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MINI-REVIEW

# A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value

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**Abstract** As fungal infections are becoming more prevalent in the medical or agricultural fields, novel and more efficient antifungal agents are badly needed. Within the scope of developing new strategies for the management of fungal infections, antifungal compounds that target essential fungal cell wall components are highly preferable. Ideally, newly developed antimycotics should also combine major aspects such as sustainability, high efficacy, limited toxicity and low costs of production. A naturally derived molecule that possesses all the desired characteristics is the antifungal protein (AFP) secreted by the filamentous ascomycete *Aspergillus giganteus*. AFP is a small, basic and cysteine-rich peptide that exerts extremely potent antifungal activity against human- and plant-pathogenic fungi without affecting the viability of bacteria, yeast, plant and mammalian cells. This review summarises the current knowledge of the structure, mode of action and expression of AFP, and highlights similarities and differences concerning these issues between AFP and its related proteins from other Ascomycetes. Furthermore, the potential use of AFP in the combat against fungal contaminations and infections will be discussed.

**Keywords** Antifungal protein · *Aspergillus giganteus* · Pathogenic fungi · Cell wall integrity · Chitin biosynthesis · Antifungal treatment

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## Introduction

Filamentous fungi represent an increasing public health problem worldwide. Around 300 fungal species are reported to be origins of major diseases, whereby, the filamentous fungus *Aspergillus fumigatus* and the dimorphic yeast *Cryptococcus neoformans* and *Candida albicans* are the three predominant causative agents of human diseases (Gupte et al. 2002; Sanz Alonso et al. 2006). In addition, indoor moulds have been recognised as important risk factors for allergic diseases and for the damage of building materials (Straus et al. 2003; Li and Yang 2004). Another important aspect is that many filamentous fungi such as *Fusarium* species and *Magnaporthe grisea* are destructive pathogens of plants and are thus responsible for enormous crop losses worldwide (Talbot 2003; Edwards 2004).

Fungal infections and contaminations have, therefore, led to an increasing demand for antifungal drugs. Still, antifungal treatment is nowadays limited to only a small number of antifungal drugs such as azoles, echinocandins and polyenes (Gupte et al. 2002; Hector 2005; Wiederhold and Lewis 2007). The application of these antifungals is, however, often restricted because of several reasons. Antifungal compounds have often low efficacy rates, as their activity is rather fungistatic than fungicidal. They show frequently severe side effects and can even be toxic to man and, moreover, frequently interact unfavourably with other medications (Gupte et al. 2002; Hector 2005). In addition, a steady increase in fungal pathogens that do not longer respond to antifungal treatment is observed presumably because of resistance mechanisms evolved in consequence of long-term antifungal treatment (Masia Canuto and Gutierrez Rodero 2002; Mellado et al. 2006; Wagner et al. 2006; Cappelletty and Eiselstein-McKitrick 2007). The development of new antifungal drugs applied in the

agricultural and/or medical field is therefore mandatory. Ideally, they should meet several criteria to overcome the limitations described above, such as (1) specific mode of antifungal action; i.e., structures or functions unique to fungi have to be targeted to prevent any side effects on man and environment, (2) high efficacy and (3) inexpensive and sustainable way of production.

Interestingly, filamentous fungi themselves can be considered as valuable sources for the production of antifungal compounds. One prominent example is the commercial use of mycoparasitic *Trichoderma* strains that antagonise plant pathogens. Here, the fungus itself is applied as biocontrol agent in the combat against soil-borne fungal plant pathogens. The mechanisms behind biocontrol are complex and involve the synthesis and secretion of synergistically acting agents such as cell-wall degrading enzymes (chitinases, glucanases and proteases) and antibiotics (Benitez et al. 2004). Hence, filamentous fungi do have significant capacities to secrete compounds with antifungal activities (Mouyna et al. 2004). In general, it is thought that these compounds are part of their defence system to protect themselves against other fungi that might act as nutrient competitors in the same environment.

Among the antifungal compounds produced by filamentous fungi, small-sized antifungal peptides with suppressive effects on fungal growth have attracted considerable interest. One prominent peptide bearing great potential for future antifungal strategies is the antifungal protein AFP secreted by *Aspergillus giganteus*. In the following, the current knowledge on structural features, mode of action and expression of AFP will be summarized, and its potential biotechnological applications discussed. Moreover, the review will also address some new important findings on AFP-related proteins such as the antifungal protein PAF from *Penicillium chrysogenum*. For an earlier review on AFP and its related proteins, the readers are referred to (Marx 2004). For a specialised review that focuses on the mechanism of PAF action, the readers are directed to (Marx et al. 2007).

