Review

The small molecular mass antifungal protein of *Penicillium chrysogenum* – a mechanism of action oriented review

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The β-lactam producing filamentous fungus *Penicillium chrysogenum* secretes a 6.25 kDa small molecular mass antifungal protein, PAF, which has a highly stable, compact 3D structure and is effective against a wide spectrum of plant and zoo pathogenic fungi. Its precise physiological functions and mode of action need to be elucidated before considering possible biomedical, agricultural or food technological applications. According to some more recent experimental data, PAF plays an important role in the fine-tuning of conidiogenesis in *Penicillium chrysogenum*. PAF triggers apoptotic cell death in sensitive fungi, and cell death signaling may be transmitted through two-component systems, heterotrimeric G protein coupled signal transduction and regulatory networks as well as *via* alteration of the Ca^{2+}-homeostasis of the cells. Possible biotechnological applications of PAF are also outlined in the review.

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Introduction

New-type antifungal drugs are urgently needed in both human medication and the agriculture to combat invading fungi [1, 2]. Meanwhile many molecules still in use in the clinical wards were discovered more than 3 decades ago [3] fungi are able to develop resistance against these chemicals [4–7], and the extensive use of antimycotics stimulate the emergence of new pathogen species with innate resistance towards these agents [4, 8–10]. Spreading resistance against widely used fungicides is also an emerging problem in plant protection [11–14].

Fortunately, new effective approaches are now available to find promising future targets and molecules for antifungal drug design. These novel tools include functional and comparative genomics, chemical genomics, proteomics and metagenomics based assays [15–22] as well as molecular modeling based on 3D characterization of antifungal targets [3, 23, 24]. Importantly, the available information on natural products and chemically synthesized molecules with antifungal activity are being systematized to allow more complex, e.g. combinatorial chemical, drug design methodologies to flourish [2, 25–28].

One of the most promising strategies to develop a completely new class of antifungal drugs relies on the

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small, cationic, cysteine-rich antifungal proteins produced commonly by eukaryotes including plants, fungi and animals [29–39]. Some of the antifungal proteins have been reported to elicit apoptosis-like cell death in sensitive fungi [30, 38, 40–43], which may provide us with excellent and novel tools to control pathogenic fungi through the modulation of their own cell death programs [30].

Considering availability and exploitability, small basic antifungal proteins are quite wide-spread in ascomycete fungi including biotechnologically important and well-studied *Aspergillus* and *Penicillium* spp. [44–52], which makes both up-stream and down-stream processing steps reproducible, safe and profitable with good yields [30, 53, 54]. Heterologous expression of antifungal proteins in resistant yeasts like *Pichia pastoris* is also applicable [55, 56].

Possible future applications of antifungal proteins may cover (i) antifungal therapies of humans [30, 57, 58], (ii) protection of plants against phytopathogenic fungi by spraying or soaking with antifungal protein solutions [59–63], (iii) construction of transgenic plants less susceptible to fungal infections [64–68] and (iv) prevention of secondary growth of plant pathogens during storage and in food technological processes [49, 69].

This review focuses on the mode of action of the antifungal protein (PAF) secreted by the β-lactam producing fungus *Penicillium chrysogenum* [29, 30, 47], and current knowledge on the structure, physiological function and biotechnological exploitability of PAF is also summarized. At the end, the exciting question whether this antifungal protein can give rise to a family of new-type antimycotics for biomedical and/or agricultural use is addressed.

Production, physiological function and structure

P. chrysogenum produces PAF, a small, cationic, cysteine- and lysine-rich antifungal protein, which was first described about 15 years ago [47]. Undoubtedly, PAF is the best characterized antifungal protein of fungal origin [29, 30, 47] in addition to the ortholog produced by *Aspergillus giganteus* (AFP; for review see [31]).

PAF is synthesized as a prepro-protein and after the removal of the N-terminal pre- and pro- sequences (18 + 19 amino acids) the mature protein is released abundantly into the culture medium with a relative molecular mass of 6.25 kDa (55 amino acids) [30, 47]. Prepro-sequences are of pivotal importance to reach proper folding and full biological activity [70].

The production of PAF is subjected to carbon and nitrogen repression, and both the mRNA and the protein maxima can be observed in the stationary phase of growth at 70–90 h cultivation times in submerged cultures [29, 47]. PAF can easily be purified using cation exchange chromatography [47], and the yields may exceed 10 mg l^{-1} fermentation broth (Dr. É. Leiter, unpublished data).

