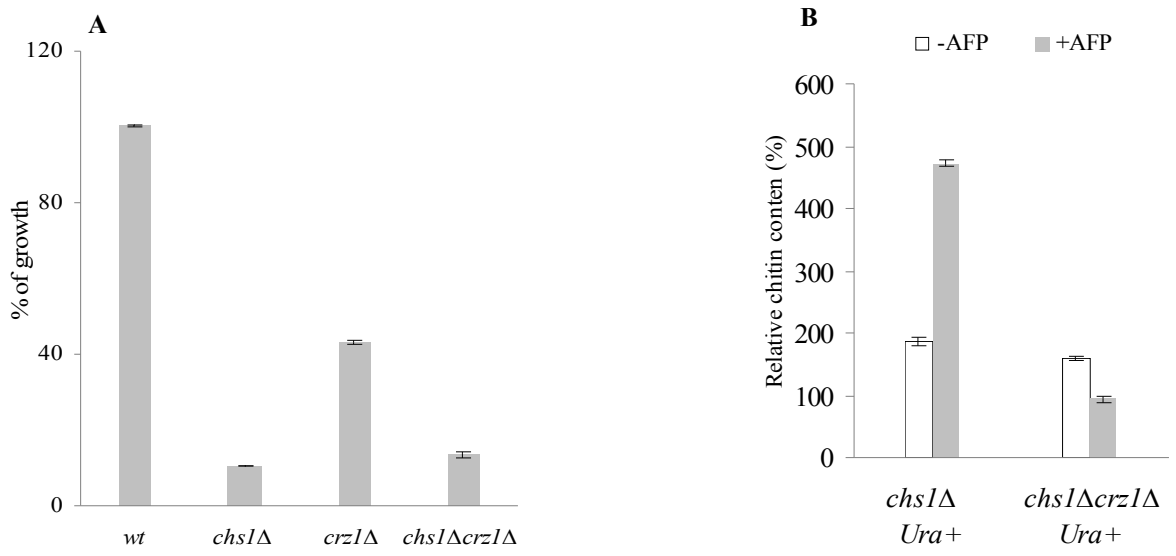

Figure 5: Crz1p-dependent chitin response in *S. cerevisiae*. *A*, growth of BY4741 and derived mutants in the presence of 400 µg/ml AFP. Growth is expressed as percentages compared with the negative control, which consisted of the strains cultivated in the absence of AFP. *Error bars*, S.D. for triplicate experiments. *B*, amount of chitin in the *chs1*Δ*crz1*Δ double mutant (strain JPSc1.4) when cultivated in the absence or presence of 150 µg/ml AFP. As a control, a *chs1*Δ strain was used in which the *URA3* selection marker, used to delete *CRZ1* (see "Experimental Procedures"), was integrated heterologously into the genome of the *chs1*Δ strain (strain JPSc1.6). We used this strain as a reference to avoid artifacts due to nonmatching auxotrophies between mutant and reference strain (Aronova, 2007). The data are given relative to the chitin amount determined in the WT strain BY4741 in the absence of AFP (set as 100%). Mean values of a duplicate experiment are given.

4.3.5. Increased chitin synthesis is a consistent response of moderate-sensitive and resistant fungi.

Previously, we have shown that chitin synthase activities are inhibited by AFP in sensitive fungi such as *A. niger*, *A. oryzae* and *F. oxysporum* (Hagen et al., 2007). As the present work revealed that resistant and moderate-sensitive *S. cerevisiae* strains responded with increased chitin synthesis to AFP, we speculated that elevated chitin synthesis may be a general cellular strategy of moderate-sensitive and resistant fungi to defend themselves against AFP. To prove or refute this assumption, we determined chitin levels in response to AFP in the AFP-resistant strain *P. chrysogenum* and in an *A. niger* mutant strain, in which we have deleted chitin synthase *csmB* (An02g02340, predicted class V chitin synthase). We have selected this chitin synthase because class V chitin synthases are specific for filamentous fungi (Roncero, 2002) and because its

expression level is modulated in response to cell wall stress (Meyer et al., 2007b). Indeed, for all yeast and fungal strains tested, we found a correlation between susceptibility and chitin synthesis (Table 2), i.e. reduced sensitivity is paralleled by increased chitin levels in response to AFP treatment in *S. cerevisiae*, *F. oxsporum chsV* mutant, *A. niger* Δ*csmB* mutant and *P. chrysogenum*. A higher sensitivity towards AFP, however, is observed in strains with down-regulated chitin synthesis, e.g. in *A. niger* WT, *A. oryzae* and *F. oxysporum*.

Table 2: Relative chitin response in selected fungal strains in response to the addition of AFP

Strain		Relevant genotype / Remark	Fold change[§]	MIC* (µg/ml)	Reference
A. niger	15/1801	wt	0.28	1	(Hagen et al., 2007)
	JP1	Δ*csmB*	1.33	10	This work
S. cerevisiae	BY4741	wt	1.69	> 400	This work
	Y02020	*chs1*Δ	7.19	> 400	This work
	Y05149	*vps34*Δ	4.37	> 400	This work
F. oxysporum	4287	wt	0.62	1	(Hagen et al., 2007)
	ChsV	Δ*chsV*	1.57	> 400	(Hagen et al., 2007)
A. oryzae	A1560	wt	0.60	1	(Hagen et al., 2007)
P. chrysogenum	ATCC 10002	wt	4.6	> 400	This work

*MIC: minimal inhibitory concentration; [§]: Chitin amounts were determined after AFP addition as described under Experimental procedures and related to the respective untreated controls (set as 1). Standard deviation is less than 15 %

4.3.6. Stimulation of cell wall salvage pathways lowers AFP sensitivity of A. niger

Recently, it was shown that pre-treatment of *Candida albicans* with activators of the calcium signaling pathway activated chitin synthesis and reduced the susceptibility of *C. albicans* towards the cell wall stressor caspofungin (Walker et al., 2008). We thus questioned whether the probability of survival also increases for a wt *A. niger* strain, when pre-treated with calcium. We

thus allowed spores of a wt *A. niger* strain to germinate, added $CaCl_2$ for defined incubation times, after which we removed it by washing the cells with fresh medium. After this procedure, growth of *A. niger* was assessed in the absence or presence of 10 µg/ml AFP. As shown in Figure 6, AFP-induced growth inhibition was less severe when germlings were primed with $CaCl_2$ - longer pre-incubation times even resulted in better survival rates - suggesting that a stimulated calcium signaling machinery can confer a higher protection against AFP in a filamentous fungus.

We finally wished to test the idea whether an artificially stimulated chitin synthesis can also improve survival rates of a wt *A. niger* strain. To examine this, we constructed an *A. niger* strain (JP3-K4), in which the *chsD* gene encoding the predicted Chs3p ortholog of *A. niger* (An09g02290, (Pel et al., 2007)) was put under control of the inducible Dox-dependent promoter. The addition of 5µg/ml Dox provoked about 31.2 ± 3.1 % more chitin in JP3-K4. As the MIC of AFP against JP3-K4 increased as well (from 1 µg/ml in the absence of Dox to 3 µg/ml in the presence of Dox), we concluded that reinforced chitin synthesis has indeed the potential to protect *A. niger* against AFP attack.

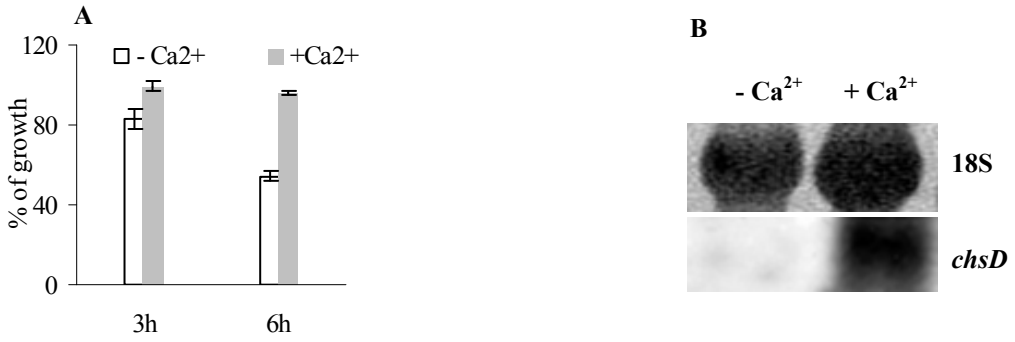

Figure 6: Effects of stimulated calcium signaling on AFP susceptibility of *A. niger*.