## Sequence, structure and bioactivity of AFP

AFP is expressed by *A. giganteus* as a 94 amino acid long preproprotein that contains a predicted signal secretion sequence and a prosequence that is thought to be removed during the process of secretion by a KexB-like protease (Wnendt et al. 1990; Wnendt et al. 1994; Martínez-Ruiz et al. 1997). However, the biological significance of these sequences, as well as the exact cleavage mechanisms, still remains to be experimentally verified. The mature form of AFP, which can be isolated from the culture supernatant of *A. giganteus*, is a 51 amino acid long peptide with a high content of cysteine (8), tyrosine (6) and lysine (12) residues (Fig. 1). The isoelectric point was estimated to be 8.8; thus, the protein is positively charged under physiological conditions (Nakaya et al. 1990). Interestingly, Martinez-Ruiz et al. described a larger form of the mature AFP (Lf-AFP) comprising six extra residues at the N-terminal end (Martínez-Ruiz et al. 1997). This protein was transiently found early in cultivation of *A. giganteus* but disappeared later. As suggested by the authors, maturation of AFP might involve an additional cleavage step by an as yet unidentified extracellular protease. As Lf-AFP exerts considerably lower antifungal activities compared with AFP suggests that Lf-AFP is an inactive precursor form of the mature protein (Martínez-Ruiz et al. 1997).

Basic local alignment search tool (BLAST) searches revealed that proteins displaying sequence homology to AFP can only be found in *Ascomycetes* (Fig. 1). Antifungal properties have been ascribed to the homologous proteins ANAFP from *A. niger* and to the identical PAF and NAF proteins from *Penicillium chrysogenum* and *P. nalgiovense*, respectively (Marx et al. 1995; Gun Lee et al. 1999; Geisen 2000). All other proteins listed in Fig. 1 are hypothetical proteins deduced from nucleotide sequences deposited in public databases and have not been analysed yet. A high degree of homology can be found within the secretion signal sequence, the predicted prosequence cleavage site and the N-terminal domain of the (predicted) mature proteins (region encompassing the first 22 amino acids of the mature AFP). Moreover, the cysteine residues 7, 14, 26, 33, 40, and 51 (numbering refers to the mature AFP) are conserved within all proteins. With respect to the mature proteins, AFP shows 35.6 and 47.1% homology to ANAFP and PAF/NAF, respectively. For the PAF protein, it has been reported that the signal secretion sequence efficiently directs secretion of pro-PAF in a transgenic *A. nidulans* strain. As intracellular expression of pro-PAF did not cause any detrimental effects on *A. nidulans* (note that the *A. nidulans* wild-type is PAF-sensitive), it was concluded that the function of the prosequence is to prevent any premature activity of PAF (Marx et al. 2005). This finding suggests that the prosequences present in AFP and its related proteins might in general be necessary to keep the proteins in an inactive form until they have not passed the plasma membrane.

AFP is the only protein of this group for which the tertiary structure has been resolved. The protein is folded into five highly twisted antiparallel β-strands (Fig. 2) that adopt a barrel topology (Martínez Del Pozo et al. 2002). This architecture resembles an oligonucleotide/oligosaccharide binding (OB)-fold domain that presents a binding face for different ligands such as RNA, single-stranded DNA, oligosaccharides and proteins (Murzin 1993; Arcus 2002). A hydrophobic core is formed, whereas the remaining hydrophobic, aromatic, and polar residues are distributed on the surface of AFP, resulting in a high solubility of the


**Fig. 1** Alignment of (putative) antifungal proteins with homology to AFP derived from (predicted) complementary DNA sequences. The following abbreviations were used: Agig, *Aspergillus giganteus* (AFP); Acla, *Aspergillus clavatus* (hypothetical protein, accession number ABR10398); Anig1, *Aspergillus niger* strain CBS 513.88 (hypothetical protein, accession number XP_001391221); Anig2, *Aspergillus niger* strain KCTC 2025 (ANAFP); Nfis, *Neosartorya fischeri* (hypothetical protein, accession number XP_001262586); Gzea, *Gibberella zeae* (hypothetical protein, accession number XP_384921); Pchr, *Penicillium chrysogenum* (PAF); Pnal, *Penicillium nalgiovense* (NAF). Amino acids sharing 50% or at least 75% homology are highlighted in *grey* and *black*, respectively. The predicted secretion signal cleavage site is marked by an *arrow*; the putative KexB-like protease recognition site is *overlined*. The first amino acid of the mature AFP, ANAFP and PAF proteins is *underlined*. The putative chitin-binding motif of AFP (CKYKAQ) is indicated with an *open bar*

protein (Campos-Olivas et al. 1995). The tertiary structure of AFP is stabilised by four disulfide bridges, consequently granting a remarkable resistance to heat and protease degradation (Lacadena et al. 1995; Theis et al. 2005). As shown in Fig. 2, AFP adopts an amphipathic structure that can be attributed to the presence of a cationic domain (K9, K10, K32) in conjunction with a hydrophobic stretch (Y29, V30, Y45, V50; Campos-Olivas et al. 1995).