Although the exact physiological function(s) of PAF remain(s) to be verified a *P. chrysoegnum Δpaf* strain displayed a repressed conidiospore development phenotype together with the reduced expression of *P. chrysogenum*'s *brlA* gene in surface cultures [71]. The physiological function of BrlA (a C2H2-type transcription factor) was first described in *Aspergillus nidulans*, where this protein is a crucial early regulator of conidiogenesis [72–74], and a similar function for the *P. chrysogenum* ortholog has been confirmed [75]. Hence, PAF may function upstream of BrlA and promote conidiospore development [71].

Interestingly, BrlA also regulates the age-dependent appearance of autolysis markers in carbon-depleted *A. nidulans* cultures [76, 77], including the production of cell-wall-degrading hydrolases like ChiB endochitinase and EngA β-1,3-endoglucanase [78, 79]. Cell wall autolysate may supply nutrients for sporulation and, therefore, it is understandable that these physiological processes share common regulatory elements [77]. Contrary to all expectations, there was no difference in the autolysis rates recorded in carbon-starving submerged cultures of wild-type and *Δpaf* mutant strains (N. Hegedűs, unpublished data). This observation may indicate that the physiological function of PAF is limited to surface cultures or is overridden by environmental conditions blocking the conidiogenesis of filamentous fungi under submerged cultivation [72].

Although the contribution of PAF to the modulation of development may provide the producer strains with some advantage in conidiospore production we should not disregard the possible role played by PAF in antibiosis. Obviously, PAF may give its producing organism an ecological advantage when *P. chrysogenum* competes for nutrients with other microorganisms in its habitats [29]. Most likely, *Penicillium* spp. enjoy manifold, physiological and ecological, advantages when harboring the *paf* gene.

As far as the primary sequence of PAF is concerned, it contains 13 lysines giving this protein a highly basic character (pI = 8.9), and 6 cysteines resulting in a highly stable and compact tertiary structure fortified by 3 disulphide bridges [29, 47, 55]. As demonstrated in Fig. 1, the 3D solution NMR structure of PAF consists of

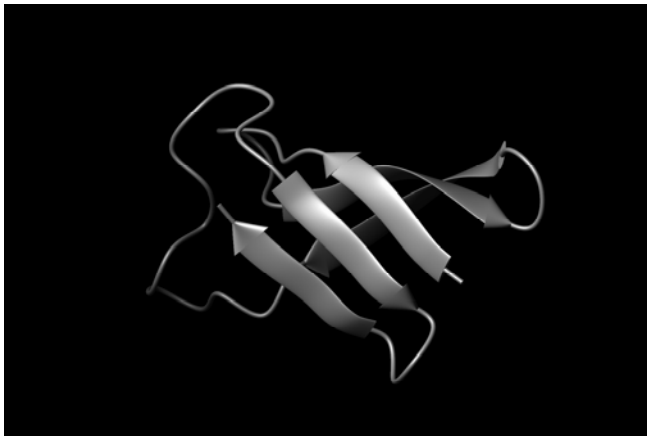

Figure 1. The tertiary structure of PAF. The structure and molecular dynamics of PAF has been determined by solution NMR methods [55]. The most representative structure of PAF is shown here using the PYMOL visualisation software (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). The structural ensemble without disulfide bond constraints has been deposited in Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB ID code: rcsb100954; PDB ID code: 2kcn). NMR chemical shift assignments are deposited in the BioMagResBank with accession number 16087.

five β-strands connected by three small loops involving β-turn motifs and by a big loop. The antiparallel β-strands create two orthogonally packed β-sheets and the six conserved cysteines form three disulfide bonds surrounded by them, creating a hidden hydrophobic central core. The ambiguity in the assignment of two disulfide bonds out of the three however could not be resolved [55]. Importantly, PAF exhibits an amphipatic surface with positively charged lysine residues (Lys9, Lys11, Lys34, Lys35) concentrating at one side of the protein molecule, and the residues Lys9, Lys35 and Lys38, which reside in conserved regions, are needed to exert full biological activity on sensitive fungi as demonstrated by site-directed mutagenesis studies [55] (Fig. 1).

