FINAL PROJECT REPORT

THE GAMMA-CORE MOTIF DETERMINES THE ANTIFUNGAL MODE OF ACTION OF SMALL, CYSTEINE-RICH, CATIONIC PROTEINS FROM ASCOMYCETES

Institutions (in time-order of contract modifications):
University of Szeged, Faculty of Science and Informatics, Department of Microbiology
01.09.2017. – 31.12.2017. (ANN 122833)
Biological Research Centre, Institution of Plant Biology
01.01.2018. – 15.12.2020. (ANN 131341)
Project leader: László Galgóczy
Beginning of the project: 01.09.2017. - End of the project: 15.12.2020.
Project duration in month: 39.5

The main aims of the present project were (1) to prove that the evolutionary conserved gamma(γ)-core motif determines the antifungal mode of action of antifungal proteins from filamentous ascomycetes (AFPs), and rationally designed synthetic peptides spanning the γ-core motif have antifungal activity; furthermore, (2) to provide a proof-of-principle for the applicability of AFPs, their γ-core engineered variants, and their rationally designed γ -core peptide derivatives in the agriculture and in the medicine to counteract the increasing problems of antifungal drug resistance.

Background of the study: The increasing incidence of fungal infections and contaminations due to drug-resistant filamentous fungi in medicine, agriculture and food industry urges the development of new antifungal strategies. The highly stable, extracellular, cysteine-rich AFPs offer an alternative, safely applicable solution. Our previous *in silico* investigations revealed that AFPs contain an evolutionary conserved [GXC]-[X_{3-9}]-[C] consensus so-called γ-core motif; and preliminary results demonstrated that the antifungal efficacy of AFPs depends on the physical and chemical properties of amino acids spanning the γ-core motif. We also demonstrated that chemically synthesized peptides spanning the γ-core motif possess antifungal activity. Based on these observations we hypothesized that: (1) The γ-core motif determines the antifungal mode of action of AFPs from Ascomycetes. (2) The antifungal activity and the selectivity of AFPs can be modulated/improved by the mutation of the γ-core motif. (3) Short synthetic peptides consisting of the γ-core motif have antifungal activity. (4) The rational design of new γ-core peptides improves their stability, antifungal activity and specificity. (5) Improved AFPs and synthetic γ-core peptides are suitable to prevent and combat fungal infections in plants and humans/animals.

Considering these hypotheses, the main aims of the project were the followings:
(1) Investigating the role of the γ-core motif in AFP function and structure.
(2) Investigating the antifungal potential of synthetic γ-core peptides.
(3) Providing a proof-of-principle for the applicability of AFPs, their γ-core engineered variants, and rationally designed γ-core peptides to counteract the increasing problems of antifungal drug resistance in the medicine and agriculture.

AFPs from *Penicillium chrysogenum* (such as PAF, PAFB, PAFC); and from *Neosartorya* (*Aspergillus*) *fischeri* (such as NFAP, NFBP, NFAP2) were studied in the above mentioned aspects during the project period.

Based on the NKFIH regulations, these aims in the ANN project are tightly connected with that of the parallel and related PD 135248 project. The results of both projects were adopted and utilized *vice versa*. Considering that present project was a bilateral FWF-NKFIH cooperation project; the Hungarian partner (principal investigator: László Galgóczy) was mainly responsible for the protein engineering experiments with AFPs from *N. fischeri*, and for the utilization of AFPs in the agriculture as potential biofungicides and biopreservatives.

During the project period, we achieved the results discussed below:

(1) Investigating the role of the γ-core motif in AFP function and structure

Before the beginning of the project, we already developed a *P. chrysogenum*-based expression system, and reported that it is a potential tool to produce AFPs and their rationally designed variants for functional and structural analyses. Furthermore, *P. chrysogenum*-based expression systems for bulk production of correctly folded and functional PAF, NFAP, and PAFB were already available at the beginning of the project (Sonderegger et al., 2016; Huber et al., 2018). Considering these results, in the present project we adopted this expression system for bulk production of recombinant AFPs and their γ-core engineered variants to investigate the role of the γ-core motif in AFP function and structure.
First, we provided a detailed phylogenetic analysis that proved the presence and conservation of the γ-core motif in all AFP classes from Eurotiomycetes (including all *Penicillium* and *Neosartorya* antifungal proteins). We reported the important role of the γ-core motif in the biological function of PAF. We created its γ-core engineered variant, the PAF^{opt}, in which specific amino acids in the positively charged and hydrophilic γ-core motif were substituted to elevate the positive net charge and the hydrophilicity of the protein. PAF^{opt} showed improved growth inhibitory efficacy against the opportunistic human pathogenic yeast, *Candida albicans*. Furthermore, the applied amino acid substitutions in the γ-core motif influenced the antifungal spectrum of the PAF against phytopathogenic filamentous ascomycetes. The applied amino acid substitutions in the γ-core region did not influence dramatically the secondary and tertiary structure of the protein. Based on these results, we emphasized the potential of common improved γ-core protein motif for the design of short antifungal peptides and as a protein motif in which targeted amino acid substitutions enhance the antimicrobial activity or influence the antifungal spectrum. We adopted and utilized these results to generate γ-core improved variants of NFAP and NFAP2.
These results were published in the following peer-reviewed papers.

Sonderegger C, Váradi G, **Galgóczy L**, Kocsubé S, Posch W, Borics A, Dubrac S, Tóth GK, Wilflingseder D, Marx F. The evolutionary conserved γ-core motif influences the anti-*Candida* activity of the *Penicillium chrysogenum* antifungal protein PAF. FRONTIERS IN MICROBIOLOGY 9: Paper 1655. (2018), DOI: 10.3389/fmicb.2018.01655. IF2018: 4.259 (Q1 Microbiology)

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide $P\gamma^{opt}$ in plant protection. MICROBIAL BIOTECHNOLOGY. 13(5): pp. 1403-1414. (2020), DOI: 10.1111/1751-7915.13559. IF2019: 5.328 (Q1 Applied microbiology and biotechnology)

Applying the above-mentioned *P. chrysogenum*-based expression system, the average yield of recombinant NFAP2 was 40-times higher than in the native producer reported before (Tóth et al., 2016). Analyses by mass spectrometry, reversed-phase high performance liquid chromatography, electronic circular dichroism (ECD) and nuclear magnetic resonance (NMR) spectroscopies revealed that the recombinant NFAP2 is correctly processed and folded. The antifungal efficacy of recombinant NFAP2 was comparable to the native protein. Beside the recombinant production, correctly folded and functional NFAP2 was synthesized using a native chemical ligation method. The bulk NFBP production was failed with the *P. chrysogenum*-, and the alternatively applied *Pichia pastoris*-based expression systems due to the structural instability of recombinant NFBP. Taking into consideration the unstable recombinant protein structure, we omitted NFBP in the project.
These results were published in the following peer-reviewed paper

Tóth L, Váradi G, Borics A, Batta G, Kele Z, Vendrinszky Á, Tóth R, Ficze H, Tóth GK, Vágvölgyi C, Marx F, **Galgóczy L**. Anti-candidal activity and functional mapping of recombinant and synthetic *Neosartorya fischeri* antifungal protein 2 (NFAP2). FRONTIERS IN MICROBIOLOGY 9: Paper 393. (2018), DOI: 10.3389/fmicb.2018.00393. IF2018: 4.259 (Q1 Microbiology)

and conference proceedings.

Váradi G, Tóth L, Nedeczky K, Vendrinszky Á, Borics A, Kele Z, Tóth KG, Vágvölgyi C, Marx F, **Galgóczy L**. Synthesis and functional mapping of the *Neosartorya fischeri* anti-yeast protein (NFAP2) ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 64:(Supplement 1) pp. 186-187. (2017) 5th Central European Forum for Microbiology. Conference place and time: Keszthely, Hungary: 18.10.2017.-20.10.2017.

Váradi G, Tóth L, Vendrinszky Á, **Galgóczy L**, Batta G, Borics A, Kele Z, Tóth GK: Chemical synthesis and investigation of the native form and an improved gamma-core analogue of *Neosartorya fischeri* antifungal protein 2 (NFAP2). JOURNAL OF PEPTIDE SCIENCE 24: (Supplemet 144), Suppl. 2, p. 177 (2018) Conference place and time: Dublin, Ireland, 26.08.2018.-31.08.2018.

In NFAP and NFAP2, we substituted the slightly hydrophilic negatively charged (NFAP) or neutral (NFAP2) γ-core motif for the same optimized γ-core, that we applied in generation of PAF^{opt}. This structural modification increased the positive net charge and hydrophilicity of the NFAP and NFAP2 (**Table 1**).

TABLE 1. Amino acid sequence and *in silico* predicted physicochemical properties of mature *Neosartorya* antifungal proteins, their γ-core optimized variants (NFAPγ^{opt} and NFAP2γ^{opt}), and the respective γ-core regions.

