POTENTIAL APPLICATIONS OF FILAMENTOUS FUNGUS DERIVED β-DEFENSIN-LIKE ANTIFUNGAL PROTEINS IN AGRICULTURE

LÁSZLÓ GALGÓCZY, MÁTÉ VIRÁGH, LAURA KOVÁCS, LILIÁNA TÓTH, CSABA VÁGVÖLGYI

University of Szeged, Faculty of Science and Informatics
Department of Microbiology,
Hungary, Szeged 6726 Közép fasor 52.
galgoczi@gmail.com

ABSTRACT
Many filamentous fungi are postharvest and destructive plant pathogens and are thus responsible for enormous crop losses worldwide. The antifungal proteins secreted by filamentous fungi are promising agents for prevention of fungal diseases in the agriculture. The extracellular β-defensin-like antifungal proteins derived from ascomycetous filamentous fungal species are especially interesting in this respect because of their chemical and biological properties. The main features of these extracellular proteins 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. The tertiary structure of these proteins is very similar to the β-defensins, it contains five antiparallel β-sheets connected by three loops. In spite of the fact that they are very different in their amino acid sequences; conserved homologous regions can be identified. Based on it they can be divided into two main groups: peptides which contain the *Penicillium chrysogenum* antifungal protein (PAF) cluster in their amino acid sequences, and peptides with *Penicillium brevicompactum* bubble protein (BP) cluster. Both of them have a potent antifungal activity, but the peptides with PAF-cluster are effective against filamentous fungi. These proteins secreted by taxonomical distinct species cause similar symptoms on the susceptible fungus, but they have different mode of action and species specificity, nevertheless, their structure is very similar. They have high stability and efficacy; their limited toxicity and low costs of production could make them suitable for use in practical respects in agricultural fields, especially in plant protection on the field and crop protection after the harvest.

Keywords: β-defensin, ascomyceteous filamentous fungi, antifungal effect, phytopathogenic fungi, postharvest pathogenic fungi

INTRODUCTION

Many filamentous fungi are postharvest and destructive plant pathogens and are thus responsible for enormous crop losses worldwide. Therefore there is a substantial demand for new compounds with antimicrobial activity. The proteins with similar structure like β-defensins are interesting from this respect, as they have effective inhibitory potential as concerns both the bacteria and fungi. The β-defensins are part of the secondary defence system, innate immunity, which was discovered in the early 1980s in the higher organisms (GANZ, 2003). From the second half of the 1990s several peptides with highly similar structure to the β-defensins have been isolated and characterized from filamentous fungal species belonging to Ascomycetes *(Figure 1.)* (GALGÓCZY ET AL., 2013A).
The main features of these extracellular proteins are a low molecular mass, a basic character, and the presence of 6-8 cysteine residues and several intramolecular disulfide bonds. 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 (GALGÓCZY ET AL., 2010).