Owing to its structural compactness, PAF exhibits a remarkable pH (1.5–11), heat (80 °C, 60 min) and proteolytic (pepsin, proteinase K, pronase) stability [55]. Nevertheless, PAF preparations lose their biological activities fast when contaminating bacteria are feeding on them (Dr. É. Leiter, unpublished data).

Antifungal activity and mode of action

Not surprisingly, the PAF-producing *P. chyrsogenum* strain exhibited innate resistance against PAF [80]. On the other hand, PAF was active against a wide array of phytopathogenic and human pathogenic fungi including well-known *Aspergillus*, *Mucor*, *Rhizomucor* and *Trichophyton* spp. [29, 47, 80–83] as well as the obligate biotrophic plant pathogen fungi *Blumeria graminis* f. sp. *hordei* and *Puccinia recondita* f. sp. *tritici* [63]. Importantly, PAF-statin combinations inhibited sporangiospore germination synergistically in several zygomycetes [82], and PAF acted synergistically or additively with fluconazole on different dermatophyte *Microsporum* and *Trychophyton* spp. [83].

Sensitive fungi undergo severe morphological changes when exposed to PAF. Swollen crippled hyphae with multiple branches and accumulation of nuclei at broken hyphal tips are typical and spectacular features of the morphological distortions elicited by PAF [63, 80]. In terms of cell physiology, increased K^+-efflux with plasma membrane hyperpolarization at the hyphal tips, shortage of actin and misdistribution of chitin at the hyphal tips, endocytosis-like internalization of PAF, intracellular accumulation of reactive oxygen species, the appearance of fungal cells with typical markers of apoptosis (Annexin V and TUNEL positivity) and the disintegration of subcellular structures including mitochondria are clearly observable [29, 30, 42, 80, 84, 85].

Outstandingly, PAF toxicity seems to require FadA-SfaD-GpgA (G protein α-, β- and γ-subunits, respectively) dependent heterotrimeric G-protein signaling [42], which pathway normally maintains vegetative growth in *A. nidulans* [73, 86–89]. The dominant interfering $fadA^{G203R}$ mutation affects the guanidine binding domain of FadA and, as a consequence, prevents the FadA Gα subunit to dissociate from SfaD-GpgA Gβγ associations, resulting in reduced growth and a hypersporulation phenotype [90]. The $fadA^{G203R}$ blockage of FadA-SfaD-GpgA signaling also reduced considerably the PAF induced cytotoxicity in *A. nidulans* [42], and a similar cytoprotective effect of $fadA^{G203R}$ mutation had been recorded in *A. nidulans* cultures exposed to tobacco osmotin (PR-5 antifungal plant defense protein) [91].

These observations raise the interesting question which G protein subunit is responsible for the transduction of PAF initiated apoptotic cell death signals. As shown in Fig. 2A, the deletion of any of the genes coding for the FadA-SfaD-GpgA subunits resulted in a reduction in PAF sensitivity. This is in good accordance with previous observations by Seo *et al*. [88], who found that elimination of any G protein subunit hindered vegetative growth considerably in *A. nidulans*. This means that both vegetative growth and apoptotic cell death signalings equally require functional heterotrimeric G protein units [42, 88]. FadA-FlbA signaling (FlbA increases the intrinsic GTPase activity of FadA and, hence, is a negative regulator of heterotrimeric G protein signaling; [90]; Fig. 2A) also regulates the degradation of glutathione reserves in carbon starvin *A. ni-*