Protein/ peptide	Number of amino acids	Molecular weight (kDa)	Number of Cys	Number of Lys/Arg/His	Theoretical pI	Estimated charge at pH 7	GRAVY
LEYK**GECFTKDNTC**KYKIDGKTYLAKCPSAANTKCEKDGNKCTYDSYNRKVKCDFRH							
NFAP	57	6.6	6	11/2/1	8.93	+5.0	−1.214
LEYK**GKCKTKKNKC**KYKIDGKTYLAKCPSAANTKCEKDGNKCTYDSYNRKVKCDFRH							
NFAPγ^{opt}	57	6.7	6	15/2/1	9.56	+11.0	−1.402
GECFTKDNTC							
NFAP γ-core	10	1.1	2	1/0/0	4.37	−1.1	−0.840
IATSPYYACNCPNNCKHKKGSGCKYHSGPSDKSKVIS**GKCEWQGGQLNC**IAT							
NFAP2	52	5.6	6	7/0/2	9.01	+5.2	−0.731
IATSPYYACNCPNNCKHKKGSGCKYHSGPSDKSKVIS**GKCKTKKNKC**IAT							
NFAP2γ^{opt}	50	5.4	6	11/0/2	8.83	+10.2	−0.918
GKCEWQGGQLNC							
NFAP2 γ-core	12	1.3	2	1/0/0	5.99	−0.2	−0.933
GKCKTKKNKC							
optimized γ-core	10	1.1	2	5/0/0	9.90	+4.8	−1.910

The γ-core motif is indicated in bold and by the underlined letters. Molecular weight (Mw), isoelectric point (pI) and the grand average of hydropathy (GRAVY) value were calculated by the ExPASy ProtParam tool (Gasteiger et al., 2005). The total net charge at pH = 7.0 was estimated using the Protein Calculator v3.4 server (The Scripps Research Institute; http://protcalc.sourceforge.net/).

The *P. chrysogenum*-based heterologous expression system and chemical synthesis (solid phase peptide synthesis and native chemical ligation of peptide fragments of the full length proteins as described by Váradi et al., 2013) were applied to generate recombinant or synthetic γ-core modified variants of NFAP (NFAPγ^{opt}) and NFAP2 (NFAP2γ^{opt}), respectively (**Table 1**). Both protein variants were degraded during the recombinant production in *P. chrysogenum*. This result let us assume that the modification renders the protein structure accessible for extracellular proteases. ECD spectroscopy demonstrated that synthetic NFAPγ^{opt} and NFAP2γ^{opt} have unordered structures compared to the respective wild-type proteins, which show a β-pleated conformation (**Figure 1**). The γ-core modification improved the antifungal efficacy of synthetic NFAPγ^{opt} against the plant pathogenic fungus *Cladosporium herbarum* FSU 1148 compared to the wild-type protein (**Figure 2A**). In contrast, a strong decrease in antifungal activity was observed with NFAP2γ^{opt} against the human pathogen *C. albicans* ATCC 10231 in comparison to the wild-type NFAP2 (**Figure 2B**). These results suggest a supporting role of the negatively charged or neutral γ-core motif of NFAP and NFAP2, respectively, in correct protein folding.

These results were published in the following conference proceedings.

Váradi G, Tóth L, Vendrinszky Á, **Galgóczy L**, Batta G, Borics A, Kele Z, Tóth GK: Chemical synthesis and investigation of the native form and an improved gamma-core analogue of *Neosartorya fischeri* antifungal protein 2 (NFAP2). JOURNAL OF PEPTIDE SCIENCE 24: (Supplemet 144), Suppl. 2, p. 177 (2018) Conference place and time: Dublin, Ireland, 26.08.2018.-31.08.2018.

Tóth L, Váradi Gy, Boros É, Nagy I, Marx F, **Galgóczy L**. Potential role of the evolutionary conserved γ-core motif in the efficacy and structural stability of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins. Acta Microbiol Immunol Hung, 2019, Volume 66, Supplemet 1 p. 204. 18th International Congress of the Hungarian Society for Microbiology. Conference place and time: Budapest, Hungary, 03.07.2019.-05.07.2019.

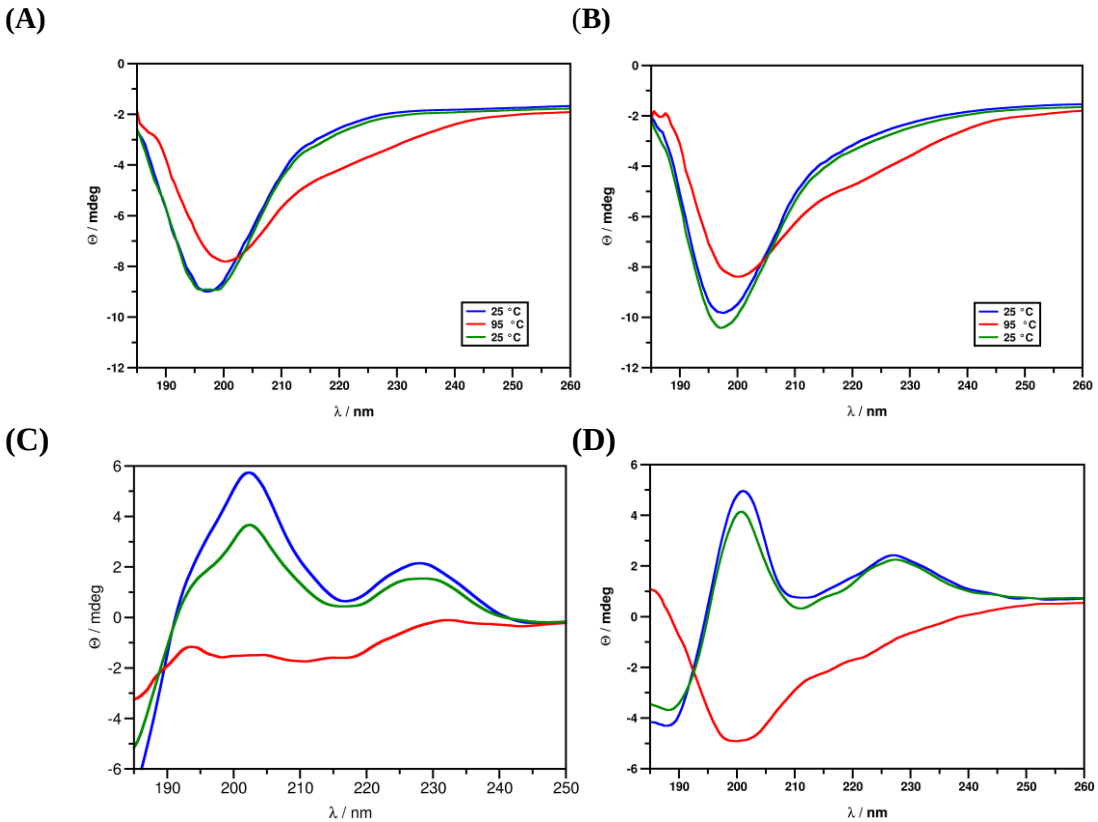

Figure 1. ECD spectra of synthetic NFAPγ^{opt} (**A**), synthetic NFAP2γ^{opt} (**B**), recombinant native NFAP (**C**), and recombinant native NFAP2 (**D**) recorded at 25°C (blue), 95°C (red), and 95°C with immediate cooling to 25°C (green).

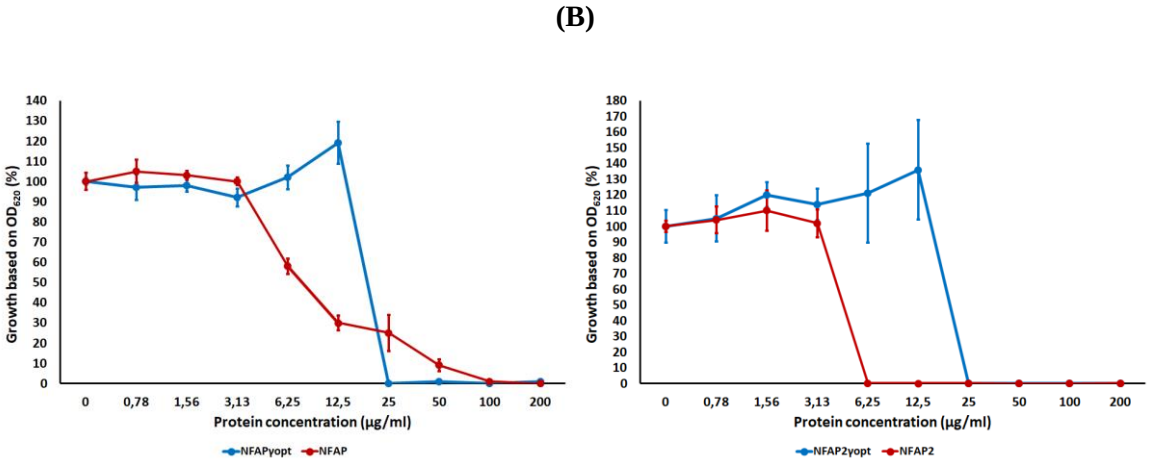

Figure 2. Antifungal activity of synthetic NFAPγ^{opt} and recombinant NFAP against *Cladosporium hebarum* FSU 1148 (**A**); and synthetic NFAP2γ^{opt} and recombinant NFAP2 against *Candida albicans* ATCC 10231 (**B**) in broth microdilution assay. Minimal inhibitory concentration is 25 µg/ml for NFAPγ^{opt}, 100 µg/ml for recombinant NFAP, 25 µg/ml for NFAP2γ^{opt}, and 6.25 µg/ml for recombinant NFAP2. The absorbance (OD_{620}) of untreated control culture was defined as 100% of growth.

The generated recombinant NFAP producing *P. chrysogenum* strain was utilized during the project period to determine the solution structure of NFAP and to get novel insights into its mode of action.
These results were published in the following peer-reviewed paper

Hajdu D, Huber A, Czajlik A, Tóth L, Kele Z, Kocsubé S, Fizil Á, Marx F, **Galgóczy L**, Batta G. Solution structure and novel insights into phylogeny and mode of action of the *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein (NFAP). INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES 129: pp. 511-522. (2019), DOI: 10.1016/j.ijbiomac.2019.02.016. IF2019: 5.162 (Q1 Medicine (miscellaneous))

and conference proceeding.

