Examination of antimicrobial effect of *Neosartorya fischeri* Antifunal Protein (NFAP) on an NFAP-producing *Aspergillus nidulans* strain

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Objectives

Neosartorya fischeri (anamorf: *Aspergillus fischerianus*) NRRL 181 isolate secretes a β-defensin-like molecule, the *Neosartorya fischeri* antifungal protein (NFAP) [1].
NFAP effectively inhibits the growth of filamentous fungi including potentially human pathogens in a dose dependent manner, and maintains its antifungal activity within wide pH and temperature range, furthermore exhibits relevant resistance to proteolysis [1,2].
These previous results suggest that NFAP is potential antifungal peptide for a practical purpose; however the deeper manifestation and the mode of its antifungal effect has not been studied so far. For this purpose we investigated the antimicrobial effect of NFAP with heterologous expression of the *nfap* gene in an NFAP-sensitive fungus, *Aspergillus nidulans*.

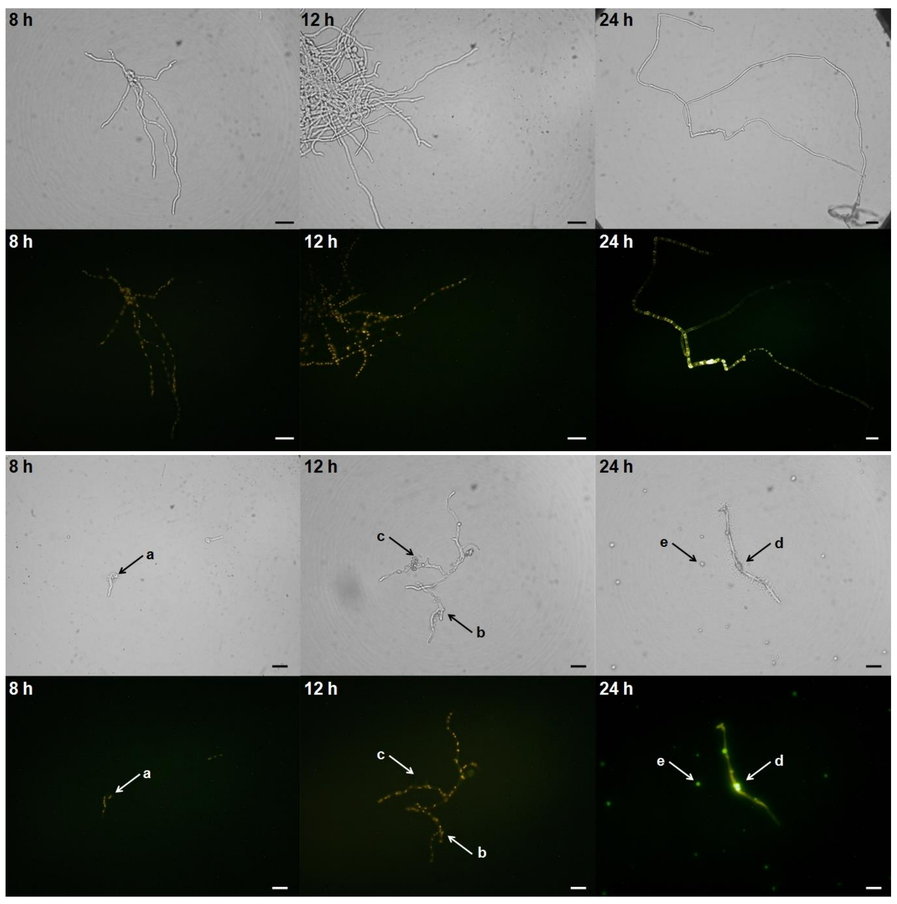

Fig. 2. DAPI-staining of germinating conidiospores of untransformed (up) and transformed (down) *A. nidulans* CS2902.
a: germination tube destruction, **b**: accumulation of nuclei at broken hyphal tips, **c**: swollen hyphal tips with hyphal deatachments, **d**: destructed hyphal fragments, **e**: small mycelial remnants with nuclei Scale bars represent 20 µm.

Table 3. Dry weight (mg) of *A. nidulans* CS2902 mycelia grown from 10^5 conidia/ml in presence of 50 and 100 mM mono- and divalent cations.

Medium / Strain	CM	CM+KCl	CM+Na_2SO_4	CM+$MgSO_4$
		50 mM		
untransformed	132.8(±5.6)	128.9(±7.3)^{ns}	133.9(±3.8)^{ns}	138.2(±8.9)^{ns}
transformed	93.1(±5.0)	103.0(±4.7)*	126.0(±1.4)***	116.7(±1.9)***
		100 mM		
untransformed	129.1(±14.1)	121.2(±7.2)^{ns}	124.0(±4.1)^{ns}	132.6(±10.6)^{ns}
transformed	92.1(±5.2)	118.4(±3.5)**	158.9(±12)***	138.7(±1.5)***

Standard deviations of three replicates (N=3) at three times are indicated in the brackets. Significant differences (p-values) were determined based on the comparison with the dry weight from CM medium. ***: p<0.0001, **: p<0.005, *: p<0.05, ns: no significant differences.