10^7 spores/ml of the WT strain N402 were allowed to germinate in YPD medium for 5 h, after which 100 mM of CaCl2 were added (as a negative control, H2O was added). After 3 or 6 h of further cultivation, germlings were washed twice with YPD and thereafter incubated in YPD supplemented without or with 10 µg/ml AFP. *A*, growth was assessed by measuring *A*600 after 24 h and is expressed as percentages compared with the growth of N402 in the absence of AFP (set as 100%). *Error bars*, S.D. for triplicate experiments. *B*, Northern analysis of *chsD* gene expression after 6 h of calcium priming. 5 µg of total RNA from calcium treated and non-treated samples were hybridized with a *chsD* probe. Methylene blue-stained 18 S RNA confirmed equal loading.

4.4. Discussion

With the hypothesis that the susceptibility of fungi towards AFP is also dependent on the cell's capacity to counteract AFP inhibitory effects, we sought to screen the yeast genomic deletion collection for AFP-sensitive mutants. The isolation of AFP-susceptible strains and their analysis in the present study strongly support the view that *S. cerevisiae* protects itself against AFP via stimulation of chitin synthesis which is mainly accomplished on transcriptional level via the calcium/calcineurin/Crz1p signaling pathway. A very likely candidate protein for the chitin rescue response is Chs3p as the chitin response was lost in a *bni4*Δ background. Most importantly, reinforcement of chitin synthesis is a response not merely specific for *S. cerevisiae* but is a distinctive feature of other AFP-moderate sensitive and AFP-resistant fungi, supporting the conclusion that an elevation of chitin levels provides the necessary response to survive an AFP attack. Such a defense strategy seems not to be realized in AFP-sensitive fungi. Here, the classical CWI pathway becomes activated whose output is an increase in glucan but not chitin synthesis (Hagen et al., 2007). This response, however, obviously fails to counteract AFP.

4.4.1. The damage-response framework of AMP - microbial interactions

The microbial defense strategy is thus an important parameter that determines the susceptibility of fungi against AFP, a conclusion that most likely can be extrapolated to the interactions of microorganisms with other AMPs. If the most adequate response has been initiated, the microorganism can resist an AMP attack, if a too weak or an inappropriate response has been opted; the microorganism becomes damaged or killed. Importantly, many signaling mechanisms such as calcium signalling meant to rescue from cell stress have the capacity to damage the microorganism itself (Berridge et al., 2000; Zhang et al., 2006). Thus, a microbial response has to be envisioned as a tightrope walk between survival and death. To comprehensively understand the inhibitory effect of AFP and in general of AMPs, we therefore propose that not only the defense strategy has to be taken into account but also a potentially self-induced damage by the microorganism. We therefore recommend the adoption of the concept of the 'damage-response framework of microbial pathogenesis' put forward by Pirofksi and Casadevall in 1999 (Casadevall and Pirofski, 1999; Casadevall and Pirofski, 2000; Casadevall and Pirofski, 2003) to the analysis of interactions between AMPs and microorganism. When translating the tenets of

this conceptual approach to microbial-AMP interactions, one can postulate that the outcome of an AMP attack is dependent on i) the innate susceptibility of the microorganism, ii) the damage potential of the AMP defined by its concentration and target-(non)specific molecular interactions and iii) the microbial response, which can be appropriate, too weak or too strong and thus detrimental to the host. Consequently, the damage-response framework plots the microbial damage as a function of the microbial response (Fig. 7A) and predicts that the cytotoxic capacity of a given AMP is relative and depends not only on the genetic background and AMP concentration but also on other variables such as microbial survival strategies.

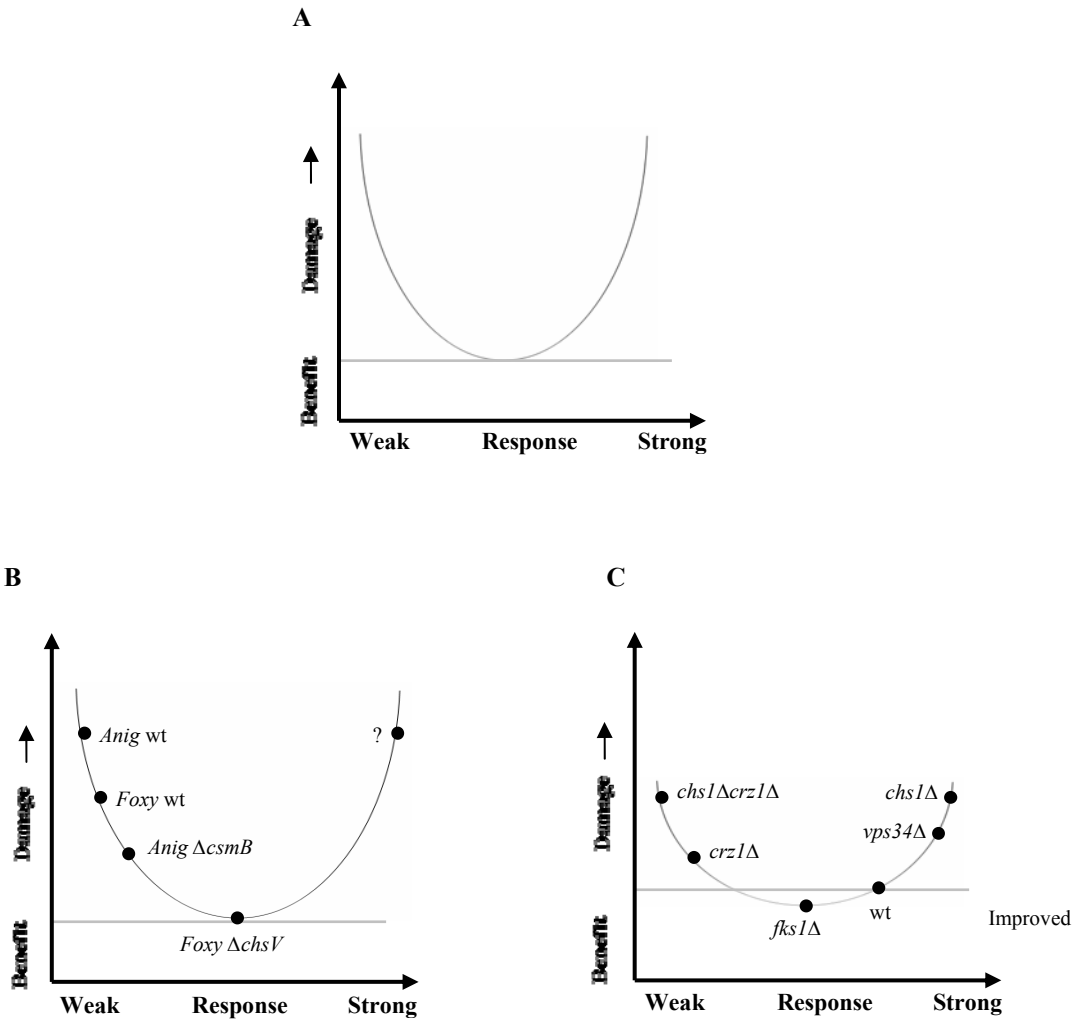

Figure 7: Cytotoxic activities of AMPs and the microbial response. *A*, the damage-response framework of microbial pathogenesis is reflected by a parabolic curve (Casadevall and Pirofski, 1999; Casadevall and Pirofski, 2000; Casadevall and Pirofski, 2003). When translating this concept to the AMP-microorganism interaction, the *y* axis denotes microbial damage, which is defined as a perturbation of cell homeostasis. The microbial damage is a function of the microbial response, which, in terms of quality and quantity, can be considered as weak, appropriate, or (too) strong. Both a too weak and a too excessive response will damage or kill the microorganism. *B*, the *csmB* and the *chsV* mutation are protective for *A. niger* and *F. oxysporum*, respectively, where by the latter makes *F. oxysporum* insensitive against AFP. With a *question mark* we denote the possible scenario in which a signaling pathway meant to protect the microorganisms causes self-damage when deregulated (*e.g.* increased cytoplasmic calcium concentrations might induce apoptosis). *C*, the *curve* in *C* is more flattened compared with *B* to indicate that´*S. cerevisiae* is in general less susceptible to AFP than filamentous fungi. Plotted are different *S. cerevisiae* deletion mutants, which we observed to enhance or diminish AFP-induced damage based on their chitin response. Most importantly, the damage-response framework also opens the possibilities to include the paradoxical effect of improved growth in the presence of AFP, which we have observed for three *S. cerevisiae* mutants (Table 1, group F).