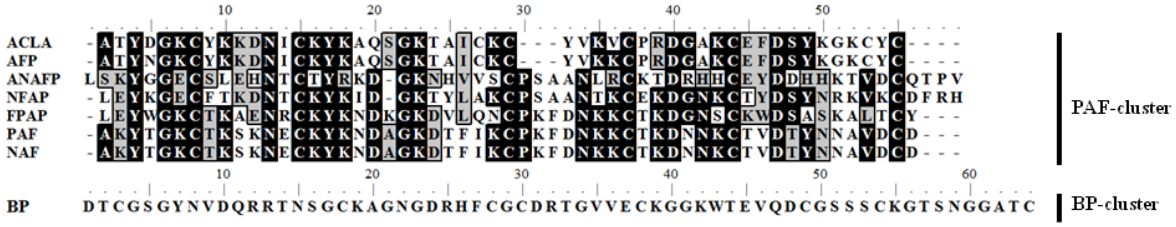

Figure 1. Isolated β-defensin-like antifungal proteins from filamentous fungi

ABBREVIATIONS: ACLA, *ASPERGILLUS CLAVATUS* ANTIFUNGAL PROTEIN (ACC. NO.: ABR 10398); AFP, *ASPERGILLUS GIGANTEUS* ANTIFUNGAL PROTEIN (ACC. NO.: X60771); ANAFP, *ASPERGILLUS NIGER* ANTIFUNGAL PROTEIN (STRAIN KTC 2025); NFAP, *NEOSARTORYA FISCHERI* ANTIFUNGAL PROTEIN (ACC. NO.: CAQ42994); FPAP, *FUSARIUM POLYPHIALIDICUM* ANTIFUNGAL PROTEIN (ACC. NO.: CAR 79015); PAF, *PENICILLIUM CHRYSOGENUM* ANTIFUNGAL PROTEIN (ACC. NO.: AAA 92718); NAF, *PENICILLIUM NALGIOVENSE* ANTIFUNGAL PROTEIN (STRAIN BFE 66, 67, 474); BP, *PENICILLIUM BREVICOMPACTUM* BUBBLE PROTEIN (ACC. NO.: AEQ 36754)

In spite of the fact that these proteins are different in their amino acid sequences; conserved homologous regions can be identified *(Figure 1.)*. Based on this they can be divided into two main groups: peptides which contain the *Penicillium chrysogenum* antifungal protein (PAF) cluster in their amino acid sequences, and peptides with *Penicillium brevicompactum* bubble protein (BP) cluster (GALGÓCZY ET AL., 2013A). Representatives of the PAF group were isolated and characterized from taxonomical distinct species, such as *Aspergillus clavatus*, *Aspergillus giganteus*, *Aspergillus niger*, *Fusarium polyphialidicum*, *Neosartorya fischeri*, *Penicillium chrysogenum*, and *Penicillium nalgiovense* (GALGÓCZY ET AL., 2010, 2013A; KOVÁCS ET AL., 2011) *(Figure 1.)*. Only one peptide with BP-cluster was isolated until now from *Penicillium brevicompactum* (SEIBOLD ET AL., 2011) *(Figure 1.)*. The isolated β-defensin-like antifungal proteins from ascomyceteous filamentous fungi are listed in the *Table 1*.

Table 1. The isolated β-defensin-like antifungal protein from ascomyceteous filamentous fungi

Protein	Fungus	Number of amino acids	Molecular weight (kDa)	Number of Cys	Number of Lys/Arg	Theoretical pI
ACLA	*Aspergillus clavatus*	51	5.8	8	11/1	9.06
AFP	*Aspergillus giganteus*	51	5.8	8	12/1	9.27
ANAFP	*Aspergillus niger*	58	6.6	6	5/3	7.14
BP	*Penicillium brevicompactum*	64	6.6	8	4/4	7.70
FPAP	*Fusarium polyphialidicum*	55	6.4	6	12/1	9.10
NAF	*Penicillium nalgiovense*	55	6.3	6	13/0	8.93
NFAP	*Neosartorya fischeri*	57	6.6	6	11/2	8.93
PAF	*Penicillium chrysogenum*	55	6.3	6	13/0	8.93

Source: SKOURI-GARGOURI ET AL., 2008; WNENDT ET AL. 1994; LEE ET AL., 1999; SEIBOLD ET AL., 2011; GALGÓCZY ET AL., 2013A; GEISEN, 2000; KOVÁCS ET AL., 2011; MARX ET AL., 1995.

Both groups have a potent antifungal activity, but the peptides with PAF-cluster are effective against filamentous fungi (GALGÓCZY ET AL., 2013A). The peptides with BP-cluster can inhibit only the growth of yeasts (SEIBOLD ET AL., 2011).

Peptides containing PAF-cluster generate similar symptoms in the susceptible filamentous fungi: they inhibit the germination of spores and the growth of the hyphae, they cause retardation of the hyphae lengthening, membrane perturbation, they induce intracellular oxidative stress and an apoptosis-like phenotype (GALGÓCZY ET AL., 2010). In spite of the similar symptoms, their mode of action could be different. For example *Aspergillus giganteus* antifungal protein (AFP) disturbs the cell wall biosynthesis by specific inhibition of chitin synthases (MEYER, 2008), and PAF evokes programmed cell death via G-protein signal transduction pathway (MARX ET AL., 2008). The antifungal spectra of the peptides are also differing; nevertheless, their tertiary structure is very similar (GALGÓCZY ET AL., 2010).