Galgóczy L, Huber A, Farkas A, Tóth L, Sonderegger C, Marx F. First insights into potential molecular targets of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein (NFAP). In: Anita Slavica, Renata Teparic, Andrej Lebos Pavunc, Domagoj Kifer (eds.) Power of Microbes in Industry and Environment 2019: Book of Abstracts. 130 p. Conference place and time: Sveti Martin na Muri, Croatia, 15.05.2019.-18.05.2019. Zagreb: Croatian Microbiology Society, 2019. p. 52. (ISBN:978-953-7778-17-0)

The *P. chrysogenum*-based expression system was applied to generate homologous recombinant PAFB and PAFC; the second and the third representatives of AFPs from *P. chrysogenum*. These proteins were characterized regarding their structure, antifungal mode of action, and their potential in the development of new treatment strategies of fungal infections.

These results were published in the following peer-reviewed papers

Huber A, **Galgóczy L**, Váradi G, Holzknecht J, Kakar A, Malanovic N, Leber R, Koch J, Keller MA, Batta G, Tóth GK, Marx F. Two small, cysteine-rich and cationic antifungal proteins from *Penicillium chrysogenum*: A comparative study of PAF and PAFB. BIOCHIMICA ET BIOPHYSICA ACTA-BIOMEMBRANES 1862: 8 Paper 183246 (2020), DOI: 10.1016/j.bbamem.2020.183246. IF2019: 3.411 (Q1 Biochemistry)

Holzknecht J, Kühbacher A, Papp C, Farkas A, Váradi G, Marcos JF, Manzanares P, Tóth GK, **Galgóczy L**, Marx F. The *Penicillium chrysogenum* Q176 antimicrobial protein PAFC effectively inhibits the growth of the opportunistic human pathogen *Candida albicans*. JOURNAL OF FUNGI 6: 141 (2020), DOI: 10.3390/jof6030141. IF2019: 4.621 (Q1 Microbiology (medical))

and conference proceedings.

Holzknecht J, Dubrac S, **Galgóczy L**, Marx F. The small, cysteine-rich, cationic protein PAFC from *Penicillium chrysogenum* is a promising antifungal therapeutic agent. In: Anita Slavica, Renata Teparic, Andrej Lebos Pavunc, Domagoj Kifer (eds.) Power of Microbes in Industry and Environment 2019: Book of Abstracts. 130 p. Conference place and time: Sveti Martin na Muri, Croatia, 15.05.2019.-18.05.2019. Zagreb: Croatian Microbiology Society, 2019. p. 109. (ISBN:978-953-7778-17-0)

Holzknecht J, Papp Cs, Farkas A, **Galgóczy L**, Marx F. PAFC: the third small, cysteine-rich, cationic antifungal protein from *Penicillium chrysogenum* effectively inhibits the growth of *Candida albicans*. Acta Microbiol Immunol Hung, 2019, Volume 66, Supplemet 1 pp. 140-141. 18th International Congress of the Hungarian Society for Microbiology. Conference place and time: Budapest, Magyarország, 03.07.2019.-05.07.2019.

(2) Investigating the antifungal potential of synthetic γ-core peptides

Peptides were synthesized on solid phase applying 9-fluorenylmethyloxycarbonyl chemistry. Regarding the stability and antifungal efficacy the structure of the synthetic γ-core peptides was optimized. The synthesized peptides contained three additional amino acids at the N-terminus and ended in an extra C-terminal lysine or threonine residue. The applied N-terminal acetylation and C-terminal amidation mimicked the propagating native protein backbone, neutral terminals and provided stability against proteolysis. We observed that cyclisation through the disulphide bridge formation impaired antifungal efficacy; thus, all cysteines in γ-core peptide derivatives possessed free sulfhydryl (–SH) groups. The studied γ-core peptide derivatives and their physicochemical properties are listed in the **Table 2**. ECD spectroscopy indicated that all γ-core peptide derivatives have unordered structure (data not shown).

During the project period, first, we reported the optimization of PAF γ-core peptide derivatives for antifungal efficacy and structural stability. We also reported the antifungal efficacy of two synthetic 14-mer peptides, Pγ and $P\gamma^{opt}$ (**Table 2**), that span the γ-core motif of wild-type PAF, and the above mentioned γ-core modified PAF^{opt}, respectively. A higher anti-*Candida* efficacy of the more positively charged and more hydrophilic $P\gamma^{opt}$ was proven. The $P\gamma^{opt}$ peptide inhibited the growth some plant pathogenic filamentous ascomycetes *in vitro*; while Pγ did not show any antifungal activity against them.

The native and optimized γ-core peptide derivatives of PAFB were also synthesized (PBγ and $PB\gamma^{opt}$ in **Table 2**, respectively). The neutral, and less hydrophilic PAFB γ-core peptide derivative (PBγ) did not inhibit the growth of *C. albicans*; while its rationally designed cationic and more hydrophilic variant ($PB\gamma^{opt}$) was able to reduce the growth of this fungus in *in vitro* susceptibility tests.

These results were published in the following peer-reviewed papers.

Sonderegger C, Váradi G, **Galgóczy L**, Kocsubé S, Posch W, Borics A, Dubrac S, Tóth GK, Wilflingseder D, Marx F. The evolutionary conserved γ-core motif influences the anti-*Candida* activity of the *Penicillium chrysogenum* antifungal protein PAF. FRONTIERS IN MICROBIOLOGY 9: Paper 1655. (2018), DOI: 10.3389/fmicb.2018.01655. IF2018: 4.259 (Q1 Microbiology)

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide $P\gamma^{opt}$ in plant protection. MICROBIAL BIOTECHNOLOGY. 13(5): pp. 1403-1414. (2020), DOI: 10.1111/1751-7915.13559. IF2019: 5.328 (Q1 Applied microbiology and biotechnology)

Huber A, **Galgóczy L**, Váradi G, Holzknecht J, Kakar A, Malanovic N, Leber R, Koch J, Keller MA, Batta G, Tóth GK, Marx F. Two small, cysteine-rich and cationic antifungal proteins from *Penicillium chrysogenum*: A comparative study of PAF and PAFB. BIOCHIMICA ET BIOPHYSICA ACTA-BIOMEMBRANES 1862: 8 Paper 183246 (2020), DOI: 10.1016/j.bbamem.2020.183246. IF2019: 3.411 (Q1 Biochemistry)

The peptide derivatives spanning the native γ-core motifs of *Neosartorya* antifungal proteins (γ^{NFAP}, γ^{NFBP}, γ^{NFAP2} in **Table 2**) were designed based on the findings regarding the stability and antifungal efficacy of the γ-core peptides from *Penicillium* AFPs reported in the above mentioned papers. Specific amino acids were substituted in the derivatives of native γ-core motif to create the γ^{NFAP}-opt, γ^{NFBP}-opt γ^{NFAP2}-opt exhibiting an elevated positive net charge and increased hydrophilicity (**Table 2**). Antifungal susceptibility tests indicated that designed peptide derivatives spanning the native γ-core motif do not show any antifungal activity on phytopathogenic filamentous ascomycetes; while their optimized variants with elevated net charge and hydrophilicity effectively inhibit the fungal growth.

These results were partially published in the following peer-reviewed paper

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2019: 4.235 (Q1 Microbiology)

and conference proceedings.

Tóth L, Váradi G, Ficze H, Tóth, KG, Marx F, **Galgóczy L**. Antifungal effect of *de novo* designed peptides according to the y-core motif of *Neosartorya fischeri* NRRL 181 antifungal proteins. In: Abrama, M; Bielen, A; Kifer, D; Vlahovicek, GM; Klaric, MS (ed.) Central European Symposium on Antimicrobial Resistance - Book of Abstracts (2018) p. 111. Conference place and time: Sveti Martin na Muri, Croatia, 19.09.2018.-22.09.2018. (ISBN:978-953-7778-16-3)

Tóth L, Váradi G, Ficze H, Tóth, KG, Marx F, **Galgóczy, L**. Examination of antifungal activity and mechanism of *de novo* designed y-core peptide motifs from *Neosartorya fischeri* NRRL 181 antifungal proteins. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 66: (S1) pp. 105-106., 2019. A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium. Conference place and time: Eger, Hungary, 17.10.2018.-19.10.2018.

Tóth L, Váradi Gy, Boros É, Ficze H, Nagy I, Marx F, **Galgóczy L**. Agricultural applicability of *Neosartorya fischeri* antifungal protein and *de novo* designed peptide derivatives. In: István Majzinger, Tamás Monostori, Monica Ocnen, Elena Pet, Sorin Mihai Stanicu, Judit Szűcsné Péter, Lajos Tanás (eds.) 17th Wellmann International Scientific Conference "Agriculture Without Borders": Book of Abstracts. 106 p. Conference place and time: Hódmezővásárhely, Hungary, 08.05.2019. Hódmezővásárhely: University of Szeged, Faculty of Agriculture, 2019. pp. 75-76. (ISBN:978-963-306-653-9)

TABLE 2. Amino acid sequence and *in silico* predicted physicochemical properties of *Penicillium* and *Neosartorya* antifungal protein γ-core peptide derivatives and their rationally designed variants.