AFP shows high antifungal activity against filamentous fungi at micromolar concentrations, whereas the growth of yeast (e.g. *Saccharomyces cerevisiae*, *Candida albicans*) or bacterial cells (e.g. *Escherichia coli*, *Bacillus subtilis*) is not affected by AFP (Lacadena et al. 1995; Theis et al. 2003). Similarly, specific antifungal activities were reported for PAF, ANAFP and NAF. For a comprehensive list of different pro- and eukaryotes tested so far and their susceptibilities towards AFP, PAF, ANAFP and NAF, readers are referred to a previous review (Marx 2004). AFP acts fungistatically when applied at concentrations below the minimal inhibitory concentration (MIC) but fungicidally at concentrations equal or higher than the MIC (Theis et al. 2005). Among the AFP-sensitive filamentous fungi are opportunistic human-pathogenic species, such as *A. fumigatus* and *A. niger*, and also important plant pathogens such as *F. oxysporum*, *F. moniliforme*, *M. grisea*, *Erysiphe graminis* and *Phytopthora infestans* (Oldach et al. 2001; Vila et al. 2001; Theis et al. 2003; Moreno et al. 2006). However, not all filamentous fungi are susceptible towards AFP (e.g. *P. chrysogenum* and *A. clavatus*), suggesting that the protein acts in a species-specific manner (Theis et al. 2003). Similarly, the growth-inhibitory activity of PAF and ANAFP towards filamentous fungi is species-specific (Gun Lee et al. 1999; Kaiserer et al. 2003; Galgóczy et al. 2005). The phenomenon that filamentous fungi show varying sensitivities towards AFP and its related proteins might suggest that the proteins specifically interact with structures that are either present, modified or absent in different fungi. Alternatively, filamentous fungi might differ in their capacities to efficiently counteract any detrimental effects provoked by the proteins. Most importantly, AFP neither exerts cytotoxic nor immunogenic effects on mammalian cell types (Szappanos et al. 2006), an observation that underlines the excellent potential of AFP for future medical applications. As with AFP, it was shown that PAF does not cause any detrimental effects on mammalian cells (Szappanos et al. 2005).

## Mode of action of AFP

As discussed above, AFP is a small-sized protein, is amphipathic, has a positive net charge and adopts a compact structure (Fig. 2). In terms of these characteristics, AFP and its homologous fungal proteins share similarities with other cationic antimicrobial peptides found in organisms ranging from bacteria to plants, invertebrates and vertebrates including mammals (De Lucca and Walsh 1999; Theis and Stahl 2004; Jenssen et al. 2006). Most of these antimicrobial proteins, probably facilitated by their amphipathic structure, interact with bacterial and/or fungal plasma membranes, and their physiological activities are related to membranolytic properties. However, other antifungal pep-

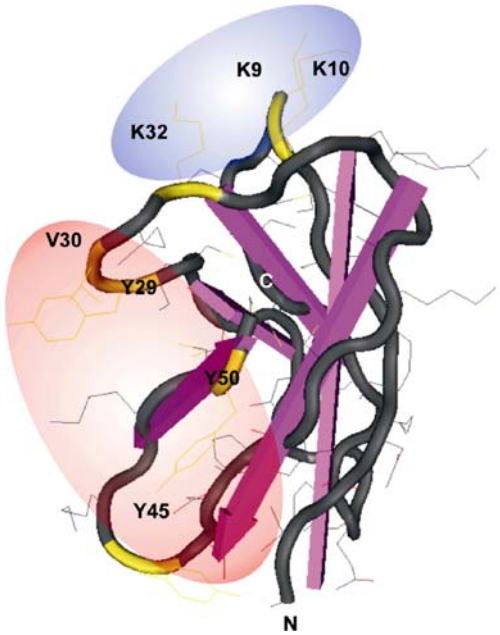


**Fig. 2** Cartoon conformation of AFP according to (Campos-Olivas et al. 1995; Lacadena et al. 1995). The orientation of the β-strands is symbolised by *arrows*. The amphipathic structure of AFP is defined by a cationic domain (K9, K10, K32) and a hydrophobic domain (Y29, V30, Y45, V50). The *image* was drawn using the Cn3D4.1 software

tides have been shown to interfere with cell wall biosynthesis to induce the formation of reactive oxygen species or to depolymerise the actin cytoskeleton (De Lucca and Walsh 1999; Thevissen et al. 2003; Theis and Stahl 2004; Jenssen et al. 2006).

