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Antifungal peptides homologous to the *Penicillium chrysogenum* antifungal protein (PAF) are widespread among Fusaria

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ABSTRACT

Putative antifungal peptide encoding genes containing *Penicillium chrysogenum* antifungal protein (PAF) characteristic amino acid motifs were identified in 15 *Fusarium* isolates, representing 10 species. Based on the predicted sequences of mature peptides, discrepancy in one, two or three amino acids was observed between them. Phylogenetic investigations revealed that they show high amino acid sequence similarity to PAF and they belong to the group of fungal derived antifungal peptides with PAF-cluster. Ten from the 15 partially purified <10 kDa peptide fraction of *Fusarium* ferment broths showed antifungal activity. The presence of approximately 6.3 kDa molecular weight peptides was detected in all of the antifungally active ferment broths, and this peptide was isolated and purified from *Fusarium polyphilaidicum*. The minimal inhibitiory concentrations of *F. polyphilaidicum* antifungal protein (FPAP) were determined against different filamentous fungi, yeasts and bacteria. Filamentous fungal species were the most susceptible to FPAF, but some yeasts were also slightly sensitive.

1. Introduction

From the second part of the 1990s several peptides with similar structure to the β-defensins have been isolated and characterized from filamentous fungal species belonging to Ascomycetes [12,21,26]. The main features of these extracellular peptides are a low molecular mass, a basic character, and the presence of 6–8 cysteine residues and several intramolecular disulfide bonds which provide them with a high stability against protease degradation, high temperature and within a broad pH range [12,21,26]. The tertiary structure of the peptides is very similar to the β-defensins, it contains five antiparallel β-sheets connected by three loops, and showing a β-barrel topology in general [3,26]. In spite of that they are very different in their amino acid sequences, conserved homologous regions can be identified in them [21,26,30]. Based on these conserved regions, these molecules can be divided into two groups [30]: peptides which contain the *Penicillium chrysogenum* antifungal protein (PAF) cluster in their amino acid sequences, and peptides with bubble protein (BP) cluster. Both types have a potent antifungal activity [21,26,30]. Peptides with PAF cluster are effective against filamentous fungi [12,21,26], while peptides with BP cluster may effectively inhibit the growth of yeasts [30]. Representatives of the first group were isolated and characterized from taxonomical distinct species, such as *Aspergillus clavatus*, *Aspergillus giganteus, Aspergillus niger, Neosartorya fischeri, P. chrysogenum*, *Penicillium nalgiovense* [12,13,15,19,21,26,31], and further homologs to them are supposed in *Gibberella zeae* (accession no.: XP_384921), *Pyrenophora tritici-repentis* (accession no.: XP_001934325) [12]. Only one peptide with BP cluster was isolated until now from *Penicillium brevicompactum* [30], but similar putative peptides were revealed in the genome of *Aspergillus fumigatus* (accession no.: XP_731495), *Chaetomium globosum* (accession no.: XP_001227832), *Myceliophthora thermophila* (accession no.: XP_003666363), *P. chrysogenum* (accession no.: XP_002568323), *N. fischeri* (accession no.: XP_001257339) [30]. Additionally, a third different group is also hypothesized represented by *Botryotinia fuckeliana* (accession no.: XP_001548954) [12].

Peptides containing PAF-cluster generate similar symptoms in the susceptible organisms (inhibition of spore germination and hyphal growth, retardation of the hyphae lengthening, membrane perturbation, induction of intracellular oxidative stress and an apoptosis-like phenotype), in spite of the fact that their mode of action could be different. For example AFP disturbs the cell wall biosynthesis, and PAF evokes programmed cell death *via* a

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G-protein signal transduction pathway [22,26]. The antifungal spectra of the peptides are also differing; nevertheless, their tertiary structure is very similar [12].

The β-defensin-like antimicrobial peptides with PAF-cluster are interesting in practical respect, and their potential applications are intensively studied both in medical and agricultural fields. Their following features that they have potent antifungal activity against potential human and plant pathogenic fungal species [12], that they could not have any toxic effects on plant and mammalian cells *in vitro* [32,33], that they can interact synergistically with other antifungal drugs and peptides [10,11,23], and their low costs of production could make them suitable as active ingredients of commercial biopesticides and medicines [21,26].

Previous *in silico* investigation of genomic databases revealed peptides with high homology to the PAF in the genome of other ascomyceteous filamentous fungi, such as in the genome of *Gibberella zeae* (anamorf: *Fusarium graminearum*) [21]. Based on this observation the aims of the present study were the followings: (i) to investigate the presence of *G. zeae* hypothetical antifungal protein (GAMA, accession no.: XP_384921) and its homologs in *Fusarium* species, (ii) to reveal their phylogenetic relationship with the other peptides containing the PAF-cluster, and (iii) to isolate the most effective one of them and to characterize it in regard to the *in silico* predicted structure and the antifungal activity.

2. Materials and methods

2.1. Strains and media

An antifungal protein induction medium (AFPIM: 1.5% starch, 1% beef extract, 2% pepton, 0.5% NaCl, 1% ethanol) was used for production of antifungal peptides [19].

The following 15 *Fusarium* isolates representing 10 different species were involved in the screening of antifungal peptide encoding genes: *F. asiaticum* (Szeged Microbial Collection, University of Szeged, Szeged, Hungary; SzMC 3891), *F. avenaceum* (SzMC 11044), *F. boothi* (SzMC 3885, SzMC 386), *F. cerealis* (SzMC 11048), *F. culmorum* (SzMC 3890, SzMC 11039, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA; NRRL 29354, NRRRL 29388), *F. graminearum* (SzMC 2887, SzMC 3888), *F. poae* (SzMC 11045), *F. polyphialidicum* (SzMC 11042), *F. pseudograminearum* (NRRL 28069), and *F. sporotrichioides* (SzMC 11043). All Fusaria derived from infected plant.