The β-defensin-like antimicrobial peptides with PAF-cluster are interesting in practical respect both in medical and agricultural fields. Their features that they have potent antifungal activity against potential human and plant pathogenic fungal species, that they could not have any toxic effects on plant and mammalian cells *in vitro*, that they can interact synergistically with other antifungal drugs and peptides, that they have high stability protease degradation, high temperature and within a broad pH range, and their low costs of production could make them suitable as active ingredients of commercial biopesticides and medicines (GALGÓCZY ET AL., 2010). Their potential applications in the agriculture are intensively studied.

IN VITRO SUSCEPTIBILITY OF PLANT AND POSTHARVEST PATHOGENIC FILAMENTOUS FUNGI TO β-DEFENSIN-LIKE ANTIFUNGAL PROTEINS WITH PAF-CLUSTER

It was demonstrated previously that the β-defensin-like antifungal proteins secreted by filamentous fungi have different species specificity (MARX, 2004), and their antifungal effect show dose-dependent characteristic. They exhibit growth retardation as long as they are applied in sublethal concentrations; and they become fungicidal with increasing concentrations (MARX, 2004; KAISERER ET AL., 2003; THEIS ET AL., 2005). It was also demonstrated the relative high presence of mono- and divalent cations decreases their inhibitory potency (MARX, 2004; GALGÓCZY ET AL., 2013B). Antifungal effect of the proteins also depends on the applied culture medium. The highest inhibitions were recorded on minimal culture medium compared to complete media (GALGÓCZY ET AL., 2010). It can be explained with the different demands for nutrients of fungi as well as with the presence of certain constituents in the media, which may interfere with the activity of PAF (MARX, 2004; KAISERER ET AL., 2003).

The antifungal spectrum of β-defensin-like antifungal proteins containing PAF-cluster is different, but there is similarity among the sensitive species to them (MARX, 2004) *(Table 2.)*. They have strong inhibitory potency against plant and postharvest fungal pathogens (GALGÓCZY ET AL., 2010) (*Table 2*).

POTENTIAL APPLICATION OF THE β-DEFENSIN-LIKE ANTIFUNGAL PROTEINS WITH PAF-CLUSTER IN THE AGRICULTURE

As it is demonstrated in the *Table 2*, the β-defensin-like antifungal peptides secreted by filamentous ascomyceteous fungi inhibit the germination of spore and growth of agriculturally harmful fungal species (GALGÓCZY ET AL., 2010). Based on this observation they could be applicable in the agriculture in the following fields.

Table 2. The investigated susceptible fungal species to β-defensin-like antifungal proteins with PAF-cluster

Antifungal protein	Sensitive fungal species	Reference
***Aspergillus clavatus* antifungal protein (ACLA)**	Ascomycetes *Alternaria solani; Aspergillus* spp. (*A. nidulans, A. niger*); *Botrytis cinerea*; *Fusarium oxysporum, F. solani*	SKOURI-GARGOURI ET AL., 2008
***Aspergillus giganteus* antifungal protein (AFP)**	Oomycetes *Phytophthora infestans* Ascomycetes *Alternaria alternata*; *Aspergillus* spp. (*A. flavus, A. nidulans, A. niger, A. terreus*); *Botrytis cinerea*; *Erysiphe graminis*; *Gliocladium roseum*; *Fusarium* spp. (*F. aquaeductum, F. bulbigenum, F. culmorum, F. equiseti, F. lactis, F. lini, F. moniliforme, F. oxysporum, F. poae, F. proliferatum, F. sambucinum, F. solani, F. sporotrichioides, F. vasinfectum*); *Magnaporthe grisea*; *Penicillium purpurogenum*; *Phytophtora infenstans*; *Trichoderma* spp. (*T. harzianum, T. koningii)*	GALGÓCZY ET AL., 2010; BARAKAT ET. AL. 2010A; BINDER ET AL., 2011
***Aspergillus niger* antifungal protein (ANAFP)**	Ascomycetes *Aspergillus* spp. (*A. flavus, A. fumigatus*); *Fusarium* spp. (*F. oxysporum, F. solani*)	GALGÓCZY ET AL., 2010
***Fusarium polyphialidicum* antifungal protein (FPAP)**	Zygomycetes *Gilbertella persicaria* Ascomycetes *Aspergillus* spp. (*A. nidulans, A. niger, A. terreus*); *Cladosporium herbarum; Trichoderma harzianum*	GALGÓCZY ET AL., 2013A
***Neosartorya fischeri* antifungal protein (NFAP)**	Ascomycetes *Aspergillus* spp. (*A. flavus, A. nidulans, A. niger, A. tamarii, A. terreus, A. tubingenis*); *Fusarium solani*	KOVÁCS ET AL., 2011
***Penicillium chrysogenum* antifungal protein (PAF)**	Zygomycetes *Mucor piriformis* Ascomycetes *Aspergillus* spp. (*A. flavus, A. fumigatus, A. nidulans, A. niger, A. terreus*); *Blumeria graminis* f. sp. *hordei*; *Botrytis cinerea; Cladosporium herbarum; Cochliobolus carbonum*; *Fusarium oxysporum*; *Gliocladium roseum*; *Trichoderma harzianum* Basidiomycetes *Puccinia recondita* f.sp. *tritici*	GALGÓCZY ET AL., 2010
***Penicillium nalgiovense* antifungal protein (NAF)**	Ascomycetes *Aspergillus flavus*; *Fusarium solani; Geotrichum candidum*	GALGÓCZY ET AL., 2010