4.4.2. The damage-response framework of AFP - fungal interactions.

A common feature of all AFP-sensitive and moderate-sensitive fungi analyzed so far is that their plasma membranes get permeabilized within minutes ((Hagen et al., 2007; Theis et al., 2005; Theis et al., 2003) and Fig. 2), suggesting that the plasma membrane represents the Achilles heel of yeast and filamentous fungi when exposed to AFP. The damage-response framework of AFP-fungal interactions opens up many possible explanations why some fungi survive the plasma membrane attack and some not or only partially (Fig. 7B, C). For example, a fungus can harbor a hypothetical AFP target but is resistant because of being fully competent in mitigating AFP (e.g. via increased chitin synthesis in *S. cerevisiae* and in *P. chrysogenum*) or because the target might be shielded because the cell wall is remodeled (e.g. as supposed for the Δ*chsV* mutant of *F. oxysporum* (Madrid et al., 2003) and as also possible in the *A. niger* Δ*csmB* strain). Alternatively, a fungus may contain a modified AFP target or less of it but is nevertheless susceptible because of being incapable to counteract AFP (e.g. the *chs1*Δ*crz1*Δ mutant of *S. cerevisiae*). One imaginable example for a too strong response would be an excessive calcium response, a hypothetical scenario that remains to be scrutinized. However, for the AFP-related protein PAF it has been shown that the PAF-sensitive fungus *Neurospora crassa* reacts with strongly increased intracellular calcium levels to PAF exposure (Binder et al., 2010). It is thus conceivable that such a response might be causally linked to the induced programmed cell death phenotype observed in *A. nidulans* after treatment with PAF (Leiter et al., 2005).

4.4.3. The damage-response framework of AFP - S. cerevisiae interactions

Out from 100 yeast deletion strains selected, growth of 35 became compromised by AFP. These mutants were affected in different cellular processes (mainly chitin synthesis and endocytosis) and also in different signaling pathways (calcium signaling, TOR signaling, cAMP-PKA signaling, CWI signaling; Table 1). On the one hand this suggests that interference with chitin synthesis and endocytosis might render *S. cerevisiae* vulnerable to AFP and on the other hand that the concerted activities of these signaling pathways might be important to resist AFP.

Why are chitin synthesis and endocytosis important for the resistance of *S. cerevisiae* against AFP? The *chs1*Δ mutant showed highest susceptibilities towards AFP, suggesting that *S.*

cerevisiae can become attacked by AFP especially during mother-daughter cell separation. This process is indeed very critical for cell integrity maintenance as the controlled activity of chitin synthesis (Chs1p) and chitin degradation (Cts1p) is necessary to separate both cells (Cabib et al., 1992). If the chitin layer is not timely closed, the plasma membrane might not be shielded sufficiently and could potentially be permeabilized by AFP. This scenario could also explain why the *wsc1*Δ strain is very sensitive towards AFP, because the main function of the mechanosensor Wsc1p (but not Mid2p) is to sense cell wall stress during cell separation (Rodicio and Heinisch, 2010). If such as stress signal is not generated, *S. cerevisiae* might not sufficiently or not timely enough respond to the AFP attack. Interestingly, Chs1p and Chs3p follow the same exocytotic and endocytotic route using chitosomes as transport vesicles (Lesage and Bussey, 2006; Ziman et al., 1996). They become mobilized to the plasma membrane in a cell cycle-dependent manner to synthesize chitin at the bud neck after which they become endocytosed into chitosomes. Hence, endocytosis is essential to maintain the chitosome pool (Lesage and Bussey, 2006; Ziman et al., 1996). Our growth-inhibitory screen uncovered nine endocytotic mutants (group C, Table 1), two of which, *end3*Δ and *sla2*Δ, have been shown to contain reduced Chs3p levels in purified chitosomes (Ziman et al., 1996; Ziman et al., 1998) and also *vps34*Δ, whose function is important for endocytic sorting (Strahl and Thorner, 2007). It is thus tempting to speculate that the blockage of endocytosis might interfere with the chitosomic reservoir of Chs1p and Chs3p which in turn cannot be mobilized during AFP attack.

Notably, not all *S. cerevisiae* signaling mutants were similarly impaired in growth, indicating that the respective pathways contribute to a varying extent to AFP counteraction (Fig. 8). Based on the growth inhibition assays, we conclude that the CWI pathway is only of minor importance to protect *S. cerevisiae* against AFP (residual growth of 80-100%; the only exception is Wsc1p with 25%). We caution however that Rho1p and Pkc1p could still be involved in the survival responses as both proteins escaped from our analysis (*rho1*Δ and *pkc1*Δ strains are not viable). However, if an AFP stress signal - supposedly recognized by Wsc1p - is transmitted to both proteins, the signal is likely not channeled into the MAP kinase cascade of the CWI pathway. A clear growth inhibition (residual growth 60-89%) was observed when strains deleted for components of the cAMP-PKA signaling pathway (*GPA2*, *PKA1*, *MSN2*) were subjected to AFP. cAMP-PKA signaling is in fact a nutrient-sensing pathway that controls the cell cycle and transmits a glucose signal to various effectors, one of which can be the stress transcription factor

Msn2p (Ohdate et al., 2010; Tamaki, 2007). Interestingly, cAMP-PKA signaling is intertwined with both calcium and TOR signaling via Msn2p under nutrient starvation conditions (Beck and Hall, 1999; Ohdate et al., 2010) and with Wsc1p under thermal stress conditions (Fuchs and Mylonakis, 2009).

Calcium signaling and TOR signaling seemed to be the most crucial pathways for the rescue of *S. cerevisiae* from AFP attack (residual growth of 20-50%). Our data showed that stimulation of the chitin defense response is virtually completely dependent on the calcium signaling machinery, emphasizing the importance of calcium signaling for survival. Remarkably, all deletion strains of this signaling cascade (*cch1*Δ, *mid1*Δ, *cna1*Δ, *cna2*Δ, *cnb1*Δ, *rcn1*Δ and *crz1*Δ) showed similar susceptibilities towards AFP, suggesting that once a calcium signal had entered the pathway (hypothetically as calcium influx via the plasma membrane channels Cch1p and/or Mid1p), the signal was directly channeled to its effector Crz1p.

The *tor1*Δ mutant was among the most susceptible strains probably due to the fact that Tor1p occupies a central position in the regulatory network that balance growth, proliferation and survival of *S. cerevisiae* (Fuchs and Mylonakis, 2009; Inoki et al., 2005). 229 genetic and 175 physical interactions are recorded for Tor1p in the Saccharomyces Genome Database, demonstrating its broad scope of cellular and regulatory functions. Among the AFP-susceptible strains identified in this study were also mutants deleted in Tor1p effector proteins such as Sla2p, She4p, Vps34p, Ypk1p (endocytosis, (Aronova et al., 2007; Zurita-Martinez et al., 2007)), Mpk1p (CWI signaling, (Levin, 2005)) and Pka1p (cAMP-PKA signaling, (Tamaki, 2007)), hinting at the possibility that Tor1p might contribute to the cellular defense against AFP via multiple pathways.