Materials and Methods

Strains and media
Heterologous expression of *nfap* gene was carried out in *Aspergillus nidulans* CS2902 (*pyrG89, riboB2, pyroA4, biA1*) [3].
Transformation pAMA-NFAP vector
The autonomously replicating pGLnfap construction carries the *nfap* cDNS with gpdA promoter and trpC terminal region of the pANGFP vector and the pUC19, pyr4 region of pAMA (Fig. 1).
Transformation of pGLnfap into *A. nidulans* CS2902 was achieved based on the method described by Tilburn et al. with the use of MM as selection medium [7].
Expression of NFAP in *A. nidulans CS2902*
For the investigation of self-poisoning effect of transformants 10^4, 10^5, 10^6 CFU/ml conidiospore was inoculated in 10 ml CM medium and incubated at 37 °C with shaking (160 rpm) for 7 days. The dry weight of the mycelia was measured after harvesting by centrifugation (30 min, 10.000×g) and lyophilization for 24 h. Untransformed *A. nidulans* CS2902 was used as control.
Investigation of the antifungal effect of NFAP on *A. nidulans* CS2902 transformants
Effect of the produced NFAP on the germination of NFAP-producing *A. nidulans* conidia was investigated with 4'-6-Diamidino-2-phenylindole- (DAPI, Serva; 0.1 µg/ml 30 min, room temperature) and calcofluor white-staining (CFW, Sigma-Aldrich; 1 µg/ml, 10 min, room temperature). Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) was used for revealing the possible apoptotic effect.

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Results

Investigation the antifungal effect of NFAP on *Aspergillus nidulans* CS2902 transformants
Macroscopic observations revealed the reduction of hyphal growth in case of the transformants expressing the *nfap* gene compared to the untransformed *A. nidulans* CS2902 strain (Table 1). This effect was absent in presence of mono- and divalent cations (50 and 100 mM KCl, Mg_2SO_4, Na_2SO_4) (Table 2).
The self-poisoning effect of NFAP on *A. nidulans* CS2902 transformants depended on the amount of conidia (CFU/ml) used for the inoculation (Table 1). Based on this observation 10^5 conidia/ml was used for further studies.
Microscopic observation revealed that transformants displayed abnormal and delayed germination compared to the untransformed strain: conidiospores formed very short, swelled hyphae with multiple branches (Fig. 2). The germination tubes were unstructured or destructed after 8 hours of cultivation (Fig. 2, Fig. 3, Fig. 4). Later, membrane damage and accumulation of nuclei at the broken hyphal tips were detected by DAPI-staining (Fig. 2). After incubation for 12 and 24 h, accumulation of destructed hyphal fragments with or without nuclei was observed in the medium (Fig. 2).
CFW-staining revealed differences in the building up of the chitin filaments between the transformed and untransformed strain. Chitin filaments were destructed and diffused among the germinating conidia at NFAP-producing *A. nidulans* CS2902 (Fig. 3). After incubation for 12 and 24 h cell walls were unstructured and the destructed chitin filaments were gathered around the mycelia in case of transformants (Fig. 3).
Apoptotic events were also detected after 8 h of incubation in case of NFAP-producing *A. nidulans* conidia during its germination (Fig. 4).

Table 1. Dry weight (mg) of *A. nidulans* CS2902 mycelia depending on the concentrations of conidia.

CFU / Strain	10^4	10^5	10^6
untransformed	77.3(±4.4)	131.9(±3.8)	182.1(±4.9)
transformed	65.0(±3.0)^{ns}	88.1(±5.8)***	00.0(±0.0)***

Standard deviations of three replicates (N=3) are indicated. Significant differences (p-values) were determined based on the comparison with the non-transformed strain. ***: p<0.0001, ^{ns}: no significant differences.

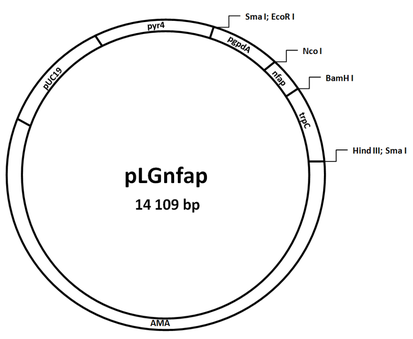

Fig. 1. Schematic representation of pGLnfap vector construction.
pgpdA: promoter of gpdA, nfap: cDNA of *Neosartorya fischeri* antifungal protein, trpC: terminator of trpC, AMA: part of pAMA vector, pUC19: pUC19 region, pyr4: pyr4 region.