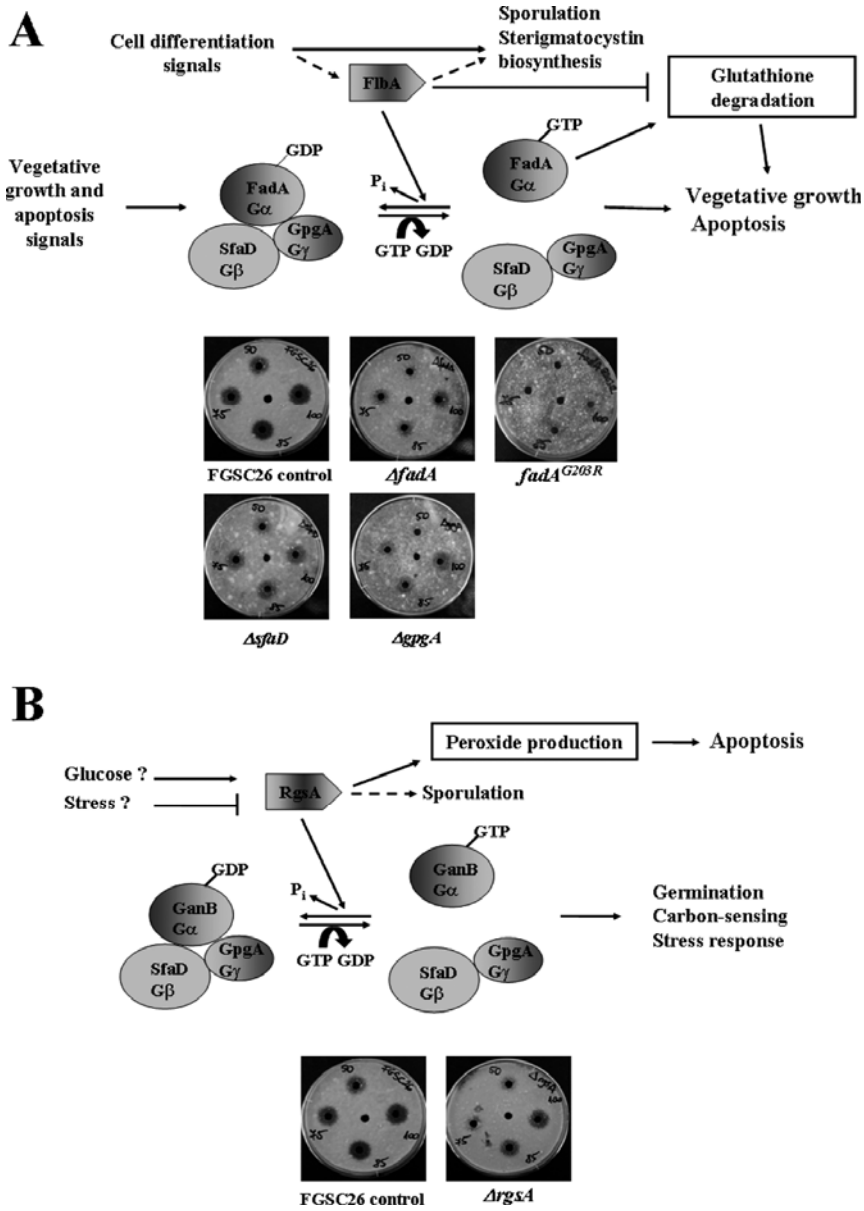

Figure 2. Initiation of cell death by PAF in *Aspergillus nidulans* – the involvement of FadA-dependent (**Part A**) and GanB-dependent (**Part B**) heterotrimeric G protein signaling pathways [88, 89, 95, 96]. FlbA and RgsA are G protein regulators, which facilitate G protein $\alpha\beta\gamma$ subunit associations by stimulating the intrinsic GTPase activities of the appropriate Gα subunits [89, 90]. The construction and genotypes of the mutants have been described in previous publications [87, 88, 95]. The agar diffusion assays were carried out according to Galgóczy et al. [81] on solid YPD medium for 2 d at 25 °C. The Δ*ganB* mutation did not affect the PAF sensitivity of *A. nidulans* (data not shown).

dulans cultures causing severe glutathione/glutathione disulphide redox imbalance [77, 92], which is a well-known elicitor of programmed cell death in fungi [93, 94].

In *A. nidulans*, germination, carbon-sensing and stress sensitivity are controlled by another heterotrimeric G protein signaling pathway consisting of GanB-SfaD-GpgA G protein α-, β- and γ-subunits [95, 96]. In this pathway, the RgsA regulatory protein facilitates G protein $\alpha\beta\gamma$ subunit associations by increasing the intrinsic GTPAse activity of GanB [89]; Fig. 2B). Because RgsA down-regulates oxidative stress response through the modulation of catalase activity and intracellular peroxide concentration [95, 97] the deletion of *rgsA* resulted in a PAF-tolerant phenotype (Fig. 2B). In addition to glutathione redox imbalance, increasing reactive oxygen species levels also initiate the onset of apoptotic cell death programs in different eukaryotes including fungi [93, 98].