Protein/ peptide	Number of amino acids	Molecular weight (kDa)	Number of Cys	Number of Lys/Arg/His	Theoretical pI	Estimated charge at pH 7	GRAVY
			Ac-KYTGKC(-SH)TKSKNEC(-SH)K-NH_2				
Pγ	14	1.6	2	5/0/0	9.51	+3.8	−1.814
			Ac-KYTGKC(-SH)KTKKNKC(-SG)K-NH_2				
$P\gamma^{opt}$	14	1.7	2	7/0/0	10.04	+6.8	−2.064
			Ac-KFGGEC(-SH)SLKHNTC(-SH)T-NH_2				
PBγ	14	1.5	2	2/0/1	8.06	+1.1	−0.671
			Ac-KFGGKC(-SH)KTKKNKC(-SH)T-NH_2				
$PB\gamma^{opt}$	14	1.6	2	6/0/0	10.05	+5.8	−1.521
			Ac-EYKGEC(-SH)FTKDNTC(-SH)K-NH_2				
γ^{NFAP}	14	1.7	2	3/0/0	6.26	−0.1	−1.500
			Ac-EYKGKC(-SH)KTKKNKC(-SH)K-NH_2				
γ^{NFAP}-opt	14	1.7	2	7/0/0	9.84	+5.8	−2.264
			Ac-QSNGNC(-SH)QTNQNQSN-NH_2				
γ^{NFAP}-optChZ	14	1.5	1	0/0/0	5.52	−0.1	−2.264
			Ac-EIKIKC(-SH)KIKKIKC(-SH)K-NH_2				
γ^{NFAP}-optGZ	14	1.7	2	7/0/0	9.93	+5.8	−0.557
			Ac-KC(-SH)DRTGVVEC(-SH)RGGRW-NH_2				
γ^{NFBP}	15	1.7	2	1/3/0	8.96	+1.9	−0.920
			Ac-KC(-SH)KNKKTKC(-SH)KGGRW-NH_2				
γ^{NFBP}-opt	14	1.7	2	6/1/0	10.32	+6.8	−2.057
			Ac-VISGKC(-SH)EWQGGQLNC(-SH)K-NH_2				
γ^{NFAP2}	16	1.8	2	2/0/0	8.02	+0.8	−0.450
			Ac-VISGKC(-SH)KTKKNKC(-SH)K-NH_2				
γ^{NFAP2}-opt	14	1.6	2	6/0/0	10.05	+5.8	−1.079

Molecular weight (Mw), isoelectric point (pI) and the grand average of hydropathy (GRAVY) value were calculated by the ExPASy ProtParam tool (Gasteiger et al., 2005). The total net charge at pH = 7.0 was estimated using the Protein Calculator v3.4 server (The Scripps Research Institute; http://protcalc.sourceforge.net/).

(3) Providing a proof-of-principle for the applicability of AFPs, their γ-core engineered variants, and rationally designed γ-core peptides to counteract the increasing problems of antifungal drug resistance in the medicine and agriculture

One of the requirements for new antifungal compounds designed for medical or agricultural applications is their harmlessness in the host. As a proof-of-principle, we tested the cytotoxic potential of PAF, NFAP, NFAP2, their engineered variants, and their antifungally active γ-core peptide derivatives on human keratinocytes, human dermal fibroblasts and colonic epithelial cells. These cell types were in direct contact with them if AFPs are applied as topical drugs in the treatment of superficial fungal infections; or as biofungicides, and the treated agricultural products were considered for human consumption. Keratinocytes are the predominant cell type in the epidermis, human dermal fibroblasts are the most common cells of connective tissue synthesizing the extracellular matrix and collagen, whereas colonic epithelial cells play a role in nutrient absorption and the innate and adaptive mucosal immunity. Monocytes were also subjected to toxicity tests. They are important parts of the human body's defence system against infectious organisms and non-self-molecules.

In vitro viability staining of primary keratinocytes and human dermal fibroblasts after exposure to NFAP2 revealed no change in the number of dead cells even after treatment with twice the minimum inhibitory concentration (MIC). Cell viability test excluded any toxic effects of PAF and PAF^{opt} on keratinocytes, colonic epithelial cells, and monocytes *in vitro*, even when the proteins were applied at concentrations much above their MICs. In contrast, $P\gamma^{opt}$ significantly decreased the viability of monocytes at concentration above the MIC, whereas keratinocytes and colonic epithelial cells remained unaffected. NFAP and γ^{NFAP}-opt did not reduce the viability of keratinocytes, epithelial cells, and monocytes in the tested concentration range up to their 2 × MIC. In contrast, a significant reduction in the viability of the keratinocytes exposed to γ^{NFAP}-optGZ in comparison with the untreated control was observed. The viability of the other cell lines was not significantly affected by this peptide. The cell membrane disruption ability of NFAP, PAF, PAF^{opt} and the antifungally active γ-core peptide derivatives was investigated on erythrocytes. None of the tested proteins and peptides caused haemolysis.

Cytotoxic potential of PAF, PAF^{opt}, NFAP and γ-core peptide derivatives on plant was investigated on *Medicago truncatula* seedlings, which is a fast-growing, small legume, easily cultivable on water agar in Petri dishes (Barker et al., 2006), and an appropriate model organism to investigate the harmful effects of antifungal peptides and proteins on the growing plants. Treatment with PAF, PAF^{opt}, $P\gamma^{opt}$, NFAP and γ^{NFAP}-opt and γ^{NFAP}-optGZ at concentrations much above their MICs did not cause any changes to the plant morphology Furthermore, no significant changes in the primary root length and the number of evolved lateral roots were observed following the treatment period.

Based on the results of the cytotoxicity tests NFAP2 was selected for *in vivo* animal model experiments, while PAF, PAF^{opt}, $P\gamma^{opt}$ NFAP, and γ^{NFAP}-opt were selected for plant and crop protection assays.

These results were published in the following peer-reviewed papers

Sonderegger C, Váradi G, **Galgóczy L**, Kocsubé S, Posch W, Borics A, Dubrac S, Tóth GK, Wilflingseder D, Marx F. The evolutionary conserved γ-core motif influences the anti-*Candida* activity of the *Penicillium chrysogenum* antifungal protein PAF. FRONTIERS IN MICROBIOLOGY 9: Paper 1655. (2018), DOI: 10.3389/fmicb.2018.01655. IF2018: 4.259 (Q1 Microbiology)

Kovács R, Holzknecht J, Hargitai Z, Papp C, Farkas A, Borics A, Tóth L, Váradi G, Tóth GK, Kovács I, Dubrac S, Majoros L, Marx F, **Galgóczy L**. *In vivo* applicability of *Neosartorya fischeri* antifungal protein 2 (NFAP2) in treatment of vulvovaginal candidiasis. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY 63: (2) Paper e01777-18. (2019), DOI: 10.1128/AAC.01777-18. IF2019: 4.684 (D1 Pharmacology (medical))

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide $P\gamma^{opt}$ in plant protection. MICROBIAL BIOTECHNOLOGY. 13(5): pp. 1403-1414. (2020), DOI: 10.1111/1751-7915.13559. IF2019: 5.328 (Q1 Applied microbiology and biotechnology)

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2018: 4.259 (Q1 Microbiology)

and conference proceedings.

Tóth L, Sonderegger C, Boros É, Nagy I, Bratschun-Khan D, Marx F, **Galgóczy L**. Applicability of *Penicillium chrysogenum* antifungal protein and its rational designed variant in plant protection. In: István Majzinger, Tamás Monostori, Monica Ocnen, Elena Pet, Sorin Mihai Stanicu, Judit Szűcsné Péter, Lajos Tanás (eds.) 17th Wellmann International Scientific Conference "Agriculture Without Borders": Book of Abstracts. 106 p. Conference place and time: Hódmezővásárhely, Magyarország, 08.05.2019. Hódmezővásárhely: University of Szeged, Faculty of Agriculture, 2019. pp. 73-74. (ISBN:978-963-306-653-9)

Tóth L, Váradi Gy, Boros É, Ficze H, Nagy I, Marx F, **Galgóczy L**. Agricultural applicability of *Neosartorya fischeri* antifungal protein and *de novo* designed peptide derivatives. In: István Majzinger, Tamás Monostori, Monica Ocnen, Elena Pet, Sorin Mihai Stanicu, Judit Szűcsné Péter, Lajos Tanás (eds.) 17th Wellmann International Scientific Conference "Agriculture Without Borders": Book of Abstracts. 106 p. Conference place and time: Hódmezővásárhely, Magyarország, 08.05.2019. Hódmezővásárhely: University of Szeged, Faculty of Agriculture, 2019. pp. 75-76. (ISBN:978-963-306-653-9)

Tóth L, Váradi Gy, Boros É, Nagy I, Marx F, **Galgóczy L**. *In vitro* cytotoxic effect of *Penicillium chysogenum* antifungal protein, its *de novo* rational designed protein variant and peptide derivative on mammalian cells and plants. Acta Microbiol Immunol Hung, 2019, Volume 66, Supplemet 1 p. 203. 18th International Congress of the Hungarian Society for Microbiology. Conference place and time: Budapest, Magyarország, 03.07.2019.-05.07.2019.

Medical application

We provided for the first time information about the *in vivo* antifungal efficacy of an AFP as topical agent for the safe treatment of mucosal fungal infection caused by a drug-resistant fungal strain. An *in vivo* murine vulvovaginitis model experiment demonstrated that NFAP2 significantly decreases the number of fluconazole-resistant *C. albicans* cells in infected vaginal tissue, and its combined application with fluconazole enhances the efficacy. This last result proposed the possibility of the fluconazole resistance reversion in the combined application. Histological examinations showed neither morphological alterations, nor pathological reactions of the vagina and vulva after NFAP2 treatment.