AFP applied on the AFP-sensitive fungus *A. niger* causes swelling of hyphal tips, as well as apical and subapical branching, and evokes cells that tend to burst exclusively at their hyphal apex. Furthermore, the protein induces the protein kinase C (PKC)-dependent cell wall integrity pathway as compensatory response (Hagen et al. 2007), indicating that AFP disturbs polarised growth of filamentous fungi by interfering directly or indirectly with fungal cell wall biosynthesis. Using immunofluorescence microscopy, it could be demonstrated that AFP binds to the cell wall and/or the plasma membrane of AFP-sensitive fungi (*A. niger*, *A. awamori*) but localizes intracellularly in AFP-resistant fungi such as *P. chrysogenum* and *A. clavatus* (Theis et al. 2003). One possible explanation for internalization of AFP by resistant fungi might be that AFP is taken up by resistant fungi and subsequently subjected to normal degradative processes. Electron microscopic analyses demonstrated that the protein localises predominantly to the cell wall outer layer, to the cell wall, and to a lesser extent to the plasma membrane of *A. niger*. In comparison, only little protein was bound to the outer layer and cell wall of an AFP-resistant *P. chrysogenum* strain but was instead found intracellularly (Theis et al. 2005). These analyses further revealed that AFP provokes severe plasma membrane alterations in *A. niger*, whereas the membrane of *P. chrysogenum* was not affected (Theis et al. 2005), suggesting a specific plasma membrane-based action of AFP in sensitive fungi. From these observations, it can be hypothesised that species-specific determinants of AFP sensitivity reside in the outer layer and cell wall of sensitive fungi—i.e. the presence (or absence) of specific binding structures determine the efficiency of AFP binding to the cell surface—and that the plasma membrane harbours additional targets of AFP. A further indication for a membrane-based action of AFP was gained from in vivo Sytox Green assays, with which the plasma membrane integrities of different fungi were studied after treatment with the protein. It could be demonstrated that AFP readily permeabilises the membrane of AFP-sensitive strains—an effect that was not observed for AFP-resistant strains (Theis et al. 2003; Hagen et al. 2007). Hence, it can be concluded that AFP disturbs plasma membrane integrity in sensitive fungi. Notably, the growth inhibitory effect of AFP, as well as its membrane permeabilisation effect, has been found to be cation-sensitive, suggesting that the presence of high amounts of cations ($Ca^{2+}$, $Na^{+}$, $K^{+}$) could lead to saturation of a putative membrane-based AFP target with cations (Theis et al. 2003).

Most recently, it was shown that AFP inhibits the in vivo activity of chitin synthases in the sensitive fungi *A. niger*, *A. oryzae* and *F. oxysporum* (Hagen et al. 2007), suggesting that AFP-mediated growth arrest is due to an inhibition of cell wall chitin biosynthesis. Supportive for this reasoning is the fact that AFP harbours at its N-terminus a putative chitin-binding domain (Fig. 1) that enables the protein to attach to chitin under in vitro conditions (Liu et al. 2002; Hagen et al. 2007). Furthermore, class V chitin synthase mutant strains of *F. oxysporum* and *A. oryzae*, as well as a class III chitin synthase mutant of *A. oryzae* were found to be less sensitive towards AFP than the corresponding wild-type strains, and their plasma membranes were not compromised after AFP treatment (Hagen et al. 2007), strongly suggesting that chitin synthases of classes III and V are targets of AFP. This is a quite interesting observation especially in view of the fact that (1) chitin synthases of both classes are unique to filamentous fungi, i.e. they are not present in AFP-resistant yeast, and that (2) these classes are essential for the maintenance of polarised growth and the virulence of plant and human pathogenic fungi (Mellado et al. 1996; Wang et al. 2001; Muller et al. 2002; Roncero 2002; Madrid et al. 2003; Mellado et al. 2003; Liu et al. 2004; Weber et al. 2006). Such a specific interaction of AFP with classes III and V chitin synthases could be one explanation for the different AFP susceptibilities of yeast and filamentous fungi. Furthermore, it points towards a

potential application of the protein in the combat against pathogenic fungi. In this context, it is important to emphasise that bacteria, plants and mammals do not possess chitin in their cell envelopes; hence, it can be assumed that a targeted employment of AFP in agriculture or pharmacology should not be harmful for man or the environment.