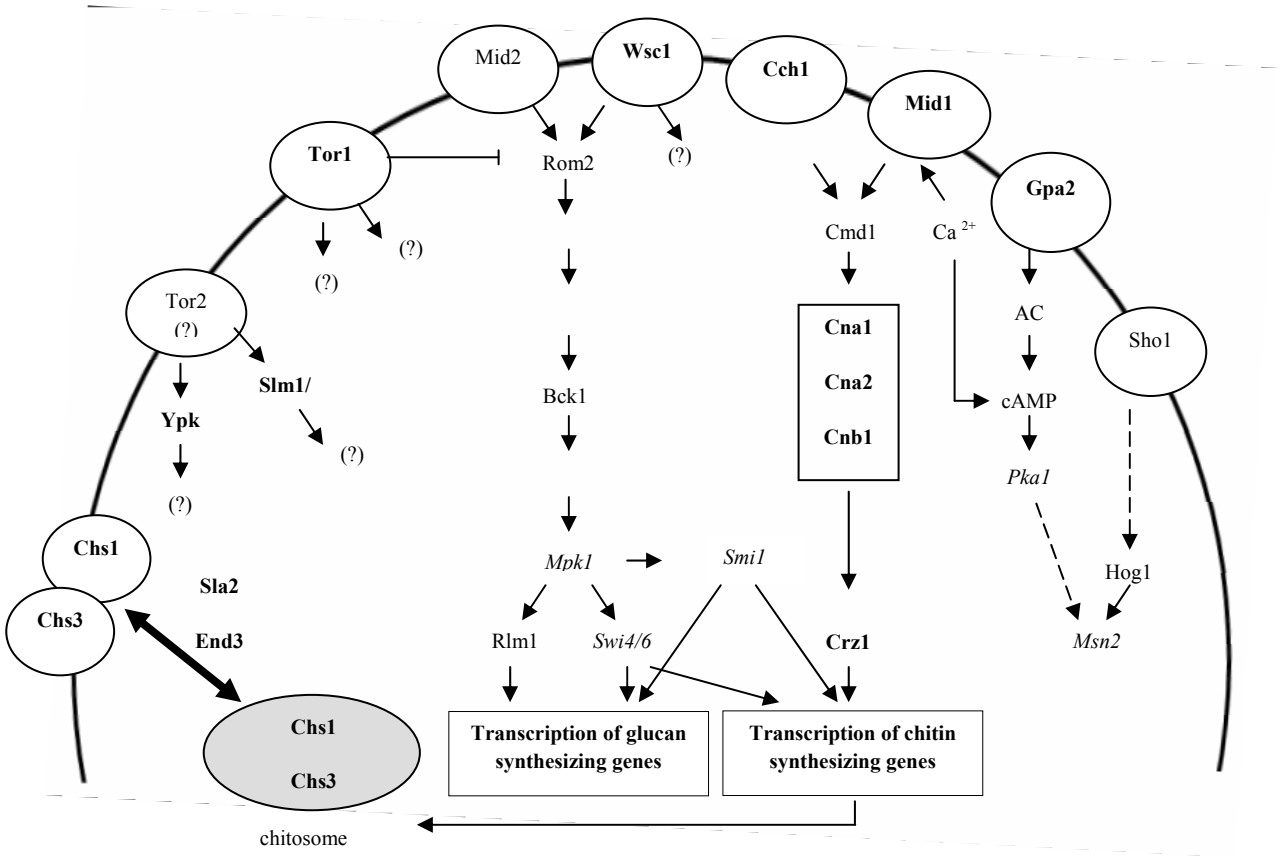

Figure 8: Proteins and signaling pathways of *S. cerevisiae* involved in AFP counteraction. Proteins that are of major importance for the defense against AFP are indicated in *boldface type*, and proteins that contribute to a lesser extent are given in *italics*. Proteins that were not included in the screening assay (*e.g.* because of essential cell functions) are indicated with a *question mark*. The interconnection of proteins into signaling networks is based on Refs. (Levin, 2005; Lesage and Bussey, 2006; Lesage et al, 2005).

4.5. Conclusions

The genetic approach followed in this work allowed the identification of a valuable collection of AFP-sensitive *S. cerevisiae* deletion strains, whose initial analysis revealed that a concerted action of calcium signaling, TOR signaling, cAMP-PKA signaling and CWI signaling is likely to safeguard *S. cerevisiae* against AFP. Biochemical analyses of selected strains uncovered that the cell wall of *S. cerevisiae* gets fortified with chitin in the presence of AFP and that this response is largely dependent on transcriptional stimulation via the calcium/calcineurin/Crz1p pathway. A

comparative analysis of different fungi showed a correlation between chitin response and AFP susceptibility, suggesting that an increase in cell wall chitin is the best strategy for yeast and filamentous fungi to survive AFP. Further analyses will decipher how other fungal survival strategies contribute to the defense against AFP and to which extent the respective signaling pathways are interconnected. We finally propose the adoption of the damage-response framework of microbial pathogenesis to the interactions of AMPs and microorganisms in order to comprehensively understand microbial survival strategies.

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SUPPLEMANTAL TABLE S1: Strains used in this study.

Strain	Relevant genotype	Source
A. niger		
N402	wild type	Lab collection
MA70.15	Δ*kusA, pyrG*$^{-}$	(Meyer et al., 2007)
MA169.4	*kusA*$^{-}$*, pyrG*$^{-}$	(Carvalho et al., 2010)
JP1	Δ*kusA,* Δ*csmB::AfpyrG*	This work
VG5.1	Singly copy of plasmid pVG2.2 at the *pyrG* locus	(Meyer et al., 2011)
JP3-K4	Singly copy of plasmid pVG2.2-chsD at the *pyrG* locus	This work
P.chrysogenum ATCC10002	wild type	Lab collection
S. cerevisiae		
BY4741	*MAT**a*****; *his3*Δ*1; leu2*Δ*0; met15*Δ*0; ura3*Δ*0*	(Brachmann et al., 1998)
Single deletion strains	BY4741 background using *kanMX4* as selection marker	EUROSCARF
JPSc1.4 (*chs1*Δ*;crz1*Δ*)*	*MAT**a*****; *his3*Δ*1; leu2*Δ*0; met15*Δ*0; ura3*Δ*0; YNL192W::kanMX4*; *YNL027W::URA3*	This work
JPSc1.6 (*chs1*Δ*)*	*MAT**a*****; *his3*Δ*1; leu2*Δ*0; met15*Δ*0; ura3*Δ*0; YNL192W::kanMX4*; *URA3*	This work

SUPPLEMANTAL TABLE S2: Primers used in this study

Primer	Target	Sequence (5´→ 3`)
csmB PP1	*csmB* promoter	TAACCGGAGCGTAAGCTAATACCTTGACAT
csmB PP2	*csmB* promoter	GATAGAGCGGAGCATAATCAGCCAACTAAC
csmB GSP3	*csmB* terminator	TCCTGCAGCACGCATACATAGATAACATAC
csmB GSP4	*csmB* terminator	AGCGTCTCGAGTTGATACATATTACGAGTC
csmB SMP1	Af-pyrG	*GTTAGTTGGCTGATTATGCTCCGCTCTATC*ACCGGTCGCC TCAAACAATGCTCT
csmB GFP2	Af-pyrG	*GTATGTTATCTATGTATGCGTGCTGCAGGA*GTCTGAGAGG AGGCACTGATGCG
Fw chs1	*chs1* ORF	GCGCACGGTGAGGCAGTTGAAGT
Rev chs1	*chs1* ORF	GCGCCACATGCGCCACCAACATT
Fw chs2	*ch2* ORF	GTTCCACAGTGCAGGCTGTA
Rev chs2	*chs2* ORF	GACTGCCATAAGCGCGTTAC
Fw chs3	*chs3* ORF	GGATACTTCCTCGCGTTCCG
Rev chs3	*chs3* ORF	GGTTGAAGCACGCTTGTGTC
Fw crz1	*crz1* ORF	ATGCCATGGCAGTACAAGGAAGATGTCATTC
Rev crz1	*crz1* ORF	GATCTGCCATCTCCAATAAC
Fw tor1	*tor1* ORF	CGTCTAGATATGGAACCGCATGAGGAG
Rev tor1	*tor1* ORF	GGAGGCAGTAAGCCATTCTAAG
Fw vps34	*vps34* ORF	GCGAGTCTGAGGACAAGCCATAT
Rev vps34	*vps34* ORF	GCGGTAGCCAAGAATACCGTGAT
Fw crz1-URA3	pGEMT-URA3	CACCCCACCTCTTCCATATCCCACGTAATGCGCCAAGC TATTTAGGTGAC
Rev crz1-URA3	pGEMT-URA3	AGTACGCTAGGGTCGATATGATCTGTCCCTGGCGATTA AGTTGGGTAACG
FW pmeI-chsD	*chsD* ORF	AGCTTTGTTTAAACGGCGCGCCGGTGACATCTGCATGC GCAATCGG
Rev pmeI-chsD	*chsD* ORF	AGCTTTGTTTAAACGGCGCGCCGGTGAGTCGCGAAACC TTACGG

Complementary overhangs used in the fusion PCR are indicated in italics. Underlined sequences were added for cloning purposes.

Chapter 5

Screening for compounds exerting antifungal activities

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Abstract

There is a strong demand for the discovery of new antifungal drugs. More and more human and plant pathogenic fungi develop resistance against currently used drugs and do therefore not respond to antifungal treatments. As humans and fungi are both eukaryotic cells in which many molecular processes are conserved, compounds that have antifungal activity are also often toxic for humans. To circumvent this, it is important to develop methods and screens for the identification of compounds which specifically kill fungi but do not affect men and environment. Here, we describe methods to screen compounds for their ability to prevent growth of the filamentous fungus *Aspergillus niger*, to monitor whether these compounds are fungicidal and whether they switch on the *agsA* reporter system, which is representative for cell wall or cell membrane stress.