These results were published in a peer-reviewed paper

Kovács R, Holzknecht J, Hargitai Z, Papp C, Farkas A, Borics A, Tóth L, Váradi G, Tóth GK, Kovács I, Dubrac S, Majoros L, Marx F, **Galgóczy L**. *In vivo* applicability of *Neosartorya fischeri* antifungal protein 2 (NFAP2) in treatment of vulvovaginal candidiasis. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY 63: (2) Paper e01777-18. (2019), DOI: 10.1128/AAC.01777-18. IF2019: 4.684 (D1 Pharmacology (medical))

and conference proceedings.

Kovács R, Tóth L, Holzkneczt J, Papp C, Borics A, Hargitai Z, Farkas A, Kovács I, Majoros L, Marx F, *Galgóczy L*. *Neosartorya fischeri* NRRL 181 antifungal protein 2 (NFAP2) as potential candidate to overcome antifungal drug resistance of *Candida* species. In: Abrama, M; Bielen, A; Kifer, D; Vlahovicek, GM; Klaric, MS (ed.) Central European Symposium on Antimicrobial Resistance - Book of Abstracts (2018) p. 71. Conference place and time: Sveti Martin na Muri, Croatia, 19.09.2018.-22.09.2018. (ISBN:978-953-7778-16-3)

Kovács, R, Tóth L, Holzkneczt J, Papp C, Borics A, Hargitai Z, Farkas A, Kovács I, Majoros L, Marx F, **Galgóczy L**. *In vivo* application of *Neosartory fischeri* NRRL 181 antifungal protein 2 (NFAP2) In: A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium: Abstract book (2018) pp. 36-37. 2 p. Conference place and time: Eger, Hungary, 17,10.2018.-19.10.2016.

Candida auris is an emerging potential multidrug-resistant human pathogenic yeast, and able to persist on indwelling devices as a biofilm, which serve as source of catheter-associated infections in the clinics. Therefore, we also studied the *in vitro* activity of NFAP2 alone and in combination with conventional antifungal drugs (such as fluconazole, amphotericin B, anidulafungin, caspofungin, and micafungin) against *Candida auris* biofilms formed by different strains. The nature of interactions was assessed utilizing the fractional inhibitory concentration index (FICI), a Bliss independence model, and LIVE/DEAD viability assay. NFAP2 was not able to eradicate all biofilms at its applied highest concentration (512 mg/l), but exerted synergy with all tested antifungals with FICIs ranging between 0.312-0.5, 0.155-0.5, 0.037-0.375, 0.064-0.375, and 0.064-0.375 for fluconazole, amphotericin B, anidulafungin, caspofungin, and micafungin, respectively. These results were confirmed using a Bliss model, where NFAP2 produced 17.54 μM2%, 2.16 μM2%, 33.31 μM2%, 10.72 μM2%, and 95.85 μM2% cumulative synergy log volume in combination with fluconazole, amphotericin B, anidulafungin, caspofungin, and micafungin, respectively. In addition, biofilms exposed to echinocandins (32 mg/l) showed significant cell death in the presence of NFAP2 (128 mg/l) (**Figure 3**). This study clearly indicated that NFAP2 displays strong potential as a novel antifungal compound in alternative combinatory therapies to combat medical device associated *C. auris* biofilms, and its potential application in antifungal lock therapy.

A peer-reviewed paper containing these results has been submitted.

Kovács R, Nagy F, Tóth Z, Forgács K, Tóth L, Váradi G, Tóth KG, Vadászi K, Borman AM, Majoros L, **Galgóczy L**. The *Neosartorya fischeri* antifungal protein 2 (NFAP2): A new potential weapon against multidrug-resistant *Candida auris* biofilms. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES (2020), under minor revision, IF2019: 4.556 (Q1 (Q1 Medicine (miscellaneous)))

The further medical potential of *Penicillium* and *Nesoartorya* AFPs and their peptide derivatives in the treatment of *Candida* infections was discussed in a review paper.

Galgóczy L, Yap A, Marx F. Cysteine-rich antifungal proteins from filamentous fungi are promising bioactive natural compounds in anti-*Candida* therapy. ISRAEL JOURNAL OF CHEMISTRY 59: pp. 360-370. (2019), DOI: 10.1002/ijch.201800168., IF2019: 2.320 (Q1 Chemistry (miscellaneous))

In an editorial note we drew attention to the potential of APs to overcome antifungal resistance.

Galgóczy L, Marx F. Do antimicrobial proteins contribute to overcoming the hidden antifungal crisis at the dawn of a post-antibiotic era? MICROORGANISMS 7: (1) E16. (2019), DOI: 10.3390/microorganisms7010016. IF2019: 4.152 (-)

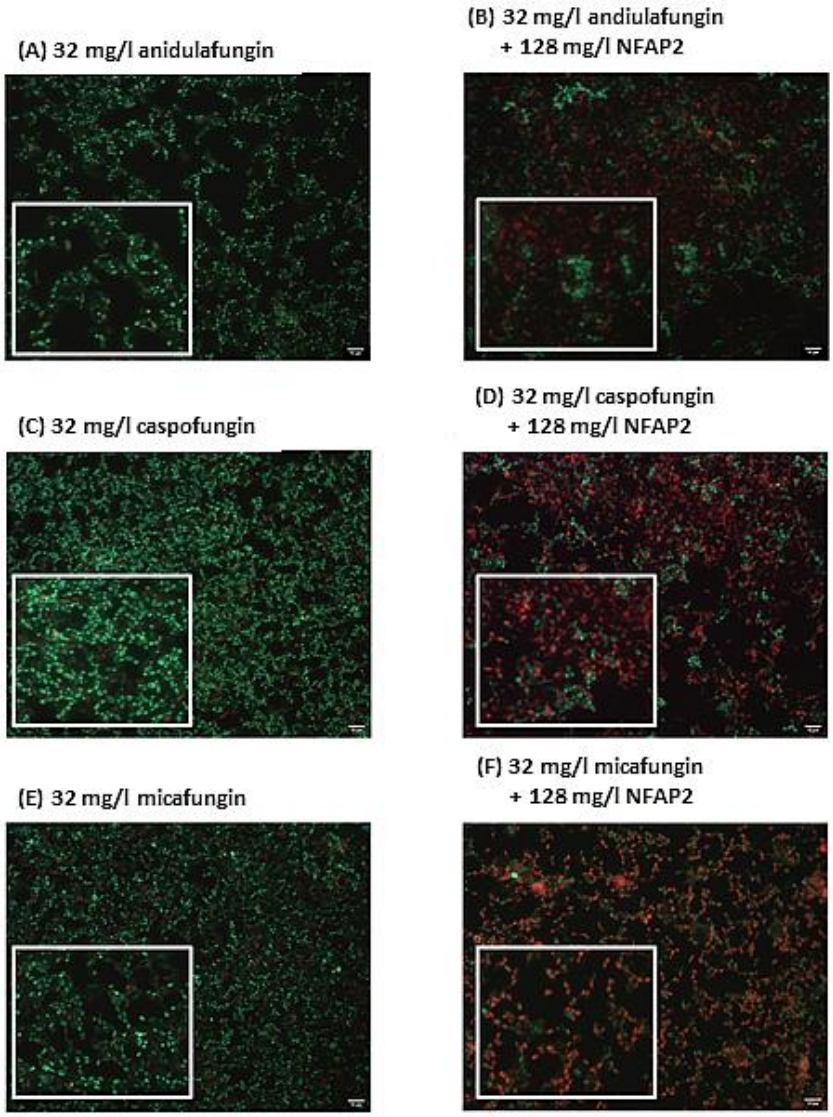

Figure 3. LIVE/DEAD fluorescence imaging of one representative *Candida auris* isolate. Images **A**, **C** and **E** demonstrate the anidulafungin, caspofungin -and micafungin-exposed biofilms (32 mg/L), respectively, while images **B**, **D**, and **F** show the anti-biofilm effect of anidulafungin, caspofungin and micafungin (32 mg/L for each drug alone) in the presence of NFAP2 (128 mg/L), respectively. Live cells (green) and nonviable cells (red) were stained with Syto9 and propidium iodide, respectively. All images show typical fields of view. Scale bars represent 10 μm.

Agricultural application

The antifungal potential of the produced recombinant *Penicillium* and *Neosartorya* AFPs and synthetic γ-core peptide derivatives (**Table 2**) were investigated in a broth microdilution susceptibility assay against pre- and postharvest plant pathogenic and mycotoxigenic filamentous fungi. The respective MICs are summarized in the **Table 3**. PAF inhibited the growth of all isolates, except for *Fusarium boothi* and *Fusarium graminearum*, in the applied concentration range showing different MICs. In contrast, PAF^{opt} was ineffective against aspergilli, while *Cladosporium* and *Fusarium* isolates proved to be more susceptible to this PAF-variant than to the native PAF, with exception of *Botrytis cinerea*. Pγ was ineffective at concentrations up to 400 μg/ml (data not shown), but the $P\gamma^{opt}$ inhibited the growth of *Botrytis*, *Cladosporium* and *Fusarium* isolates. These results indicated that the γ-core modulation of PAF influences the antifungal spectrum and efficacy of the protein. PAFB proved to be antifungal active against all tested fungal isolates at various MICs; however, aspergilli showed the so-called paradoxical effect, namely the

Table 3. Minimal inhibitory concentrations (μg/ml) of *Penicillium* and *Neosartorya* APs and their rationally designed γ-core peptide derivatives against pre- and postharvest plant pathogenic and mycotoxigenic filamentous ascomycetes.