How can AFP-mediated inhibition of chitin synthesis be explained? Unfortunately, the mechanism(s) involved in chitin biosynthesis of filamentous fungi are far from being understood, and the current knowledge is still fragmentary. Basically, it is thought that zymogenic chitin synthases are transported within specialised microvesicles (chitosomes) to the hyphal apex and become accumulated at the Spitzenkörper (Bracker et al. 1976; Ruiz-Herrera et al. 1977; Sietsma et al. 1996; Bartnicki-García 2006; Riquelme et al. 2007). Interestingly, new findings suggest that different populations of chitosomes might exist that carry different classes of chitin synthases (Riquelme et al. 2007). Chitin synthases presumably become further transported to the plasma membrane, get inserted there, and after a proteolytic activation, the polymerisation of *N*-acetylglucosamine to the β-1,4-linked homopolymer chitin initiates. Chitin molecules are subsequently translocated across the plasma membrane upon which they coalesce to form microfibrils and associate with other cell wall components (Ruiz-Herrera and Martinez-Espinoza 1999; Cohen 2001; Riquelme et al. 2007). Hence, a number of events are involved in chitin biosynthesis, and different hypotheses can thus be drawn to explain the inhibitory effect of AFP on chitin biosynthesis: Based on the data accumulated so far, it may be conceivable that AFP interferes with the release of chitin synthases of classes III and V from chitosomes or with the transport and insertion of these synthases into the plasma membrane. It is also possible that AFP interacts with membrane-localised chitin synthases by adhering, e.g. to chitin precursors or to newly synthesised chitin. Alternatively, AFP interacts with as yet undefined membrane components and, thereby, disturbs the proper anchoring of chitin synthases within the plasma membrane. Irrespectively, whether AFP interacts directly or indirectly with membrane-bound chitin synthases, such an interaction could, in turn, cause stretching of the plasma membrane and eventually results in the loss of membrane and cell wall integrity. Recent results implicate that sphingolipids and sterols form segregated microdomains (lipid rafts) in the lateral plane of the plasma membrane (reviewed by Futerman and Hannun 2004) and are involved in the stabilisation of polarity axes in *A. nidulans* (Li et al. 2006). As suggested by Li et al., lipid rafts may serve as anchoring regions for scaffold proteins such as formins, which are known to function as actin filament-nucleating proteins and play crucial roles in polarised growth of fungi (Harris et al. 2005 and references therein). As the actin-filament based transport is crucial for the transport and function of class V chitin synthase in *A. nidulans* (Takeshita et al. 2005), it is, although speculative, conceivable that inhibition of chitin synthesis in AFP-sensitive fungi is dependent on the interaction of AFP with distinct membrane components such as sphingolipids that might serve as secondary receptors for AFP. Preliminary experiments supporting this assumption have shown that depletion of cellular glucosylceramide levels in *A. niger* and *A. fumigatus* resulted in reduced AFP susceptibilities (S. Hagen and V. Meyer, unpublished data). In this context, it is interesting to note that filamentous fungi possess a putative acyl-CoA-dependent ceramide synthase (BarA) that is absent in yeast and proposed to generate a specific pool of sphingolipids necessary to maintain polarised hyphal growth (Li et al. 2006). In addition, it has recently been reported that the susceptibility of *Neurospora crassa* towards plant defensins, which share similarities to AFP in terms of size, structure and disulfide bridge formation, is strongly dependent on the sphingolipid and sterole profile of the plasma membrane (Ferket et al. 2003; Park et al. 2005). And furthermore, the chitin interacting agent Calcofluor white that increases chitin synthase activities in *S. cerevisiae* (Roncero et al. 1988) also modulates expression of two sphingolipid-dependent regulators Pil1p and Lsp1p, which have also been described to be involved in PKC-dependent cell wall integrity signalling in *S. cerevisiae* (Edlind and Katiyar 2004; Zhang et al. 2004; Delom et al. 2006). Hence, it might be imaginable that AFP-induced inhibition of chitin biosynthesis is somehow connected with the sphingolipid profile present in sensitive fungi. Further studies investigating all of the hypotheses discussed above need to be performed in the future to unravel the exact mechanism by which AFP interferes with chitin biosynthesis.

Whereas the mechanism of action of ANAFP and NAF has not been studied so far, considerable insights into the mode of action has been gained for PAF, which has been most recently summarised in an excellent review (Marx et al. 2007). Briefly, species-specific determinants for PAF susceptibility are unknown yet; however, they are also proposed to reside in the cell envelope (cell wall and/or plasma membrane) of filamentous fungi. As with AFP, PAF provokes a hyperbranching phenotype in sensitive fungi (e.g. *A. niger*, *A. nidulans*, *A. fumigatus*), and its antifungal activity can be antagonised with cations. PAF application causes multifactorial effects in sensitive fungi, such as hyperpolarisation of the plasma membrane, increase in potassium ion permeability, generation of reactive oxygen species, changes in the morphology of mitochondria and induction of an apoptosis-like phenotype (Marx et al. 2007). Remarkably, PAF cytotoxicity requires its active internalisation, which presumably occurs via an endocytosis-like mechanism, suggesting that the antifungal effects of PAF are primarily evoked from the inside of sensitive cells (Oberparleiter et al. 2003).

Thus, although AFP and PAF show considerable similarities with respect to their amino acid sequences, protein attributes and (non)target organisms, the data accumulated so far point towards different mode of actions that could be a consequence of their different localisation within sensitive fungi. AFP applied at its minimal inhibitory concentration is specifically localised at the cell envelope of sensitive fungi, thereby inhibiting chitin biosynthesis (Theis et al. 2003; Theis et al. 2005; Hagen et al. 2007), whereas PAF is localised to the cytoplasm of susceptible fungi (Oberparleiter et al. 2003), thereby evoking different responses as mentioned above (Marx et al. 2007). Only at concentrations that are several ten- to hundredfold higher than the minimal inhibitory concentration, AFP can be detected intracellularly (Theis et al. 2005; Moreno et al. 2006). This localisation pattern, however, might be unspecific because of the high protein concentrations applied. Once the cell wall and plasma membrane integrity has been disturbed by AFP, intracellular structures will disintegrate, and in turn, the protein could enter already collapsed cells and bind via its OB-fold or via its cationic domain to intracellular remnants (Theis et al. 2005).