Plant protection against fungal pathogens in the field

AFP could be a potential biopesticide in the plant protection based on its specific inhibition of chitin synthase III and V. These enzymes play a crucial role in the pathogenicity of plant pathogenic fungi (MEYER, 2008). The direct application of AFP dissolved in water on rice and *Pelargonium* plant leaves protected them against *Magnaporthe grisea* and *Botrytis cinerea* infection even after two or six weeks of infection (VILA ET AL., 2001; MORENO ET AL., 2003). It was observed that AFP did not show toxicity toward rice protoplast under conditions of total inhibition (VILA ET AL., 2001). Similarly, the pre-incubation of tomato roots with AFP protected the plants against *Fusarium oxysporum* infection (THEIS ET AL.,

2005). PAF mitigated the symptoms of barley powdery mildew and wheat leaf rust infections by the inhibition of the growths of two obligate biotrophic fungal pathogens, *Blumeria graminis* f. sp. *hordei* and *Puccinia recondita* f.sp. *tritici* (BARNA ET AL., 2008).
Transgenic wheat, rice and pearl millet plants expressing the AFP have been successfully created. These plants showed less susceptibility to the potential fungal pathogens. Significant reduction of disease symptoms were observed for transgenic wheat, rice plants and pear millet infected by *Erysiphe graminis*, *P. recondita*, and *M. grisea*, and *Sclerospora graminicola, Puccinia substriata*, respectively (OLDACH ET AL., 2001; COCA AT AL., 2002, MORENO ET AL. 2005). The transgenic plants showed normal growth behaviour and morphology. They proved to be fertile and any detrimental effects on the host organisms were not observed in case of the above mentioned external and internal applications and expression of AFP (MEYER, 2008).

Crop protection against postharvest fungal pathogens
It was observed that secondary growth of different *Fusarium* species is inhibited by AFP on barley in post harvest conditions and as a consequence of it they detected a reduction in levels of the mycotoxin deoxynivalenol after the AFP treatment compared to the untreated control (BARAKAT ET AL., 2010B). Similarly, AFP also prevented the contamination of some fruits and vegetables by some possible postharvest filamentous fungal pathogens during storage (e.g. tomato and mango fruits against *Alternaria alternata*), and the AFP treatment reduced the mycotoxin levels of the crops (BARAKAT ET AL., 2010A).

CONCLUSIONS

Based on the above discussed studies several features of the β-defensin-like antifungal proteins secreted by ascomycetous filamentous fungi correspond to the requirement of the novel, safely applicable biopesticides in agriculture. All these characteristics are supported by the fact that several antimicrobial peptides and their synthetic analogues have been used in transgenic plants and their application as biopesticides have been intensively studied. The β-defensin-like antifungal proteins could be an excellent biological alternative to combat against plant and postharvest pathogenic filamentous fungi.

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