Isolate	PAF	PAF^{opt}	Pγ^{opt}	PAFB	NFAP	γ^{NFAP}-opt	NFAP2	γ^{NFAP2}-opt	γ^{NFBP}-opt	Origin of isolate
Aspergillus flavus SZMC 3014	3.125	>400	>400	3.125	100	>200	>200	>200	>200	*Triticum aestivum*/Hungary
Aspergillus flavus SZMC 12618	3.125	>400	>400	3.125	100	>200	>200	>200	>200	*Triticum aestivum*/Hungary
Aspergillus flavus SZMC 20745	3.125	>400	>400	3.125	12.5	>200	>200	>200	>200	*Zea mays*/Hungary
Aspergillus flavus SZMC 20755	3.125	>400	>400	3.125	25	>200	>200	>200	>200	*Zea mays*/Hungary
Aspergillus niger SZMC 0145	3.125	>400	>400	3.125	50	>200	>200	>200	>200	Fruits/Hungary
Aspergillus niger SZMC 2759	3.125	>400	>400	3.125	50	>200	>200	>200	>200	Raisin/Hungary
Aspergillus nomius SZMC 22631	200	>400	>400	25	25	>200	>200	>200	>200	chees/Hungary
Aspergillus parasiticus SZMC 22727	3.125	>400	>400	3.125	200	>200	>200	>200	>200	indoor air/Croatia
Aspergillus pseudonomius SZMC 22631	200	>400	>400	>400	200	>200	>200	>200	>200	*Zea mays*/Serbia
Aspergillus welwitschiae SZMC 21821	1.56	>400	>400	3.125	25	>200	>200	>200	>200	*Allium cepa*/Hungary
Aspergillus welwitschiae SZMC 21832	1.56	>400	>400	3.125	12.5	>200	>200	>200	>200	*Allium cepa*/Hungary
Botrytis cinerea SZMC 21472	1.56	12.5	25	n.d.	6.25	200	50	>200	>200	*Rubus idaeus*/Hungary
Botrytis cinerea SZMC 21474	n.d.	n.d.	n.d.	12.5	50	50	12.5	50	25	*Fragaria* × *ananassa*/Hungary
Botrytis cinerea NCAIM F.00751	n.d.	n.d.	n.d.	12.5	50	50	12.5	50	25	Hungary
Botrytis pseudocinerea SZMC 21470	100	n.d.	25	25	100	100	12.5	50	200	*Brassica napus*/Hungary
Botrytis pseudocinerea SZMC 21471	100	n.d.	25	25	100	100	12.5	50	200	*Brassica napus*/Hungary
Cladosporium herbarum FSU 1148	100	12.5	6.25	12.5	100	12.5	12.5	100	3.125	n.d.
Cladosporium herbarum FSU 969	100	12.5	6.25	12.5	100	12.5	12.5	100	3.125	n.d
Fusarium boothi CBS 110250	>400	200	12.5	n.d.	25	50	>200	>200	12.5	*Zea mays*/South Africa
Fusarium graminearum SZMC 11031	n.d.	n.d.	n.d.	3.125	200	>200	>200	>200	>200	*Citrus sinensis*/New Caledonia
Fusarium graminearum SZMC 6236J	>400	200	12.5	n.d.	25	50	>200	>200	25	Vegetables/Hungary
Fusarium oxysporum SZMC 6237J	400	100	12.5	n.d.	25	50	>200	>200	200	Vegetables/Hungary
Fusarium solani CBS 115659	200	50	12.5	200	50	12.5	>200	50	6.25	*Solanum tuberosum*/Germany
Fusarium solani CBS 119996	200	50	12.5	200	100	50	>200	200	25	*Daucus carota*/The Netherlands
Fusarium verticillioides SZMC 11411	n.d.	n.d.	n.d.	25	200	>200	>200	>200	>200	*Zea mays*/Hungary

CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; FSU: Fungal Reference Centre University of Jena, Jena, Germany; SZMC: Szeged Microbiological Collection, University of Szeged, Szeged, Hungary; NCAIM: National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary. n.d.: data not available.

fungus resumed growth at concentrations above the MIC (data not shown). Its γ-core peptide derivatives (PBγ and PBγ^{opt}) proved to be not active in the *in vitro* antifungal susceptibility tests (data not shown). PAFC did not exert any antifungal effect on the tested isolates (data not shown). NFAP inhibited the growth of all tested isolates with various MICs in the investigated concentration range (up to 200 µg/ml). In contrast, NFAP2 was ineffective against aspergilli and fusaria, while *Botrytis* and *Cladosporium* isolates proved to be more susceptible to this protein than to NFAP. The synthetic *Neosartorya* γ-core peptide derivatives spanning the native γ-core of *Neosartorya* AFPs (γ^{NFAP}, γ^{NFBP}, γ^{NFAP2}) were ineffective at concentrations up to 200 µg/ml (data not shown), while all rationally designed variants (γ^{NFAP}-opt, γ^{NFBP}-opt, γ^{NFAP2}-opt) with elevated positive net charge and hydrophilicity inhibited the growth of *Botrytis*, *Cladosporium* and *Fusarium* isolates at various MICs, but were ineffective against aspergilli. Then, we investigated whether the net charge or the hydrophilicity influenced the antifungal activity of the rationally designed γ-core peptide derivatives. Therefore, two different variants of γ^{NFAP}-opt were synthesised. In the γ^{NFAP}-optChZ, amino acid substitutions reduced the net charge from +5.8 to neutral but maintained the GRAVY (**Table 2**). In contrast, the GRAVY was reduced to −0.557, whereas the net charge remained unchanged in the γ^{NFAP}-optGZ variant (**Table 2**). Antifungal susceptibility test indicated that the positive net charge, not the hydrophilicity of these γ-core peptides plays a major role in antifungal efficacy. ECD spectroscopy demonstrated that the antifungal activity does not require a conformational change of the β-pleated AFPs or the canonically ordered conformation of the synthetic γ-core peptide derivatives.

Based on the results of the *in vitro* susceptibility tests, the most effective antifungal proteins, namely the PAF, PAF^{opt}, NFAP and their rationally designed and optimized γ-core peptide derivatives were considered as effective plant and crop protective biocompounds. Therefore, they were selected for functional testing.

These results were partially published in the following peer-reviewed papers

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide Pγ^{opt} in plant protection. MICROBIAL BIOTECHNOLOGY. 13(5): pp. 1403-1414. (2020), DOI: 10.1111/1751-7915.13559. IF2019: 5.328 (Q1 Applied microbiology and biotechnology)

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2019: 4.235 (Q1 Microbiology)

and conference proceedings.

Tóth L, Váradi G, Ficze H, Tóth, KG, Marx F, **Galgóczy L**, Antifungal effect of *de novo* designed peptides according to the y-core motif of *Neosartorya fischeri* NRRL 181 antifungal proteins. In: Abrama, M; Bielen, A; Kifer, D; Vlahovicek, GM; Klaric, MS (ed.) Central European Symposium on Antimicrobial Resistance - Book of Abstracts (2018) p. 111. Conference place and time: Sveti Martin na Muri, Croatia, 19.09.2018.-22.09.2018. (ISBN:978-953-7778-16-3)

Tóth L, Váradi G, Ficze H, Tóth, KG, Marx F, **Galgóczy, L**. Examination of antifungal activity and mechanism of *de novo* designed y-core peptide motifs from *Neosartorya fischeri* NRRL 181 antifungal proteins. ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA 66: (S1) pp. 105-106., 2019. A Magyar Mikrobiológiai Társaság 2018. évi Nagygyűlése és a XIII. Fermentációs Kollokvium. Conference place and time: Eger, Hungary, 17.10.2018.-19.10.2018.

Tóth L, Váradi Gy, Boros É, Ficze H, Nagy I, Marx F, **Galgóczy L**. Agricultural applicability of *Neosartorya fischeri* antifungal protein and *de novo* designed peptide derivatives. In: István Majzinger, Tamás Monostori, Monica Ocnen, Elena Pet, Sorin Mihai Stanicu, Judit Szűcsné Péter, Lajos Tanás (eds.) 17th Wellmann International Scientific Conference "Agriculture Without Borders": Book of Abstracts. 106 p. Conference place and time: Hódmezővásárhely, Magyarország, 08.05.2019. Hódmezővásárhely: University of Szeged, Faculty of Agriculture, 2019. pp. 75-76. (ISBN:978-963-306-653-9)

Tóth L, Boros É, Poór P, Ördög A, Váradi Gy, Kele Z, Borics A, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth KG, Rákhely G Marx F, **Galgóczy L**. Biofungicidal potential of antifungal proteins and their peptide derivatives from filamentous ascomycetes. A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium. Book of Abstracts (2020) p.37. Conference place and time: Kecskemét, Hungary, 14.10.2020.-16.10.2020.