## Biotechnological application of AFP

The potential relevance of AFP and its related proteins as new antifungal drugs in the combat against fungal infections has as yet only been studied for AFP. As mentioned above, chitin synthesis and chitin synthases of classes III and V have been shown to play a crucial role in the pathogenicity of plant pathogenic fungi such as *F. oxysporum*, *M. grisea*, *Wangiella dermatididis* and *Ustilago maydis* (Wang et al. 2001; Madrid et al. 2003; Liu et al. 2004; Fortwendel et al. 2005; Steinbach et al. 2006; Weber et al. 2006; Odenbach et al. 2007). Thus, inhibition of the respective chitin synthase activities and, thereby, inhibition of polarised chitin synthesis and tip growth seem to be predestined approaches for efficient antifungal strategies. As AFP specifically targets these processes, AFP can be considered as a promising candidate for practical applications in crop protection or in pharmacology.

Several attempts have already been undertaken to test the suitability of AFP to substitute for classical fungicides in the agricultural field. For example, Vila et al. have reported that rice plants are protected against *M. grisea* infection when the protein is directly applied to rice leaves (Vila et al. 2001). Pre-incubation of tomato roots with AFP also secured the plants from an infection by *F. oxysporum*, a soil-borne fungus known to colonise and attack tomato plants at their roots (Theis et al. 2005). Other approaches aiming at the establishment of transgenic plants less susceptible to pathogens due to the expression of the AFP-encoding *afp* gene were successful too. Significant reduction of disease symptoms where observed for transgenic wheat artificially infected with *E. graminis* and *P. recondita* (Oldach et al. 2001) for transgenic rice plants challenged with *M. grisea* (Coca et al. 2004; Moreno et al. 2005) and for transgenic pearl millet lines confronted with *Sclerospora graminicola* and *P. substriata* (Girgi et al. 2006). Neither the external application of AFP on plants nor the constitutive or induced expression of the *afp* gene by the plants caused any detrimental effects on the host organisms. All plants tested so far showed normal growth behaviour and morphology, and were fertile, strongly suggesting that AFP can safely be applied in crop protection. Moreover, as the bioactivity of AFP is restricted to filamentous fungi and detrimental effects on bacteria, yeast, plant and human cells have not been observed so far, it can be assumed that application of AFP in the agricultural field will cause little if any side effects on the environment.

## Understanding the control of *afp* gene expression

To best exploit AFP, detailed knowledge has not only to be gathered concerning its mode of action but also concerning its expression in *A. giganteus*. Prerequisite for a biotechnological application of the protein will be an efficient and economic way of production, preferably using *A. giganteus* as homologous host, for which the growth conditions could easily be optimised for large-scale fermentations. To identify growth conditions and inducing stimuli supporting maximum protein yields, several approaches have been undertaken to investigate the transcriptional regulation of the *afp* gene.

In silico analysis of the 5′-upstream region of the *afp* gene revealed a number of putative *cis*-acting elements representing binding sites for various fungal transcription factors (Table 1), pointing at a complex transcriptional regulation of the *afp* gene. Using Northern, protein and reporter gene analyses, it was demonstrated that transcription of the *afp* gene is indeed under the control of different environmental conditions. In general, when *A. giganteus* is grown in liquid culture, transcription of the *afp* gene initiates as soon as the mid-logarithmic growth phase is reached and achieves a maximum, when the culture enters the stationary phase (Meyer et al. 2002). In comparison, rhythmic *afp* expression was observed during growth on solid medium. Here, expression occurred exclusively in the vegetative mycelium at the developmental stage when the colony was competent to form aerial hyphae. As the gene has not been found to be expressed in conidiophores or conidia suggests that *afp* expression is temporally and spatially regulated during asexual development (Meyer et al. 2002). Transcription of the *afp* gene is neither under

**Table 1** Putative *cis*-acting elements within the *afp* promoter and corresponding *trans*-acting factors

| Number of motifs present | Transcription factor | Cellular process | Organism[a] | Reference |
|---|---|---|---|---|
| 2 | AnCF (±) | Acetamide utilisation, secondary metabolism | *A. nidulans* | Kato et al. 1998 |
| 1 | CreA (–) | Carbon catabolite repression | *A. nidulans* | Kulmburg et al. 1993 |
| 2 | AreA (+) | Nitrogen metabolite repression | *A. nidulans* | Kudla et al. 1990 |
| 2 | PacC (±) | Ambient pH regulation | *A. nidulans* | Tilburn et al. 1995 |
| 1 | HSF (+) | Heat shock response | *S. cerevisiae* | Sorger 1990 |
| 5 | Msn2/4p (+) | General stress response | *S. cerevisiae* | Martinez-Pastor et al. 1996 |
| 1 | StuA (±) | Asexual development | *A. nidulans* | Dutton et al. 1997 |
| 1 | RlmA (+) | Cell wall integrity | *A. niger* | Damveld et al. 2005 |
| 5 | Crz1p (+) | Stress response (salt, pH, heat shock) | *S. cerevisiae* | Yoshimoto et al. 2002 |
| 1 | Pho4p (+) | Phosphate regulation | *S. cerevisiae* | Yoshida et al. 1989 |