Key words: antifungal, *Aspergillus*, cell wall, cell membrane, susceptibility assay

5.1. Introduction

Both the plasma membrane and the cell wall of fungi contain components which are unique to the fungal kingdom. Hence, drugs that interfere with the biosynthesis of these components are likely to be fungal-specific. Azoles, polyenes and echinocandins are three groups of drugs that are used nowadays to treat fungal infections (Ostrosky-Zeichner et al., 2010). Azoles inhibit ergosterol synthesis in fungi, which is the cholesterol equivalent of animal membranes. The four currently used azoles include fluconazole, itraconazole, voriconazole and posaconazole and these drugs block ergosterol biosynthesis by inhibiting the activity of the cytochrome P450 lanosterol demethylase (Odds et al., 2003). Fenpropimorph as a morpholine also inhibits ergosterol biosynthesis and protects plants against pathogenic fungi (Marcireau et al., 1990). Polyenes are amphiphatic drugs that strongly bind to ergosterol and create channels thereby disrupting the integrity of fungal membranes. The most often used polyene, amphotericin B, is effective against several pathogenic fungi; however, its use is restricted because of detrimental side effects on mammalian cells (Ostrosky-Zeichner et al., 2010).

In addition to the cell membrane, fungal cells are surrounded by a cell wall, which is essential for the fungus to withstand the internal turgor pressure. The fungal cell wall is composed of chitin, beta-glucans and mannosylated proteins. Depending of the fungal species, polysaccharides (e.g. alpha-glucans, galactomannans) can be present as well (Klis et al., 2007; Latge, 2007). Some of these components are covalently linked and connected to each other to ensure the rigidity and strength of fungal cell walls. Echinocandins are currently the only class of antifungals that target the biosynthesis of the fungal cell wall. The three echinocandins used in medicine (caspofungin, micafungin and anidulafungin) inhibit the function of the (1,3)-beta-D-glucan synthase, which is an essential enzyme for fungal cell wall biosynthesis (Kartsonis et al., 2003).

Whether a fungal cell is directly killed by a drug is dependent on a variety of factors. Both the concentration of the drug as well as the intrinsic resistance of the specific fungus are important factors that determine drug sensitivity. At non-lethal concentrations, the drugs can trigger stress responses that can make the fungal cell more resistant towards the drugs. It has been well established that the addition of drugs that interfere with cell membrane or cell wall biosynthesis trigger the cell wall stress response pathway (Agarwal et al., 2003; Meyer et al., 2007; Ouedraogo et al., 2011). The pathway is partially conserved in fungi such as the yeasts *Saccharomyces cerevisiae* and *Candida albicans* and the filamentous fungi such as

Aspergillus fumigatus ands *A. niger* (Damveld et al., 2005; Levin, 2005; Valiante et al., 2009). We have previously shown that the induction of the *agsA* gene from *A. niger*, which encodes a putative (1,3)-alpha-D-glucan synthase is a very suitable and specific reporter to monitor fungal cell wall stress (Damveld et al., 2005; Meyer et al., 2007).

In the following, we describe microtitre- and microscopic-based methods to identify compounds which are fungicidal and, moreover, induce the *agsA* reporter. These methods allow the set up of high-throughput approaches to identify potential drugs which very specifically disrupt fungal-specific mechanisms essential for survival.

5.2. Materials

1. Glucose (50%): For 1 liter: Boil 500 ml Milli-Q (MQ) in a 1000 ml beaker on a heated magnetic stirrer. Slowly add 500 g of D(+)-Glucose anhydrous. After glucose has been dissolved, let the solution cool down to RT, add MQ up to 1 liter and autoclave.
2. ASPA+N (50 ×): For 1 liter: Add 297.5 g (3.5 M) $NaNO_3$, 26.1 g (0.35 M) KCl and 74.8 g (0.55 M) KH_2PO_4 to 600 ml MQ in a 1 liter cylinder. When all salts are dissolved, set pH to 5.5 with KOH (use 5 M KOH). Add MQ up to 1 liter and autoclave.
3. ASPA-N (50 ×): For 1 liter: Add 26.1 g (0.35 M) KCl and 74.8 g (0.55 M) KH_2PO_4 to 600 ml MQ in a 1 liter cylinder. When dissolved, set pH to 5.5 with KOH. Add MQ up to 1 liter and autoclave.
4. $MgSO_4$ (1M): For 1 liter: Add 246.5 g $MgSO_4 \cdot 7\ H_2O$ to 600 ml MQ in a 1 liter cylinder. When all salts are dissolved, add MQ up to 1 liter and autoclave.
5. Trace element solution (1000 ×): For 1 liter: Add 10 g (26.9 mM) EDTA, 4.4 g (15.3 mM) $ZnSO_4 \cdot 7\ H_2O$, 1.01 g (5.1 mM) $MnCl_2 \cdot 4\ H_2O$, 0.32 g (1.3 mM) $CoCl_2 \cdot 6\ H_2O$, 0.315 g (1.3 mM) $CuSO_4 \cdot 5\ H_2O$, 0.22 g (0.18 mM) $(NH_4)_6\ Mo_7O_{24} \cdot 4\ H_2O$, 1.11 g (10mM) $CaCl_2$ and 1.0 g (3.6 mM) $FeSO_4 \cdot 7\ H_2O$ to 600 ml MQ. When dissolved, set pH to 4.0 with NaOH (use 1 M NaOH; 40 g/l) and HCl (use 1 M HCl; 75 ml 37% hydrochloric acid/l), fill MQ up to 1 liter and autoclave. (*see* Note 1)
6. Vitamin solution (1000 ×): For 100 ml: Add 100 mg thiamin-HCl, 100 mg riboflavin, 100 mg nicotinamide, 50 mg pyridoxine, 10 mg pantotenic acid, 2 mg biotin to 50 ml of warm MQ (about 50-60°C) in a 100 ml cylinder. When all vitamins are dissolved, add MQ up to 100 ml, sterilize by filtration and store at 4°C under dark conditions.

7. Minimal medium (MM): For 500 ml: Add under sterile conditions to 480 ml of sterile MQ: 10 ml of 50% glucose , 10 ml of 50 × ASPA+N, 1 ml of 1 M $MgSO_4$ and 500 µl of 1000 × trace element solution. For MM+agar, autoclave 480 ml of MQ with 7.5 g of agar (Scharlau) and add all components after autoclaving under sterile conditions.
8. 2 x Minimal medium (2 x MM): 2% glucose, 2 x ASPA+N, 4 mM $MgSO_4$, 2 x trace element solution, 0.06 % Yeast extract.
9. Complete medium (CM): For 500 ml: Add 0.5 g casamino acids, 2.5 g yeast extract and if required, 7.5 g agar to 480 ml of MQ and autoclave. Afterwards, add under sterile conditions: 10 ml of 50% glucose, 10 ml of 50 × ASPA+N, 1 ml of 1 M $MgSO_4$, 500 µl of 1000 × trace element solution.
10. Saline solution: For 1 liter: Add 9 g (0.9% w/v) NaCl to 900 ml MQ in a 1 liter cylinder. When NaCl is dissolved, add MQ up to 1 liter and autoclave.
11. YPD medium: 0.3% yeast extract, 1% bactopeptone, 2% glucose
12. Myracloth (Calbiochem)
13. Cotton sticks (Hecht)
14. Flat bottom 96 wells plate (transparent, Sarstedt)
15. V-bottom 96 wells plate (Sarstedt)
16. Polystyrol 96 well plate, black (Greiner)
17. Multichannel pipette (20-300 µl) (Rainin)
18. Microtitre plate reader (e.g. Victor^3, Perkin Elmer)
19. Incubator (Heraeus)
20. Inverted microscope (Leica ICC50)
21. Microscope counting chamber
22. SYTOX-Green (Invitrogen, Paisley, UK)
23. Fluorescence microscope allowing both light and fluorescence imaging (GFP).

5.3. Methods

3.3.1. Inhibitory testing using growth assays

This method can be used to generally test growth inhibitory effects of compounds towards fungi like *A. niger*. To study growth inhibition, the optical density of the cultures is followed

and visualized by microscopic means. Figure 1 depicts growth inhibition of *A.niger* in a 96-well plate incubated with different concentrations of the antifungals caspofungin and fenpropimorph.