For the plant protection assay, we adopted the pathogenicity test method described by El Oirdi *et al.* (2010; 2011). *B. cinerea* is known as fungal necrotroph of tomato plant leaf tissue (Nambeesan et al., 2012). Considering the promising results from the *in vitro* susceptibility and toxicity tests (**Table 3**), the plant protection ability of PAF, PAF^{opt}, Pγ^{opt}, NFAP and γ^{NFAP}-opt was tested against *B. cinerea* SZMC 21472 infection of tomato plant leaves. Results with *Penicillium* AFPs and peptides are published in a peer-reviewed paper with a conclusion that PAF protects tomato plant leaves against *B. cinerea* infection, while PAF^{opt} and Pγ^{opt} are not able to inhibit the infection development. Plant protection bioassay results with NFAP and γ^{NFAP}-opt are demonstrated in the **Figure 4**. To reveal the potential toxic effect of the proteins and peptides, uninfected leaves were first treated with them. A reliable cell viability assay applying Evan's blue staining (Vijayaraghavareddy et al., 2017) was used to monitor the size of the necrotic zones after treatment. This dye can stain only those cells blue around the treatment site, which have a compromised

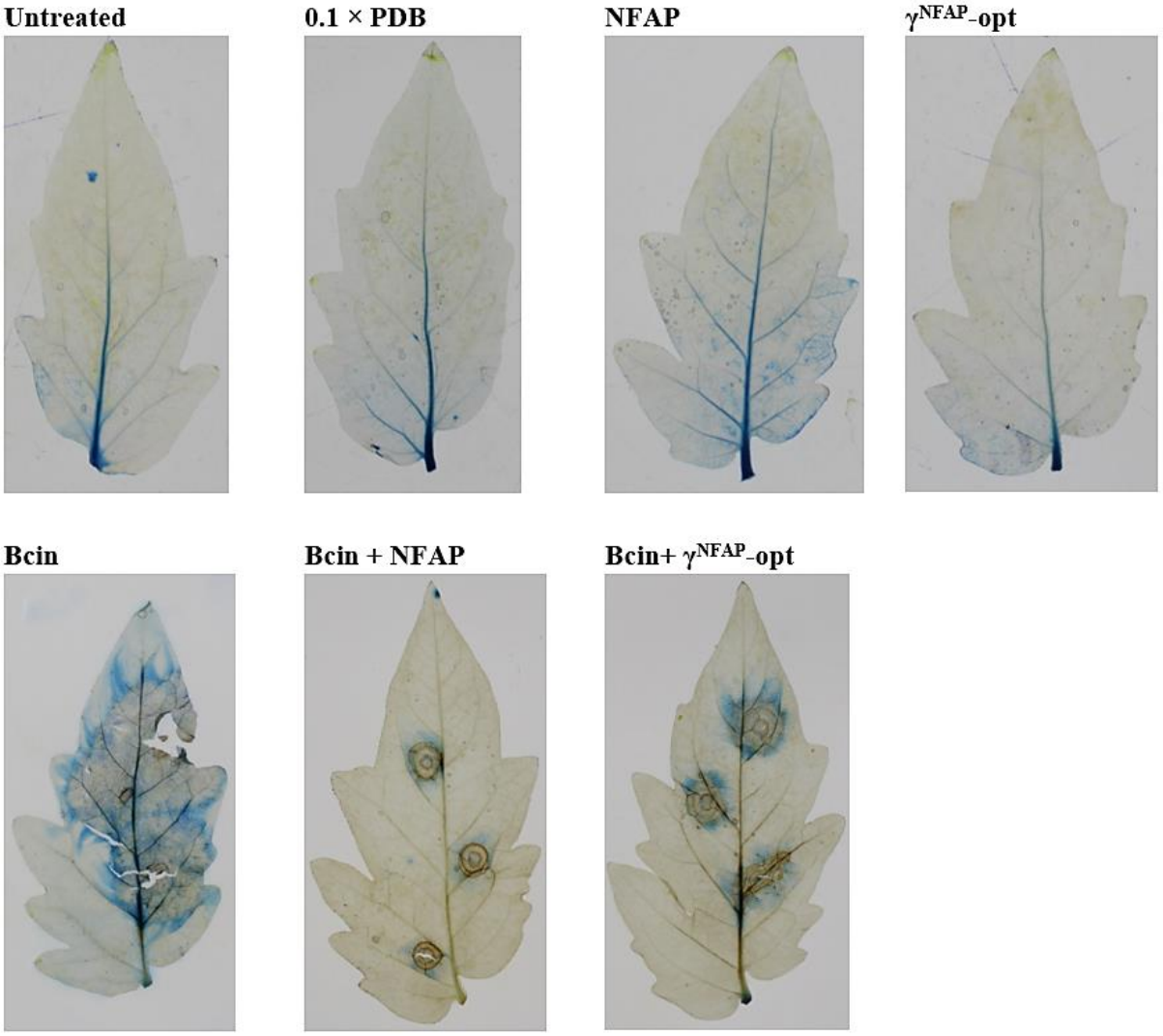

Figure 4. Evan's blue staining of untreated tomato leaves (Untreated), and tomato leaves treated with 0.1 × PDB (0.1 × PDB), 1 × MIC NFAP (NFAP), 1 × MIC γ^{NFAP}-opt (MIC γ^{NFAP}-opt), *Botrytis cinerea* (Bcin), *B. cinerea* + 1 × MIC NFAP (Bcin + NFAP), *B. cinerea* + 1 × MIC γ^{NFAP}-opt (Bcin + γ^{NFAP}-opt). Leaves were kept at 23°C, 60% humidity, and under 12 - 12 hours photoperiodic day-night simulation at 1200 lux for 4 days. Blue coloured zones or necrotic lesions on the leaves indicate cell death and established infection at site of the treatment points with *B. cinerea*.

plasma membrane due to a microbial infection or suffer from membrane disruption by the activity of proteins and peptides. All treatments were not toxic to the plants because cell death was not indicated by Evan's blue staining (NFAP and γ^{NFAP}-opt in **Figure 4**). The same was true for the 0.1 × potato dextrose broth-treated control (0.1 × PDB in **Figure 4**). This medium was used to establish the infection and to allow conidia germination. The *B. cinerea* infected but untreated leaves exhibited extensive necrotic lesions and blue coloured zones around the infection points indicating cell death in the consequence of an established and extensive fungal infection (Bcin in **Figure 4**). Next, the tomato plant leaves were infected with *B. cinerea* and treated with NFAP or γ^{NFAP}-opt. The lack of intensive blue coloured zones and necrotic lesions around the inoculation points indicated that NFAP protected tomato plant leaves against *B. cinerea* infection and the invasion of the fungus into the leaf tissue (Bcin + NFAP in **Figure 4**). In contrast γ^{NFAP}-opt was not able to fully impede fungal infection, but mitigated the symptoms. Slight blue coloured zones appeared at the inoculation points of *B. cinerea* (Bcin + γ^{NFAP}-opt in **Figure 4**).

The ability of NFAP and its antifungally active γ-core peptide derivatives to protect crops was studied on tomato fruits against *C. herbarum* FSU 1148 infection. This fungus is known as a postharvest spoilage agent of fresh fruits and vegetables, including tomatoes under storage conditions, especially when the vegetable surface was damaged (Snowden, 1992). Control treatments with NFAP, γ^{NFAP}-opt and γ^{NFAP}-optGZ did not cause any decay on the surface of the tomato fruits. The same was observed when the fruits

were treated with 0.1 × PDB, the medium used for the infection. *C. herbarum* infection, instead, was established within the applied incubation period at the sting points and the deeper tissues. Application of NFAP, γ^{NFAP}-opt and γ^{NFAP}-optGZ at *in vitro* detected MIC inhibited the development of decay. No intensive fungal growth was observed on the surface or in deeper tissues of the tomato fruits.

These results were partially published in the following peer-reviewed papers

Tóth L, Boros É, Poór P, Ördög A, Kele Z, Váradi G, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth GK, Rákhely G, Marx F, **Galgóczy L**. The potential use of the *Penicillium chrysogenum* antifungal protein PAF, the designed variant PAF^{opt} and its γ-core peptide $P\gamma^{opt}$ in plant protection. MICROBIAL BIOTECHNOLOGY. 13(5): pp. 1403-1414. (2020), DOI: 10.1111/1751-7915.13559. IF2019: 5.328 (Q1 Applied microbiology and biotechnology)

Tóth L, Váradi Gy, Boros É, Borics A, Ficze H, Nagy I, Tóth KG, Rákhely G, Marx G, **Galgóczy L**. Biofungicidal potential of *Neosartorya* (*Aspergillus*) *fischeri* antifungal protein NFAP and novel synthetic γ-core peptides. FRONTIERS IN MICROBIOLOGY 11: Paper 820. (2020), DOI: 10.3389/fmicb.2020.00820. IF2019: 4.235 (Q1 Microbiology)

and conference proceedings.

Tóth L, Boros É, Poór P, Ördög A, Váradi Gy, Kele Z, Borics A, Holzknecht J, Bratschun-Khan D, Nagy I, Tóth KG, Rákhely G Marx F, **Galgóczy L**. Biofungicidal potential of antifungal proteins and their peptide derivatives from filamentous ascomycetes. A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium. Book of Abstracts (2020) p.37. Conference place and time: Kecskemét, Hungary, 14.10.2020.-16.10.2020.

Tóth L, Ördög T, Poór P, Ördög A, Váradi Gy, Tóth KG, Rákhely G, **Galgóczy L**. Combinatorial application of *Neosartorya fischeri* antifungal proteins and their peptide derivatives in plant protection. A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium. Book of Abstracts (2020) p.38. Conference place and time: Kecskemét, Hungary, 14.10.2020.-16.10.2020.

Mycotoxin decreasing ability of NFAP and NFAP2 at their MIC and 2 × MIC was investigated on mycotoxigenic isolates of *Aspergillus flavus* (SZMC 20745) and *Fusarium verticillioides* (SZMC 11411), respectively, applying the method described by George et al. (2020). The first isolate is able to produce aflatoxin B1, while the second one is known as a producer of fumonisin B1. Significant difference in the mycotoxin production of both fungi after AFP treatments was not observed in comparison with the untreated controls (**Figure 5**).