At this point, it seems to be reasonable to assume that both FadA(FlbA)-SfaD-GpgA and GanB(RgsA)-SfaD-GpgA heterotrimer G protein signalings contribute to the PAF-initiated cell death in *A. nidulans* (Figs. 2A, B) through the modulation of intracellular glutathione and reactive oxygen species levels [92, 97]. Nevertheless, these pathways share the same G protein βγ subunits [89] and, hence, any change affecting the availability of SfaD and GpgA may affect simultaneously both FadA- and GanB-dependent signalings giving rise to rather complex regulatory patterns.

Downstream of the heterotrimer G protein signaling pathways, PkaA and PkaB protein kinases may regulate germination, vegetative growth and stress responses [89, 99]. PkaA may be activated by both FadA and GanB G protein α-subunits, and maintains germination and vegetative growth [99]. Not surprisingly, heterotrimeric G protein generated cell death signals are likely to proceed *via* the cAMP-PkaA pathway. In fact, a *pkaA* deletion mutant of *A. nidulans* exhibited a reduced sensitivity to PAF exposures, and PAF augmented or ameliorated the toxic effects of 8-Br-cAMP (activates cAMP-PkaA signaling) or caffeine (decreases cAMP concentration), respectively [85]. In addition, the PkcA-MpkA protein kinase-mitogen activated protein kinase cell wall integrity signaling pathway [100–104] mediated basal resistance towards PAF although this antifungal protein failed to activate MpkA *via* phosphorylation [85]. This finding indicates that PAF is not primarily targeting the cell wall/cell wall components of sensitive fungi and, hence, does not induce cell wall remodeling [85]. This is further corroborated by the solution NMR and surface plasmon resonance data excluding any strong PAF-chitobiose binding [55]. On the contrary, *A. giganteus* AFP is a chitin-binding protein, interferes with chitin biosynthesis in sensitive fungi and induces cell wall stress [31, 105].

Because PAF provokes hyperpolarization of sensitive hyphae [42] one might suggest that the mechanism of PAF cytotoxicity should be explained primarily by the perturbation of cation homeostasis. In addition to the K^+-efflux recorded in *A. nidulans* [80], significant increases in the resting cytosolic Ca^{2+}-concentrations of PAF-exposed *Neurospora crassa* cells have been reported most recently by Binder *et al.* [106]. Intriguingly, although the uptake of extracellular Ca^{2+}-ions was stimulated by PAF the L-type Ca^{2+}-channel CCH-1 did not play any role in this PAF-induced physiological change [106]. Further studies are needed to clarify how rapid changes in the resting cytosolic Ca^{2+}-concentrations will trigger oxidative stress and apoptotic cell death in PAF-exposed hyphae [29, 30, 42, 80, 84, 85].

There is an increasing body of evidence supporting the view that, similar to yeasts, the SskB-PbsB-HogA/SakA mitogen-activated protein kinase system is the centerpiece of stress signaling in the *Aspergilli* [107–113]. Important upstream elements of HogA/SakA signaling are NikA histidine kinase, YpdA histidine-containing phosphotransmitter and SskA response regulator, which set up a two-component histidine-to-aspartate phosphorelay [110–117]. Downstream of HogA/SakA the bZIP-type transcription factor AtfA may function and regulate global transcriptional stress responses [111, 113, 118–121]. It is noteworthy that another response regulator protein, SrrA, also functions in the *Aspergilli* and is essential in the regulation of oxidative stress response [110, 112, 113, 117]. In addition to AtfA [111, 121], NapA bZIP-type transcription factor (an ortholog of budding yeast's Yap1p and fission yeast's Pap1p) also participates in the regulation of oxidative stress response in *A. nidulans* [122], and may stimulate SrrA function in addition to His-Asp phosphorelay circuitry [117]. Considering that PAF treatments cause oxidative stress in *A. nidulans* [80] it was reasonable to assume that either the SskA-HogA/SakA-dependent or the SrrA-dependent (or both) stress signaling pathway should be activated to cope with the deleterious effects of PAF-exposures.