1. Prepare spore solution of *A. niger* wild type strain as follows: Streak spores from a single colony on a CM agar plate and incubate until the plate is abundantly covered with sporulated mycelium (3-6 days, 25-37 °C).
2. In order to harvest spores from CM agar plate, add 10 ml of saline solution to the plate and carefully release spores by scraping over the surface plate with a sterile cotton stick.
3. Pipet spore solution from the plate into a sterile 15 ml tube. If required, remove mycelial debris (vegetative mycelium, conidiophores) by filtration through a sterile myracloth filter.
4. Count spores using a microscope counting chamber.
5. Prepare a spore solution with the final titre of 7.5 x 10^5 spores/ml. (*see* Note 2)
6. Start the growth inhibition assay with letting the spores germinate first: Fill each well of a flat-bottom 96 well plate with 30 µl sterile MQ, 50 µl 2 x MM and 20 µl *A.niger* spore solution. Close the plate with a lid and incubate for 7 hours at 30°C.
7. Prepare a compound stock plate for efficient and fast addition of the compounds by using a V-bottom 96 well plate: Prepare serial dilutions of the compounds and add each 40 µl per well. Every compound should be tested in triplicate. Pipet also 40 µl water or any other solvent used as negative control to at least three wells. Store the compound plate at 4°C until germination is finished.
8. Add 45 µl sterile MQ and 75 µl 2 x MM to each well of the germination plate using a Multi channel pipette.
9. Transfer 30 µl of the compounds (and controls) from the compound plate to the germination plate using a Multi channel pipette.
10. Incubate the growth inhibition plate at 30 °C and record the kinetics of growth every hour by measuring the optical density at 620 nm (Fig. 1A). Incubate for a maximum of 24 hours. (*see* Note 3)
11. Visualize growth of *A. niger* in the 96 wells plate of each condition via microscopy (Fig. 1B).

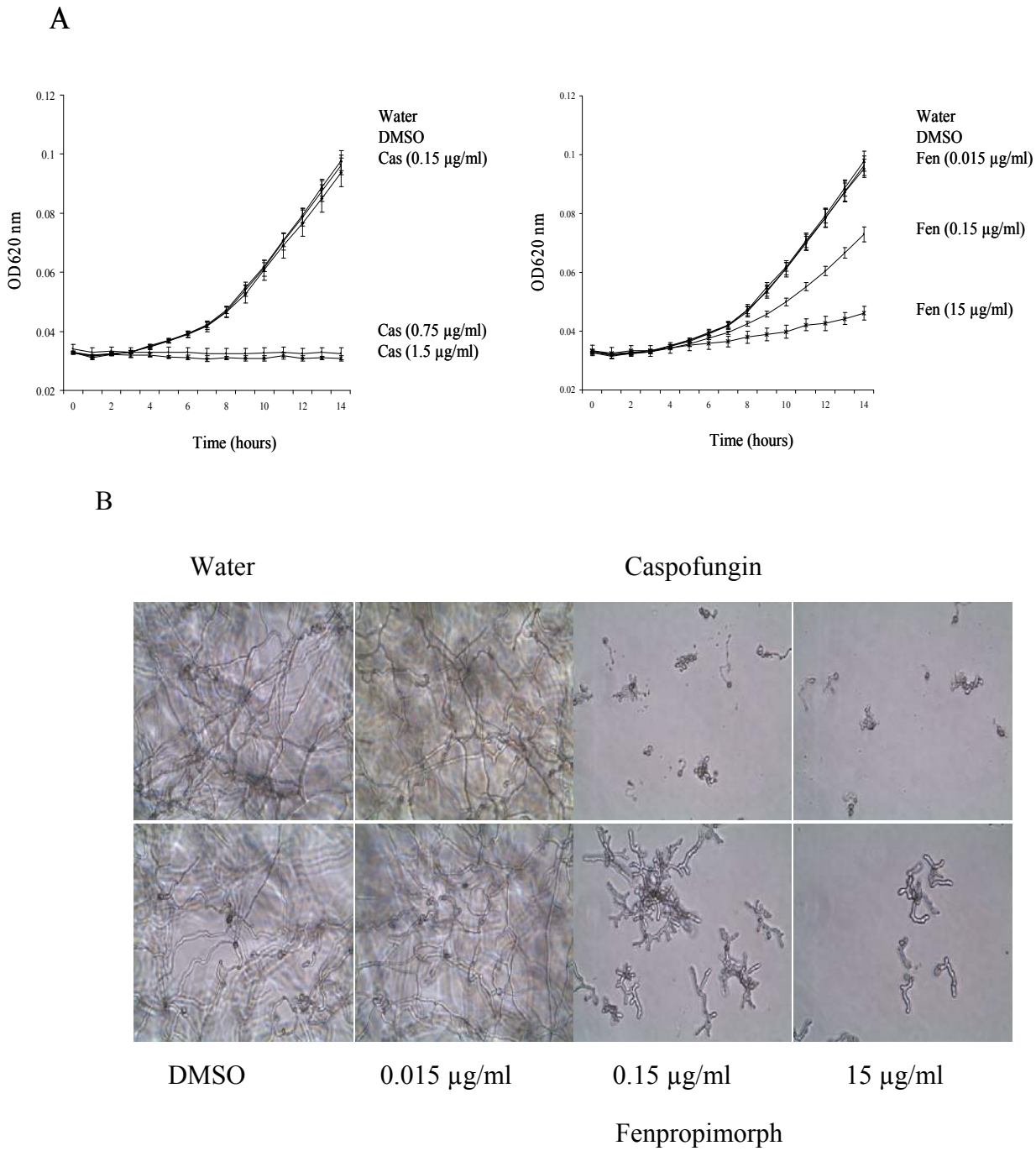

Figure 1: Growth inhibition of *A. niger* incubated with different concentrations of the antifungals caspofungin and fenpropimorph. Note that DMSO is the solvent for both compounds. Panel A shows optical density measured by a plate reader, panel B displays microscopic pictures taken with an inverted microscope.

5.3.2 Cell membrane susceptibility testing using a Sytox Green assay

The integrity of cell membrane can easily be carried out using the SYTOX-Green assay (Thevissen et al., 1999). SYTOX Green is a high-affinity nucleic acid stain that can penetrate cells having compromised cell membranes but does not cross intact membranes. The method

given below describes the procedure for testing the susceptibility of cell membranes of filamentous fungi and yeast towards potential antifungals (Ouedraogo et al., 2011).

1. Obtain fresh spores or cells of the fungal strains under investigation and count them using a microscope counting chamber.
2. Inoculate 10^2 spores or 10^5 yeast cells in black polystyrol 96 well plates containing 150 µL YPD medium and incubate at 28°C for 20 – 40 h for filamentous fungi and for 12 - 16 h for yeast cells. (*see* Note 4)
3. Add 0.2 µM final concentration of SYTOX-Green for filamentous fungi and 1 µM for yeast cells and place the plate into the dark. (*see* Note 5)
4. Add 25 µl of the antifungal under investigation using serial dilutions.
5. Continue cultivation in the dark at 28°C. Measure the kinetics of fluorescence formation in minute intervals up to 2 hours using a microtiter plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. (*see* Note 6)
6. Calculate the relative fluorescence values by subtracting the fluorescence values of a culture incubated only with SYTOX-Green without an antifungal compound.

3.3 Cell wall and cell membrane susceptibility testing using an agsA::GFP assay

The *agsA* gene coding for (1,3)-alpha-D-glucan synthase is specifically induced in response to compounds that interfere with cell wall or cell membrane integrity of *A. niger* (Damveld et al., 2005; Meyer et al., 2007). The *agsA* gene is therefore an excellent and fungal-specific marker for detecting cell surface integrity. (Note that bacteria, yeasts, plants and mammals do not have a (1,3)-alpha-D-glucan synthase). To study the effect of compounds on (1,3)-alpha-D-glucan synthesis, two *A. niger* reporter strains, containing either a cytoplasmatically (strain JvD1.1) or nuclear (strain RD6.47) targeted *gfp* gene under the control of the *agsA* promoter can be used.

1. Obtain fresh spore solutions from the reporter strains JvD1.1 (expressing PagsA-GFP) and RD6.47 (expressing PagsA-H2B-GFP) as described above.
2. Inoculate 2 x 10^4 conidia from the reporter strains in flat bottom 96 wells plate (Sarstedt) containing 100 µl 2x CM.
3. Incubate for 6 hours at 37 °C.
4. After spore germination, add 100 µl of a two-fold dilution series for each antifungal compound to individual wells. The effect of each compound shall be tested for at least

3-4 different concentrations. Include respective negative (water or other solvent) and positive (caspofungin) controls.

5. After adding the compound solution, place the microtiter plates for 3 more hours at 30 °C. (*see* Note 7)
6. Discard the medium by inverting the microtiter plate and analyze germlings that are adherent to the bottom of each well by fluorescence microscopy (*see* Note 8). Compounds which induce *agsA* expression will induce a strong GFP fluorescence even if germ tube elongation is inhibited. A wild type *A. niger* strain shall always be used as a negative control because *agsA* expression will be naturally induced after prolonged cultivation.