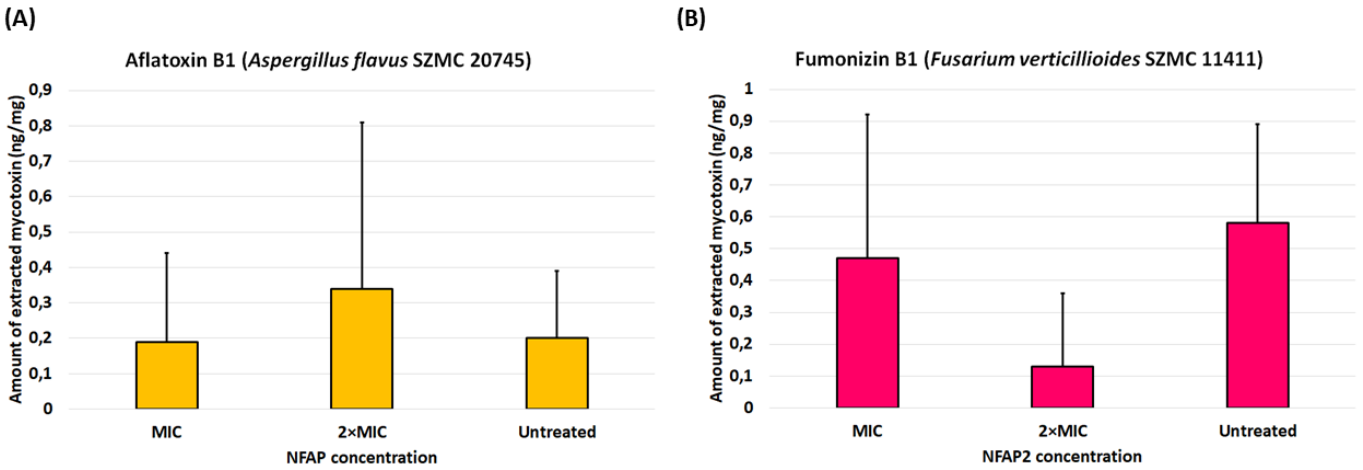

Figure 5. Impact of NFAP and NFAP2 on mycotoxin producing ability of *Aspergillus flavus* (SZMC 20745) and *Fusarium verticillioides* (SZMC 11411), respectively, in YES medium after static incubation at 25°C for 7 days. Amounts of mycotoxins (ng) are referred to 1 mg dry weight mycelium.

Conclusions

Taken together, the results of present project demonstrated that the positively charged and hydrophilic γ-core motif determines the antifungal activity and antifungal spectrum of an AFP. Specific amino acid substitutions in this peptide motif to elevate the positive net charge and the hydrophilicity of the AFP improve the antifungal efficacy or change the antifungal spectrum, and do not influence dramatically the secondary and tertiary structure. If an AFP possess a negatively charged or neutral, less hydrophilic γ-core motif, it supports the correct protein folding. Rationally designed synthetic peptides spanning the γ-core motif of AFPs are antifungally active if they are hydrophilic and have high positive net charge. The positive net charge, not the hydrophilicity of these γ-core peptide derivatives plays major role in the antifungal efficacy. Native AFPs, their γ-core engineered variants and rationally designed synthetic γ-core peptide

derivatives are promising candidates for development of new, safely applicable antifungal therapeutic strategies in the medicine; and for biopreservation in agriculture and food industry because: (1) They effectively inhibit the growth of several human pathogenic, pre- and postharvest plant pathogenic and mycotoxigenic fungi. (2) They do not show cytotoxic potential on human cell lines and intact plants. (3) They show therapeutic potential against superficial mycoses caused by antifungal drug-resistant yeasts, and have potential to eradicate antifungal drug resistant yeast biofilms in combined application with licenced antifungals. (4) They are able to inhibit the establishment of fungal infection on plant, and to protect stored crop against decay caused by postharvest moulds. However, further studies that focus on their environmental impact and address their pharmacokinetic properties in the human body are essential to push forward their applicability. Additionally, our results provide a proof-of-principle for biotechnological production of protein-based biofungicids and bioactive peptides. A patent application is therefore considered.

PhD thesis partially based on the project results

Liliána Tóth (2018) Isolation and characterization of *Neosartorya fischeri* antifungal protein 2 (NFAP). University of Szeged, PhD School of Biology

MSc theses based on the project results

Hargita Ficze (2019) Potential applications of *Neosartorya fischeri* antifungal protein and its peptide derivatives in the agriculture. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

Ördög Tamás (2020) Combinatorial application *of Neosartorya fischeri* antifungal proteins and their peptide derivatives in plant and crop protection. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

BSc thesis based on the project results

Kornél Nedeczky (2018) Anti-yeast effect of peptides designed according to the *Neosartorya fischeri* antifungal protein 2 (NFAP2) γ-core motif. University of Szeged, Faculty of Science and Informatics, Department of Micobiology.

Scientific staffs

The research team consisted the following members: (1) the principal investigator (PI, László Galgóczy, extra payment on the project's budget); (2) one postdoctoral researcher (Liliána Tóth, applied full-time on the project budge); (3) a PhD student from the University of Szeged (Liliána Tóth., not on the project's budget; but after her PhD graduation applied as a postdoctoral researcher on the project's budget); (4) BSc and MSc diploma workers from the University of Szeged (Hargita Ficze, Kornél Nedeczky, Tamás Ördög).

Project collaborators

Present project is tightly connected with the PD 135248 project based on the regulations for NKFIH Postdoctoral Excellence Programme from 2016. As the ANN 131341 was an NKFIH-FWF joint research project, most of the results were achieved in tight collaboration with **Florentine Marx's group** (Institute of Molecular Biology, Biocenter, Medical University of Innsbruck; Innsbruck, Austria). The project was conducted in collaboration with the following national partners. They supported the project with those techniques that are not routinely applied in the host institution laboratories. **Gábor K. Tóth's group** (Department of Medical Chemistry at Faculty of Medicine, University of Szeged; Szeged, Hungary): Electrospray ionization mass spectrometry, reversed-phase high performance liquid chromatography solid-phase peptide and protein synthesis. **Gyula Batta's group** (Department of Organic Chemistry, Faculty of Science and Technology, University of Debrecen; Debrecen, Hungary): Nuclear magnetic resonance spectroscopy. **Attila Borics** (Chemical Biology Group of the Institute of Biochemistry, Biological Research Centre; Szeged, Hungary): Electronic circular dichroism spectroscopy. **László Majoros** and **Renátó Kovács** (Department of Medical Microbiology, Faculty of Medicine, University of Debrecen, Debrecen,

Hungary): *In vivo* animal model experiments, biofilm eradication investigations. **Péter Poór** and **Attila Ördög** (Department of Plant Biology, Faculty of Science and Informatics, University of Szeged): plant protection model experiments. **Mónika Varga** and **Sándor Kocsubé** (Department of Microbiology, Faculty of Science and Informatics, University of Szeged): Mycotoxin decreasing experiments.

References

Barker D, Pfaff T, Moreau D, Groves E, Ruffel S, Lepetit M, et al. (2006) "Growing *M. truncatula*: choice of substrates and growth conditions", in *Medicago truncatula* Handbook. eds. E-P. Journet, and U. Mathesius (Ardmore: The Samuel Roberts Noble Fondation), 1−25.

El Oirdi M, Trapani A, Bouarab, K. (2010) The nature of tobacco resistance against *Botrytis cinerea* depends on the infection structures of the pathogen. Environ Microbiol 12: 239−253.

El Oirdi M, El Rahman TA, Rigano L, El Hadrami A, Rodriguez MC, Daayf F, et al. (2011) *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. Plant Cell 23: 2405−2421.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, et al. (2005) "Protein identification and analysis tools on the ExPASy server", in The Proteomics Protocols Handbook. ed. J.M. Walker. (Totowa, NJ: Humana Press), 571−607.

George E, Yang C, Burns L, Schrunk D. (2020) Simultaneous analysis of mycotoxins in corn-based animal feed by quadrupole-Orbitrap LC-MS/MS. Thermo Scientific Application Note. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-65867-lc-ms-quadrupole-orbitrap-mycotoxin-an65867-en.pdf

Huber A, Hajdu D, Bratschun-Khan D, Gáspári Z, Varbanov M, Philippot S, et al. (2018) New antimicrobial potential and structural properties of PAFB: A cationic, cysteine-rich protein from *Penicillium chrysogenum* Q176. Sci Rep 8:1751.

Nambeesan S, AbuQamar S, Laluk K, Mattoo AK, Mickelbart MV, Ferruzzi MG, et al. (2012) Polyamines attenuate ethylene-mediated defense responses to abrogate resistance to *Botrytis cinerea* in tomato. Plant Physiol 158: 1034−1045.

Snowden A. (1992). "Solanaceous fruit vegetables", in Post-Harvest Diseases and Disorders of Fruits and Vegetables. ed. A. Snowden (London: CRC Press), 53−93.

Sonderegger C, Galgóczy L, Garrigues S, Fizil Á, Borics A, Manzanares P, et al. (2016) A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. Microb Cell Fact 15:192.

Tóth L, Kele Z, Borics A, Nagy LG, Váradi G, Virágh M, et al. (2018) NFAP2, a novel cysteine-rich anti-yeast protein from *Neosartorya fischeri* NRRL 181: isolation and characterization. AMB Express 6:75.

Váradi G, Tóth GK, Kele Z, Galgóczy L, Fizil Á, Batta G. (2013) Synthesis of PAF, an antifungal protein from *P. chrysogenum*, by native chemical ligation: native disulfide pattern and fold obtained upon oxidative refolding. Chemistry 19:12684−12692.

Vijayaraghavareddy P, Adhinarayanreddy V, Vemanna RS, Sreeman S., Makarla, U. (2017) Quantification of membrane damage/cell death using Evan's blue staining technique. Bio-protocol 7: e2519.

Szeged, 12 January 2021

László Galgóczy
Principal investigator