As shown in Fig. 3, neither the NikA-SskA phosphorelay nor the HogA/SakA mitogen-activated protein kinase system played any role in the orchestration of the stress defense against PAF in *A. nidulans*. Quite surprisingly and paradoxically, the deletion of the *srrA* gene resulted in a PAF-tolerant phenotype. It is well-documented that the antifungal effects of iprodione and fludioxonil proceed through NikA-SskA-HogA/SakA and NikA-SrrA signaling pathways and, in accordance with this, *ΔnikA*, *ΔsrrAΔsskA* and *ΔsrrAΔhogA* mutants displayed iprodione and fludioxonil tolerant phenotypes [115]. Because PAF-tolerance resulted only in the *ΔsrrA*, *ΔsrrAΔsskA* and *ΔsrrAΔhogA* mutants without any phenotype appearing in the *ΔnikA* mutant (Fig. 3B) we assume that the antifungal effect of PAF was transmitted by a SrrA-dependent system other than NikA-SrrA phosphorelay and without the involvement of SskA-HogA/SakA signaling. The genome of *A. nidulans* harbors 15 histidine kinases and only four response regulators and one sole phosphotransmitter [116]. Therefore, the multiple involvement of SrrA in different phosphorelay networks seems to be probable. Alternatively, PAF-initiated cell death signaling might be transmitted *via* SrrA without the involvement of phosphorelay components as it has been suggested, at least in part, for the transmission of H_2O_2-generated oxidative stress signals [112, 117] (Fig. 3A).

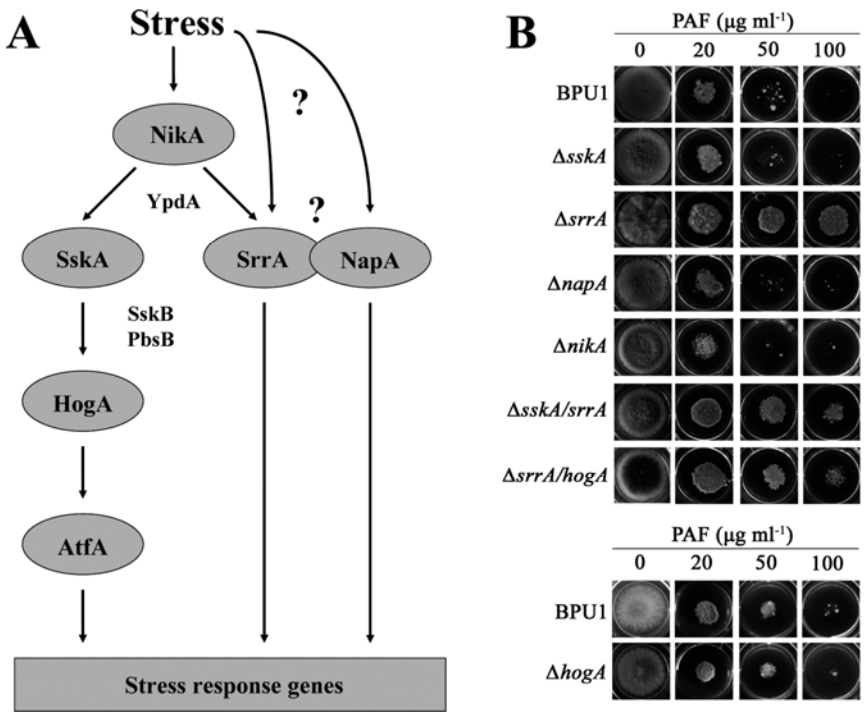

Figure 3. Transmission of the antifungal effect of PAF *via* SrrA response regulator – a sensitivity screening study. **Part A:** A brief overview of stress signaling pathways in *Aspergillus nidulans* [110–113, 115–117, 121, 122]. As demonstrated by Vargas-Pérez *et al*. [112], H_2O_2-generated oxidative stress signal proceeds *via* SrrA independently of NikA histidine kinase. The NapA transcription factor is a regulator of the oxidative stress response of *A. nidulans* [122], and it may also interact with SrrA leading to the activation of certain stress response genes in an YpdA-phophorylation-independent manner [117]. As indicated by the questions marks, upstream elements of the NikA-independent oxidative stress signaling are not known yet, and the formation of SrrA-NapA complex also needs verification. **Part B:** PAF tolerance screening in stress signaling pathway mutants of *A. nidulans* [110, 115, 122]. In all assays, the BPU1 strain was used as control, and the strains were grown in 12-well tissue culture plates for 3 d at 37 °C on appropriately supplemented solid complex medium as described elsewhere [80, 110].

Remarkably, none of the bZIP-type oxidative stress responsive transcription factors studied {neither NapA (Fig. 3B) nor AtfA [121]} took part in the orchestration of the PAF stress response, and this might contribute to the cytotoxicity of PAF [29, 30, 42, 80].