After Meyer et al. (2002) and Meyer et al. (2005) and own unpublished data.
*(+)* Positive-acting factor, *(–)* negative-acting factor, *(±)* positive- or negative-acting in a gene-dependent manner
[a] Organism from which the consensus originates.

the control of carbon catabolite repression (CreA-mediated) nor regulated by nitrogen metabolite repression (AreA-mediated; Meyer et al. 2002). However, its expression level significantly enhances under different stress conditions such as alkaline pH, salt stress, heat shock, carbon-starvation, phosphate-starvation and, to some extent, during the presence of other fungi during co-cultivation. In contrast, *afp* transcription is strongly inhibited under acidic pH conditions and in medium containing excess of phosphate (Meyer and Stahl 2002; Meyer et al. 2002; Meyer and Stahl 2003). Thus, it seems that stress conditions have in general a positive impact on *afp* transcription, probably reflecting a role of AFP in the defence response of *A. giganteus* to harmful environmental conditions. Based on the observations that application of a heat shock and use of media containing low phosphate levels considerably enhance *afp* transcription and AFP secretion (Meyer and Stahl 2002; Meyer et al. 2002), an improved cultivation protocol was developed for *A. giganteus* that finally resulted in about 15- to 20-fold increase in AFP yield (Theis et al. 2003).

The expression pattern of genes encoding AFP-related proteins has only partially been studied in the case of the *paf* gene. In contrast to *afp*, transcription of *paf* seems to be under control of carbon catabolite repression (the promoter contains four putative CreA-binding sites) and nitrogen metabolite repression (two putative AreA-binding sites are located within the promoter). Furthermore, *paf* expression is not affected by heat shock or varying phosphate concentrations in the culture medium (Marx 2004), demonstrating that expression of both genes is differently regulated. The only commonality that both genes share is that their maximum expression level is achieved during the stationary growth phase (Meyer et al. 2002; Marx 2004).

The alkaline expression pattern of *afp* strongly suggested that the gene might be under control of the pH-responsive PacC transcription factor, supported by the presence of two putative PacC binding sites within its upstream region. PacC has been shown to be involved in the control of a number of fungal genes, among which are structural genes required for the synthesis of medically important antibiotics such as penicillin and cephalosporin (Espeso et al. 1993; Espeso and Peñalva 1996; Suárez and Peñalva 1996). Although both PacC recognition sites within the *afp* promoter are specifically recognised by the *A. giganteus* and *A. nidulans* PacC proteins under in vitro conditions (Meyer and Stahl 2002 and own unpublished data), PacC is surprisingly not relevant for the up-regulation of *afp* under alkaline conditions. Instead, the pH-dependent increase in *afp* messenger RNA and AFP levels is completely prevented by the calcineurin inhibitor FK506 (Meyer et al. 2005). Calcineurin is a $Ca^{2+}$/calmodulin-dependent protein phosphatase involved in the activation of the *S. cerevisiae* transcription factor Crz1p. Crz1p is a positive-acting protein that up-regulates expression of a number of effector genes in response to exposure of *S. cerevisiae* to alkaline pH, salt stress and heat shock (Serrano et al. 2002; Cyert 2003; Viladevall et al. 2004). Studies in different *Aspergilli* implicate that calcineurin activity is important for cell cycle progression, polar growth, phosphate regulation and survival under certain stress conditions such as alkaline pH, cell wall stress and heat shock (Rasmussen et al. 1994; Juvvadi et al. 2003; Steinbach et al. 2006; Steinbach et al. 2007a, b). In view of the up-regulation of the *afp* promoter by alkalinisation, heat shock and salt stress (Meyer et al. 2002), and the presence of five putative Crz1p binding sites within the *afp* promoter, a possible involvement of the calcineurin signalling pathway in *afp* regulation might exist. Respective analyses are currently underway to experimentally verify this assumption (V. Meyer, A. Spielvogel and E. Espeso, unpublished).