The antifungal activities of the <10 kDa fraction of the ferment broths were investigated by an agar diffusion technique on low cationic agar medium (LCM; 2% glucose, 0.1% yeast extract, 0.05% peptone, 2% agar) on the hyphal growth of the PAF-sensitive *Trichoderma longibrachiatum* (University of Alberta Microfungus Collection and Herbarium, Alberta, Canada; UAMH 7955) and *Mortierella elongata* (NRRL 5513) [12,19].

The antifungal effect of the *Fusarium polyphialidicum* antifungal protein (FPAP) was examined in LCM broth against 19 zygomyceteous, 16 ascomycetous (including filamentous fungi and yeasts) fungal isolates and 4 bacterial isolates (including Gram-positive and negative strains). These isolates are listed in Table 1.

2.2. In silico investigations

The BioEdit program [16] was used to examine the antifungal protein sequences, similarity searches in the NCBI databases were performed using the Basic Local Alingment Search Tool [1]. The physical and chemical properties of the mature FPAP were examined with the primary structure analysis software of the ExPASy Proteomics Server [35]. The signal sequence, the structure, the 3D model and the disulfide bridges of the mature peptide were predicted using the SignalP 4.0 Server [27], the SCRATCH Protein Predictor [5], and the MODELER 9.9 [29] and the DISULFIND [4], respectively. The 3D model of FPAP was visualized by UCSF Chimera software [28].

For the phylogenetic analysis the available PhyML 3.0 aLRT software (with SH-like approximate likelihood-ratio test and the WAG substitution model) and the TreeDyn viewer from the website of Methodes et Algorithmes pour la Bio-informatique (http://www.phylogeny.fr) were used [2,5–8,14].

2.3. Isolation of antifungal peptide encoding genes from Fusaria

Fifteen fungal isolates representing 10 species have been screened by PCR. DNA was isolated with the E.Z.N.A. SP Fungal DNA Kit (Omega Bio-tek) from 96 h old mycelia grown up in potato dextrose broth (Sigma–Aldrich) at 25 °C under continuous shaking (220 rpm). PCR to amplify the DNA fragments of the putative antifungal protein encoding genes were performed with *gama1* (5′-GAGTCCATGTGTCTGCGTTTGCTG-3′) and *gama2* primers (5′-ACCCTAGCCCAAACTCTGCGCAATC-3′). These primers were designed to the −100–124 bp and +95–110 bp regions of the *G. zeae* hypothetical antifungal protein (GAMA, accession no.: XP_384921) encoding gene (GenBank accession no.: BK004091). PCR experiments were performed according to the following temperature regime: 94 °C, 3 m; 94 °C, 1 m; 45 °C, 1 m; 68 °C, 1 m 15 s, 10 cycles, dT/ds = 0,18; 94 °C, 45 s; 54 °C, 1 m; 68 °C, 1 m 15 s, 20 cycles, dt/ds = 5; 68 °C, 10 m. The resulting 619 bp length amplicons were checked by gel electrophoresis in 0.8% agarose gel, and after isolation with the Gen Elute Minus EtBr Column (Sigma–Aldrich) they were sequenced (Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary).

RNA and the cDNA of the putative proteins were isolated and synthesized with the E.Z.N.A. Fungal RNA Kit (Omega Bio-tek) and the Revert Aid H Minus First Strand cDNA Syntehsis Kit (Fermentas). Primers designed to the open reading frames (ORF) were used to amplify the cDNA with the following parameters: 94 °C, 2 m; 94 °C, 30 s; 53 °C, 30 s; 72 °C, 1 m, 30 cycles; 72 °C, 5 m. Amplicons were sequenced by Biological Research Center of the Hungarian Academy of Sciences (Szeged, Hungary).

The nucleic acid sequences of the hypothetical antifungal protein encoding genes were deposited in EMBL database under the following accession numbers: *F. boothi* SzMC 3885, FM212222; *F. boothi* SzMC 3886, FM212223; *F. graminearum* SzMC 3887, FM212224; *F. graminearum* SzMC 3888, FM212225; *F. pseudograminearum* NRRL 28069, FM212226; *F. cerealis* SzMC 11048, FM212227; *F. polyphialidicum* SzMC 11042, FM212228; *F. sporotrichioides* SzMC 11043, FM212229; *F. poae* SzMC 11045, FM212230; *F. avenaceum* SzMC 11044, FM212231; *F. culmorum* SzMC 11039, FM212232; *F. culmorum* SzMC 3890, FM212233; *F. culmorum* NRRL 29354, FM212234; *F. culmorum* NRRRL29388, FM212235; *F. asiaticum* SzMC 3891, FM212236.

PCR reaction mixtures (all materials from Fermentas) contained 5 pg template DNA, 2.5 μl 10 × Pfu buffer with 20 mM $MgSO_4$, 0.3 μl Pfu polymerase (2.5 U/ml) 5 μl of dNTP Mix (each 2.5 mM), and 2 μl of each primer (100 pmol/ml) in a final volume of 25 μl.

2.4. Antifungal peptide production and partial ferment broth purification

AFPIM inoculated with 10^5 conidia/ml and incubated at 220 rpm, 25 °C for 7 days and 37 °C for 1 day was used for production of the antifungal peptides [19]. Supernatants of the fungal cultures were partially purified with dialysis against 20 mM Tris–HCl/OH buffer pH 7.2 (Dialysis Tubing, Benzoilated, Sigma–Aldrich) and