As demonstrated by Fig. 3B, PAF is a valuable tool in mapping novel cell death signaling pathways in filamentous fungi [30, 42, 85], which may lead eventually to the development of new-type antifungals [123]. Mosaics of the signal transduction pathways transmitting the deleterious effects of PAF or, just the opposite, alarming genome-wide regulatory networks to combat PAF-initiated stress have been revealed [30, 42, 85] (Fig. 3), but this is only the peak of an iceberg. For example, nothing is known about the primary targets, either locating on the cell surface or in the cytoplasm, of this antifungal although G-protein coupled receptors [124], lipid rafts [30] and/or two-component histidine-to-aspartate phosphorelays are among the most promising candidates. The precise identification of the targets of antifungal proteins and the elucidation of their mode of action are of primary importance when their future biotechnological applications are considered, *e.g.* in the field of plant protection [125].

Some new hints on the mechanism of action of PAF came from the analyses of its 3D NMR solution structure [55]. The three disulphide bridges present in biologically active PAF not only maintain protein integrity but may also make this protein able to be internalized by membrane-associated oxidoreductases like protein disulfide isomerases and may also confer PAF redox activity in the cytoplasm giving rise to reactive oxygen species [55]. Evidently, further structure-function analysis studies may shed light on the precise mechanisms of action of antifungal proteins of fungal origin.

Comparative analyses of the versatile survival strategies of yeasts and filamentous fungi to combat the deleterious effects of antifungal proteins may also lead us to a deeper understanding of how these antimycotics work. The sensitivity of fungi towards PAF differs significantly, and the exclusive activity of PAF against filamentous fungi but not against yeasts suggests that specific targets (*e.g.* membrane lipids and/or receptors) or increased target accessibility may define the species-

specific efficiency of PAF [29, 30]. Most recently, Ouedraogo *et al*. [126] adopted the concept of 'damage-response framework of microbial pathogenesis' published by Casadevall and Pirofski [127] to explain the differences in the success rates of fungi and yeasts to cope with antifungal proteins. According to this hypothesis, an adequate response against antifungals causes resistance, but if the response is too weak or too strong, the target organism is damaged or killed. It is reasonable to assume that the different strategies of fungi against PAF contribute to the observed sensitivity or resistance towards this antifungal protein.

It is important to note that the outcome of fungus-antifungal protein interactions will also depend on the antifungal potential of the protein and the innate susceptibility of the organism [126]. For example, *Aspergillus terreus*, which is an amphotericin B resistant mould [128], is also resistant to PAF exposures [80, 84] most likely owing to its inherently high specific catalase activity [128].

Possible technological applications of PAF

The possible future biomedical, agricultural and food technological applications of antifungal proteins of different origin have been reviewed recently by several authors [29–39].

Because neither *P. chrysogenum* PAF nor *A. giganteus* AFP caused detrimental effects on mammalian including human cells and tissues when tested in the intended therapeutic concentration ranges *in vitro* both antimycotics are attractive and promising candidates in future antifungal drug developments [57, 58]. Obviously, further studies are needed to demonstrate the safety of these antifungal proteins in animals and their effectiveness against obligatory and opportunistic human pathogenic fungi in suitable animal models. Human dermatophytes are undoubtedly among the first possible future targets of PAF-based therapies especially when employed in combination with "in use" antimycotics like azoles [83].

An interesting new aspect of the biotechnological application of *Penicillium* PAF arose from the observation that *P. nalgiovense*, a mould used in some food technological processes, also harbors a *paf* gene and produces a PAF protein that is identical to its *P. chrysogenum* counterpart [49]. This finding raises the interesting question whether this mould (and other *Penicillia* used in the food industry) produces PAF during food ripening as well, i.e. PAF whether or not enters the digestive tract of humans as a normal part of daily nutrition. Because *P. chrysogenum* is "generally recognized as safe" (GRAS) by the US FDA [30] high-scale industrial production of PAF either by this β-lactam producer or by other *Penicillia* used routinely in the food industry is an easy challenge for today's fermentation technology. Another exiting option is to control filamentous fungus growth by PAF during storage of food ingredients or in the food technological processes themselves [49, 69].

Today, incorporation and expression of *paf* gene in crops seems to be one of the most promising ways to start agricultural applications of PAF. Previous successful attempts to construct transgenic wheat [64, 68], rice [61, 65, 66] and pearl millet [67] producing biologically active *A. giganteus* AFP are really encouraging and inspiring in this regard.

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