External pH changes have a wide-ranging physiological impact on yeast and filamentous fungi, and it is thought that one consequence of ambient alkaline pH generates a situation

of phosphate starvation (Zvyagilskaya et al. 2001; Serrano et al. 2002; Lamb and Mitchell 2003). The circumstance that *afp* expression is strongly inhibited in the presence of high external phosphate concentrations but induced as soon as phosphate becomes limiting (Meyer and Stahl 2002) suggests that pH- and phosphate-dependent regulation of the *afp* gene might be somehow interconnected. Indeed, using phosphate uptake measurements, it was shown that less phosphate is taken up by *A. giganteus* and *A. nidulans* under alkaline compared to acidic growth conditions (V. Meyer, unpublished data), indicating that one cause of increased *afp* expression under alkaline pH is a reduced intracellular level of phosphate. In agreement, expression of a reporter gene (β-galactosidase encoded by the *Escherichia coli lacZ* gene and put under control of the *afp* promoter) was significantly higher in a mutant strain of *A. nidulans* defective in phosphate acquisition compared to the wild-type strain (V. Meyer, unpublished data). In yeast, derepression of phosphate-repressible genes is mediated by the activity of the Pho4p transcription factor, the orthologue of the *A. nidulans* PalcA protein (Oshima 1997; Wu et al. 2004). As the *afp* promoter contains one Pho4p binding site, it might be speculated that the *afp* gene is a phosphate-repressible gene under control of PalcA — a speculation that awaits experimental validation.

Interestingly, the regulatory system involved in phosphate acquisition has also been implicated to play a role in programming the asexual and sexual development of *A. nidulans* (Bussink and Osmani 1998; Dou et al. 2003; Wu et al. 2004). In this context, it is conspicuous that the *afp* gene is also under developmental control, suggesting that, beside the proposed function of PalcA, also StuA might be involved in regulating *afp* transcription (the *afp* promoter comprises one StuA binding site). StuA has been found to be a positive- or negative-acting transcription factor involved in regulating the asexual development of filamentous fungi, and its transcript levels are significantly induced in vegetative hyphae at the time when the competence for asexual development is acquired (Dutton et al. 1997; Wu and Miller 1997; Borneman et al. 2002; Ohara and Tsuge 2004). Measurements of *afp::lacZ* expression in a *stuA* null mutant of *A. nidulans* revealed that their levels are twofold reduced in comparison with the reporter expression in a wild-type background (V. Meyer, unpublished data), suggesting that StuA might be positively involved in *afp* gene regulation.

Finally, also cell wall stress provoked by the presence of Congo Red, an inhibitor of cell wall glucan assembly (Kopecka and Gabriel 1992), induced a twofold increase in *afp* transcript levels in *A. giganteus* (A. Spielvogel and V. Meyer, unpublished data), implicating that the positive-acting RlmA transcription factor (one RlmA binding site is present within the *afp* promoter) might also function in *afp* gene regulation.

Based on these findings, it can be concluded that transcription of the *afp* gene is under control of complex regulatory systems, some of which might even be interconnected. Future studies will be necessary to unravel the mechanisms underlying this sophisticated regulation. However, molecular research on *A. giganteus* is still in its infancy, and hence, the dissection of *afp* gene regulation will first require the establishment of a molecular tool box suitable for targeted genetic manipulation of *A. giganteus*. Additional selection systems (beside hygromycin selection) need to be established for *A. giganteus*, which will be extremely valuable for the generation of multiple mutations. Furthermore, efficient gene targeting approaches need to be adapted for *A. giganteus*, which are a prerequisite to perform *afp* promoter deletion studies in the homologous system or to delete putative transcription factors.

## Concluding remarks and prospects

The development of antifungal agents for agricultural or clinical applications is of considerable interest, as the problem of emergence of antifungal resistant strains continues to grow. The antifungal protein AFP targeting unique characteristics of filamentous fungi is an attractive alternative to currently used drugs. First, it is a naturally occurring peptide, and biodegradability should not constitute an issue. Second, the bioactivity of the protein is restricted to filamentous fungi, i.e. the likelihood of detrimental effects on humans or the environment is considerably low. Third, the protein can be produced in a sustainable way, using *A. giganteus* as host or even another (AFP-resistant) filamentous fungus, for which optimal large-scale fermentation protocols already exist. And finally, the protein has already been shown to efficiently protect plants against fungal infections.

However, still much remains to be done. The complete clarification of the molecular targets of AFP and its mode of action are one of the most important prerequisites for a future application of the protein. Furthermore, the clinical potential of AFP has not been evaluated so far. Numerous animal model systems such as avian and rodent models have already been established (Sarfati et al. 2002; Schmidt 2002), and the antifungal activity of AFP should be investigated in these models to judge a potential use in pharmacology.

In addition, future studies addressing the mode of action of AFP and its regulation can also be of fundamental value. On the one hand, the identification of AFP targets will most probably reveal new insights into the regulation of polar growth of filamentous fungi and the discovery of cellular processes unique to filamentous fungi. For example, an

antifungal agent of bacterial origin helped in the identification of a ceramide synthase crucial for the polar growth of *A. nidulans* (Li et al. 2006). On the other hand, the detailed analysis of the complex pattern of *afp* gene regulation can contribute to a much deeper understanding of regulatory networks present in *Aspergillus* and may unravel the relationships and cross-talks between different regulatory pathways.

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