5.4. Notes

1. The color of the 1000 × trace element solution is green when freshly made. After autoclaving, the color changes from green to purple within two weeks.
2. Spore solutions of *A. niger* can be stored at 4 °C. However, all assays described work best if the spore solution used is not older than 2 weeks.
3. 24 hours is the maximum time for incubation as the wells dry out due to medium evaporation.
4. YPD is a complete medium for both, yeast and filamentous fungi. Cultivate cells until the culture reaches the mid-logarithmic growth phase. The time required is strain-dependent.
5. It is very important that all experiments involving SYTOX Green are performed in the dark. 0.2 µM and 1 µM SYTOX Green are the optimal concentration for filamentous fungi and yeast, respectively.
6. Permeabilized mycelia or cells respond with increasing fluorescence already after a few minutes of incubation with the antifungal (Theis et al., 2005).
7. It is important to cultivate at 30°C as higher temperatures negatively interfere with GFP folding.
8. Use a 40 × objective. For GFP images, use a fixed exposure of e.g. two seconds. Process images using Adobe Photoshop.

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Chapter 6

General discussion and concluding remarks

Due to significant limitations in current antifungal drugs ranging from toxicity to drug resistance, the pharmaceutical industry has relied extensively on natural products as a source of bioactive small molecules which are suitable for antifungal development. The genus *Aspergillus* and other filamentous fungi are known for their ability to secrete a variety of natural active compounds, including antibiotics, mycotoxins, immunosuppressants, cholesterol-lowering agents and antifungal proteins (Fox and Howlett, 2008; Keller et al., 2005). The term "secondary metabolite" has been used to describe these low molecular weight natural products, usually produced after the primary growth has stopped. In the case of AFP, we can also considere the protein as a secondary metabolite, as its expression can only be detected late during submerged growth and highly enhanced during different stress conditions (Meyer et al., 2002; Meyer et al., 2008).

Research on AFP has focused primarily on identifying the putative target of AFP and its mode of action. Chitin synthases have been found to be the potential target of AFP (Hagen et al., 2007). However, other putative targets such as membrane lipids (e.g. Δ3-deasturated glycosphingolipids) and proteins are still under investigation and could be targets as well.

The aim of the present work was to bring AFP closer to any application in the modern pharmaceutical industry by understanding why AFP kills some fungi and not others. Therefore, considerable effort has been directed at elucidating the survival strategies of yeast and filamentous fungi against AFP. The focus has been on the determination of the signaling pathways which are responsible for *S. cerevisiae* resistance to AFP and for characterizing the function of *A. niger* chitin synthase class III and V in the cell wall integrity maintenance by using different genetic approaches.

All antifungal agents somehow interfere with signal transduction, receptor signaling and biochemical equilibria which can be elucidated through the knowledge of the sequenced genome. In addition, the clinical relevance of an antifungal target does not comprise just a single biochemical entity of a receptor, but the simultaneous interference with a number of pathway or enzymes. In this way, it is important to know whether a signaling pathway is involved in the resistance of an antifungal agent rather than just to know the target. Many drugs or suspected drugs have been defined to interact with more than one target. At least 200 possible drugs targets have been counted. Therefore, the most prominent way to bring an antifungal protein into clinical usage is also to understand the response signaling pathways used by the microorganims to defend themselves against this antifungal and to elucidate the interaction of these pathway with the antifungal (Imming et al., 2006).

The most important signaling pathways enable eukaryotic cells to adapt to environmental change and stress is the Mitogen-Activated Protein Kinase (MAPK) cascade (Marshall, 1994; Toone and Jones, 1998) (Fig 2, Chapter 2). In addition to the MAPK cascade, other signaling pathways are involved in cell wall integrity, including the calcium/calcineurin pathway, Tor signaling pathway and the cyclic AMP protein kinase A pathway (Hirata et al., 1995; Petersen and Nurse, 2007; Thevelein and de Winde, 1999).

The maintenance of cell wall and membrane integrity in the genus *Aspergillus* has been studied to investigate the understanding of their importance as regards to the composition and survival strategies in presence of antifungal compounds (chapter 2). In *S. cerevisiae*, several signaling pathways have been triggered in the presence of AFP and the activation of the calcium/calcineurin pathway acts in proper cell wall assembly. Generally, calcium pathway activation appears as a second response to cell wall remodeling next to the classical cell wall integrity (CWI) pathway.

In our study, the yeast cells survive to AFP stress by strongly activating its second response mechanism, i.e. the calcium/calcineurin signaling, and at the same time several other independent and different stress response pathways such as Tor signaling and AMP protein kinase A pathway (chapter 4). The results are in part consistent with the finding that different signaling pathways can interplay to safeguard yeast cell during stress (Fuchs and Mylonakis, 2009; Rodriguez-Pena et al., 2010). Therefore, the activation of the CWI pathway is not a uniform response but rather depends on the agents or compounds that elicit the response. The CWI pathway can interact with the HOG pathway or TOR signaling, and the possibility that other signaling mechanisms could be recruited by different agents' stresses is well known (Fuchs and Mylonakis, 2009). In addition, the calcium/calcineurin pathway was characterized in the genome-wide expression of *S. cerevisiae* exposed to calcium, sodium and/or the specific inhibitor of this pathway, FK506 (Yoshimoto et al., 2002). This signaling pathway operates via the Crz1p transcription factor. Its activation leads to the induction of genes involved in various cellular functions, such as ion transport/homeostasis, signaling, vesicle-mediated transport and cell wall maintenance (Yoshimoto et al., 2002). In the opportunistic human pathogen *Candida albicans*, the corresponding pathway was found to be essential for the organism to survive plasma membrane stress due to presence of ergosterol-biosynthesis inhibitors (Cruz et al., 2002). The perturbation of the cell wall and membrane leads to increased expression of genes that function in cell wall/membrane biosynthesis and maintenance (Boorsma et al., 2004; Garcia et al., 2004; Jung and Levin, 1999; Lagorce et al.,

2003; Reinoso-Martin et al., 2003; Smits et al., 2001). Other studies as well as Chapter 4, indicate that the common response to cell wall stress is the remodeling of the cell wall, which results among other things, in an increase in chitin levels (Boorsma et al., 2004).

In filamentous fungi, the response of AFP by increasing α and β-1,3-glucan but not chitin is characterized by high sensitivity to the antifungal protein (chapter 3 and 4). One reason is that the regulatory pathways responsive to stress are relatively conserved, but not identical between yeast and filamentous fungi as well as in the genus *Aspergillus* (Miskei et al., 2009). This could explain the differential survival response between yeast and filamentous fungi against AFP. Recent studies have led to the discovery of new and novel functions for the MAPK cascade in *Aspergillus*, which are very different for those studied in model yeast systems. For example, the SakA/HogA osmotic stress response pathway is also involved in nitrogen sensing regulating conidial germination in *Aspergillus* (Xue et al., 2004). Furthermore, the different susceptibilities and survival strategies against AFP which are observed for filamentous fungi reinforce the idea that different factors determine the susceptibility of a fungus towards AFP (Fig 1).

Moreover, in this research study, chitin synthase mutants of class III and V of *A. niger* were constructed and the functional role of the *chsB* (class III) and *csmB* (class V) in cell wall integrity was characterized (Chapter 3). The data show that class III and V chitin synthase are involved in asexual development and cell wall integrity in *A. niger*. Previous studies have congruently determined the importance of class III and V chitin synthase in cell wall integrity and morphogenesis in other Aspergilli (Borgia et al., 1996; Fukuda et al., 2009; Horiuchi et al., 1999; Specht et al., 1996). Chapter 3 clearly shows that both chitin synthase mutants respond differently to AFP. Similar to the AFP resistant filamentous fungi, *P. chrysogenum*, the class V chitin synthase mutant (*csmB*) of *A. niger* increases its wall chitin, thereby becoming less susceptible to AFP.

Based on the results obtained with the research described in this thesis, elucidation of other targets molecules such as Δ3-deasturated glycosphingolipids and membrane proteins or biological processes is necessary to completely understand the complex mode of action of AFP. This study has revealed that the dynamic actions of the antifungal agent either stimulate, or inhibit a biological process and it is necessary to move away from the descriptions of single protein, target, receptors and so on but to view the entire cell and its defense systems as the target. The elucidation of the fungal total membrane proteome in the absence and presence of

AFP will provide for example a starting point for further in depth analyse different pathways that are involved in AFP responses.