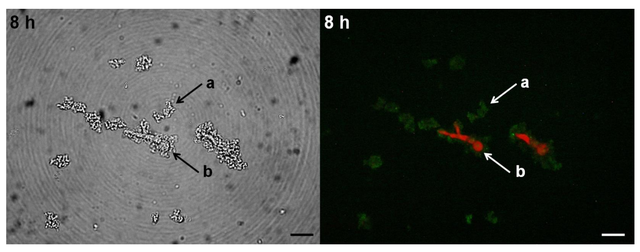

Fig. 4. Staining of germinating conidiospores of *A. nidulans* CS2902 transformant with Annexin V-FITC Apoptosis Detection Kit. Scale bars represent 20 µm.
a: Destructed mycelial fragments, **b**: with apoptotic germinating conidia

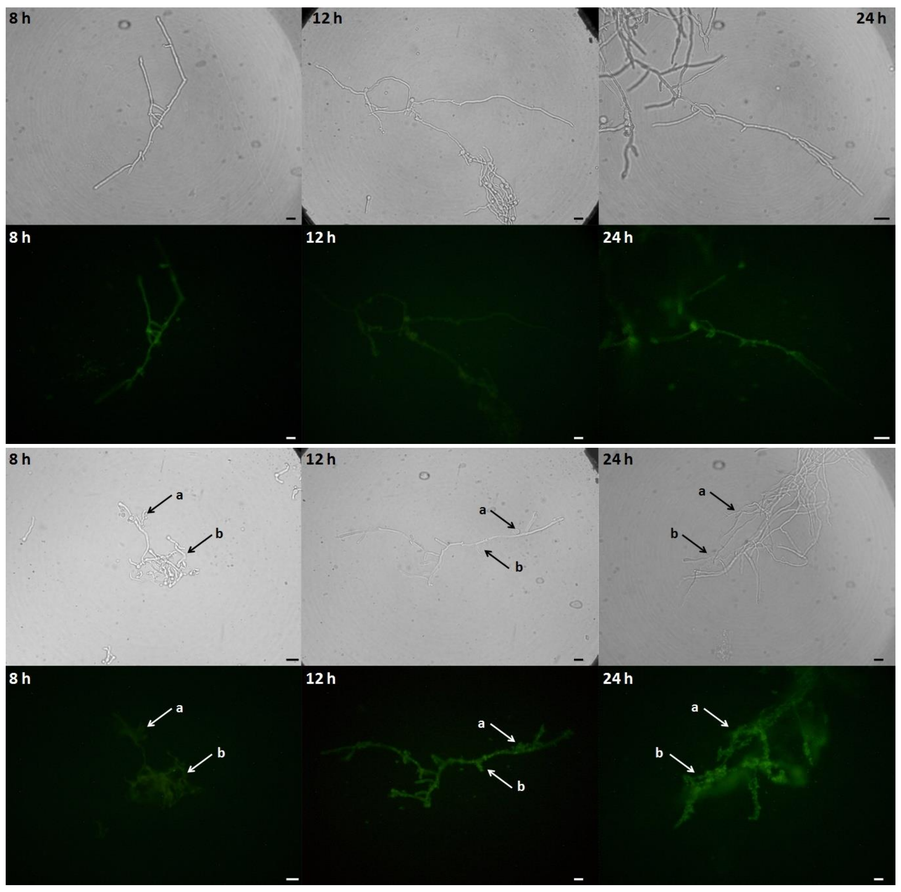

Fig. 3. CFW-staining of germinating conidiospores of untransformed (up) and transformed (down) *A. nidulans* CS2902. Scale bars represent 20 µm.
a: hyphal fragments with destructed chitin filaments, **b**: unstructured cell wall

Conclusion

The self-poisoning effect of *A. nidulans* CS2902 transformants was depended on the amount of conidia.
The manifestation of the antifungal effect of NFAP exerted on *A. nidulans* CS2902 transformants was similar to those described previously at related peptide from *Aspergillus giganteus* (AFP) and *Penicillium chrysogenum* (PAF) [8]: Based on these similar results, it is hypothesised that NFAP also has a specific binding structure in the outer layer and cell wall of the sensitive fungi and destructs their integrity.
The disturbance of the building-up of chitin filaments could be the result of the damage of cell wall integrity not of the specific inhibition of the chitin synthase, because of the the lack of N-terminal chitin biding domain in the NFAP which was described at AFP.
Further studies are needed for the confirmation of that the apoptotic phenotype in case of NFAP-producing *A. nidulans* CS2902 is consequence of a similar effect as exerted by PAF (programmed cell death trough ROS) or only the result of germination disturbance.

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