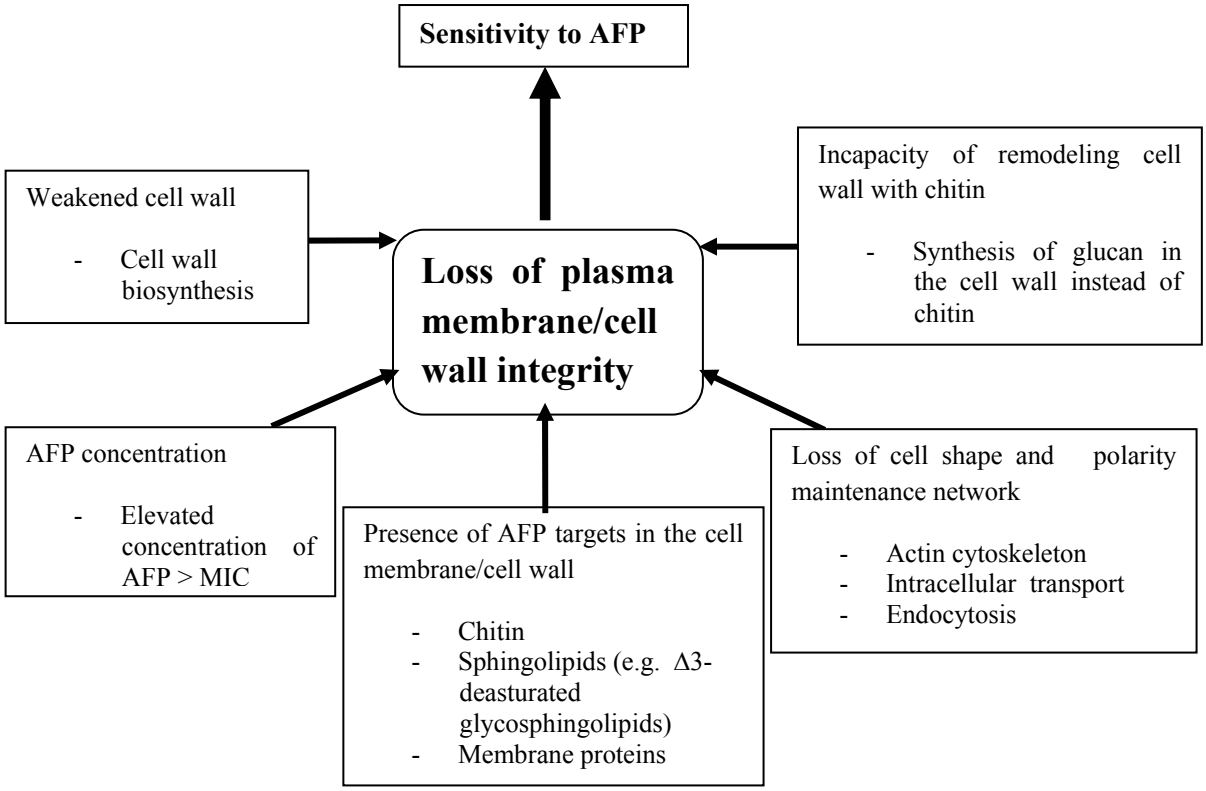

Figure 1: Factors contributing to AFP sensitivity in fungi. General overviews of strains that are sensitive or moderate sensitive to AFP show that AFP causes a permeability of the membrane in different background strains. Therefore, susceptibility of fungi to AFP depends on a variety of factors which result in loss of the plasma membrane/cell wall integrity.

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Summary

The antifungal protein (AFP) secreted by the filamentous ascomycete *Aspergillus giganteus* is a naturally occurring biomolecule which possesses the characteristics to combat fungal infection. AFP is a small, basic, cysteine-rich peptide which exerts extremely potent antifungal activity against human and plant pathogenic fungi without affecting the viability of bacteria, yeast, plants and mammalian cells. In the process of elucidating the antifungal protein mode of action, it has been shown that AFP disturbs membrane integrity and inhibits chitin synthesis in sensitive fungi.

In order to understand how resistant fungi such as yeast cells can counteract the antifungal protein, a genetic approach has been used to screen different mutants for their sensitivity to AFP. The data gained suggest that *S. cerevisiae* safeguards itself against AFP via a concerted action of several different signaling pathways, such as calcium signaling, TOR signaling, cAMP-protein kinase A signaling, and cell wall integrity signaling. The analysis of the composition of the cell wall of selected strains could show that an increased chitin level can better counteract an AFP attack and that cells showing this response become less susceptible, even resistant, to AFP.

Chitin synthase classes III and V play an important role in the chitin response in filamentous fungi. This work could consistently show that the maintenance of cell wall integrity of *Aspergillus niger* requires both chitin synthases. The examination of the corresponding knockout mutants showed that class V mutants become significantly less sensitive to AFP, which also correlates with an increased level of chitin in the cell wall; an observation which can be verified in class V chitin synthase mutants in other filamentous fungi (*A. oryzae, Fusarium oxysporum*). This suggests that one conserved fungal AFP resistance mechanism is based on the concerted interaction of several signaling pathways resulting in the reinforcement of the cell wall with chitin.

Zusammenfassung

Das Protein AFP ist ein antifungal wirksames Biomolekül, welches Pilzkontaminationen und -infektionen verhindern kann. AFP ist ein amphipathisches, sekretorisches Protein des filamentösen Pilzes *Aspergillus giganteus* und übt eine fungizide Wirkung gegenüber einer Vielzahl von human- und pflanzenpathogenen Pilzen aus, ohne jedoch die Vitalität von Hefen, Bakterien, Pflanzen oder Säugerzellen negativ zu beeinflussen. Es wird vermutet, dass die Wirkung des AFP auf einer spezifischen Interaktion des Proteins mit pilzlichen Membranbestandteilen beruht, in deren Konsequenz die Plasmamembran von AFP-sensitiven Pilzen permeabilisiert sowie die zellwandlokalisierte Chitinsynthese blockiert wird.

Im Rahmen der hier vorliegenden Arbeit wurden Studien durchgeführt, die zu einem Verständnis von AFP-Resistenzmechanismen führen sollten. Hierfür wurde in einem genetischen Screeningansatz verschiedene, rational ausgewählte, Genknockout-Mutanten der AFP-resistenten Hefe *Saccharomyces cerevisiae* auf eine mögliche veränderte AFP-Suszeptibilität untersucht. Die hier gewonnenen Ergebnisse legen den Schluss nahe, dass ein konzertiertes Zusammenspiel verschiedener regulatorischer Kaskaden, wie Calcium-, TOR-, cAMP-Proteinkinase A- und Zellwandintegrität-Signaling, die Grundlage für die AFP-Resistenz von *S. cerevisiae* bilden. Die Analyse der Zellwandzusammensetzung von ausgewählten Stämmen konnte zeigen, dass jene Stämme, die mit einer Erhöhung des Chitingehaltes auf AFP reagieren, eine verringerte Suszeptibilität oder sogar Resistenz bezüglich AFP aufweisen.

Eine wichtige Rolle in der Chitinantwort von Hyphenpilzen können Chitinsynthasen der Klassen III und V spielen. In dieser Arbeit konnte übereinstimmend gezeigt werden, dass für die Aufrechterhaltung der Zellwandintegrität von *Aspergillus niger* beide Chitinsynthasen benötigt werden. Die Untersuchung entsprechender Knockout Mutanten zeigte, dass die Klasse V Mutante eine erheblich reduzierte Empfindlichkeit gegenüber AFP aufwies, die ebenfalls mit einem Anstieg des Chitingehaltes in der Zellwand korrelierte – eine Beobachtung, die in anderen Klasse V Mutanten von Hyphenpilzen (*A. oryzae, Fusarium oxysporum*) bestätigt werden konnte. Dies lässt vermuten, dass ein konservierter AFP-Resistenzmechanismus auf einem Zusammenspiel verschiedener Signalwege beruht, deren zellulärer Output eine Verstärkung der Chitinsynthese ist.

Curriculum Vitae

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Selbstständigkeitserklärung

Ich erkläre an Eides Statt, dass die vorliegende Dissertation in allen Teilen von mir selbständig angefertigt wurde und die benutzten Hilfsmittel vollständig angegeben worden sind. Diese Arbeit wurde in der jetzigen oder einer ähnlichen Form keiner anderen Prüfungsbehörde vorgelegt. Ich habe mich weder anderwärts um einen Doktorgrad beworben, noch besitze ich einen entsprechenden Doktorgrad. Die dem Verfahren zugrunde liegende Promotionsordnung ist mir begannt.

Berlin, den 30 September 2011 Ouedraogo Jean-Paul