FINAL PROJECT REPORT

BIOFUNGICIDE AND BIOACTIVATOR POTENTIAL OF NOVEL DEFENSIN-LIKE PROTEINS FROM SOLANUM LYCOPERSICUM L.

Project leader: Liliána Tóth
Institutions: University of Szeged, Faculty of Science and Informatics, Department of Biotechnology
Beginning of the project: 01. 12. 2020. - End of the project: 30. 11. 2023.
Project duration in month: 36

The main aim of the project was to prove the fungicid potential of *Solanum lycopersicum* L. K4BPB5 (LpDef1), B1N680 (LpDef2), K4CBP6 (LpDef3) defensin-like proteins and their γ-core peptide derivatives for the agriculture as safely applicable plant and crop protective biomolecules.

Background of the study:
Phytopathogenic fungal infections in agricultural fields are a huge problem worldwide every year. These pathogens not only cause huge yield loss by infecting the plant, but also great damage to the stored crop and the food, since the consumption of mycotoxins produced by certain fungal species (e.g.: *Fusarium* spp. *Aspergillus* spp.) can cause serious health problems (kidney damage, liver cancer, etc.). The prevention of fungal infections is therefore important from economic and health point of view. Nowadays, however, the control of phytopathogenic fungal infections is a huge challenge for plant protection, as a high degree of resistance to the currently used chemical-based antifungal agents has developed in recent years, and according to European Union regulations, the use of current chemical fungicides must be halved by 2030. Based on all this, the development of novel antifungal strategies can be a solution to this problem affecting all parts of the world. The positively charged, cysteine-rich, defensins from plants and their chemically synthesized peptide derivatives based on the evolutionarily conserved [GXC]-[X3-9]-[C] consensus, so-called γ-core motifs may be suitable for the development of new antifungal agents, as they have antifungal effect and no resistance development to them has been shown so far. However, the high cost of production, the limited information about antifungal spectrum, the unrevealed long-term toxic effect on plant development and human health contradict direct, topical application of them as biofungicides or bioprotective agents in the agriculture [1].

Considering these challenges, the main aims of the project were the followings:

(1) Produce of novel defensin-like proteins from *Solanum lycopersicum* L. in heterologous expression system
(2) Synthesis of γ-core peptide derivatives of *Solanum lycopersicum* L. defensins
(3) Investigating their antifungal effect against plant pathogenic filamentous fungi
(4) Investigating their antifungal mechanism
(5) Investigating their toxicity
(6) Investigating their applicability as biofungicides
(7) Investigating their environmental stability

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

1) Production of novel defensin-like proteins from *Solanum lycopersicum* L. in heterologous expression systems

Antifungal plant defensins are produced in small amounts in the host during infections, so it is difficult to isolate them in reasonable amount [2]. In order to characterize and investigate defensins, it is necessary to use a reliable fungal-based expression system. During the previous work of our research group, a *P. chrysogenum* Q176-based expression system proved to be applicable for bulk production of small antifungal proteins from ascomycetous fungi [3]. These proteins are similar to antifungal plant defensins, as they also have a positively charged, β-folded structure stabilized by disulfide bonds [2]. Considering these results, in the present project, we generated a *P. chrysogenum*-based heterologous expression system to produce LpDef1 (**K4BPB5**), LpDef2 (**B1N680**) and LpDef3 (**K4CBP6**) (**Table 1**). The host strains proved to be sensitive to the produced K4BPB5 and B1N680; therefore, they were not able to produce them. In contrast, we were able to produce the K4CBP6, however only in small amounts (yield: 0.075 μg ml^{-1}). Mass spectrometric analysis indicated that beside the full-length K4CBP6, its shorter version (lacking one arginine at the N-terminus) also appeared. Based on electronic circular dichroism (**ECD**) spectroscopy measurements, the lack of the arginine does not influence the secondary structural elements and thermal stability of the K4CBP6. Due to the host sensitivity, and the small yield of the K4CBP6, we started to generate a *P. pastoris*-based expression system with an inducible promoter for the bulk production of the K4BPB5, B1N680 and K4CBP6. This system is commonly applied in production of different plant defensins possessing β-sheets in their structure [2].

Table 1. Amino acid sequence and *in silico* predicted physicochemical properties of predicted *Solanum lycopersicum* L. antifungal defensins from SGN database [4].

Name	Locus [4]	Number of aa	Mw (Da) [5]	Number of Cys	Number of Lys/Arg/His	pI [5]	Estimated charge at pH=7.0 [6]	GRAVY [5]
MERKIYGLLFLLVILFTSQMSIRGAEG**RVCISQSHGYKGPCVRDHNCALVCRNEYFSGGDCIGFGFNRKCFCTKAC**								
Predicted disulphide-bond pattern[7]: *adbacbdc*								
LpDef1 K4BPB5	Solyc04g009590.2.1	49	5452.27	8	3/4/2	8.73	+4.1	-0.206
MGNSLRLFATFFLVAMLLLATGPTTSVEA**RTCESQSHHFKGNCLSDTNCGSVCRTEGFTGGNCRGFRRRCFCTRNC**								
Predicted disulphide-bond pattern[7]: *adbacbdc*								
LpDef2 B1N680	Solyc07g007710.2.1	47	5293.91	8	1/7/2	8.99	+5.2	-0.845
MAQSIRFFATLFLLAMLVMATEMGPTRIVEA**RHCESLSHRFKGPCVSDKNCASVCETERFSGGNCRGFRRRCFCTKPC**								
Predicted disulphide-bond pattern[7]: *adbacbdc*								
LpDef3 K4CBP6	Solyc07g007760.2.1	47	5357.14	8	3/7/2	9.14	+6.2	-0.757

Black letters indicate the pre-pro sequence, while red and bold letters the mature protein. The SignalP1 4.1 server was used to predict the cleavage site of the signal sequences [8]. Putative γ-core motifs are underlined in the primary structure. GRAVY: grand average of hydropathy value.

These results were partially published in the following conference proceedings.

Tóth L, Papp R, Váradi G, Tóth KG, Papp C, Rákhely G, Poór P, Galgóczy L. Antifungal effect of a novel defensin from *Solanum lycopersicum* L., and it's γ-core peptide derivatives. In: Bánfalvi Zsófia; Gócza Elen; Olasz Ferenc; Pál Magda; Posta Katalin; Várallyay Éva (szerk.) „FIBOK 2022": Fiatal Biotechnológusok V. Országos Konferenciája: Program és angol nyelvű összefoglalók, Conference place and time: Gödöllő, Hungary 11.04.2022.-12.04.2022., Gödöllő, Magyarország: MATE Genetika és Biotechnológia Intézet, pp. 33-33., 2022.

Papp R, Váradi G, Kele Z, Rákhely G, Poór P, Galgóczy L, Tóth L. Production of a novel defensin from *Solanum lycopersicum* L. in fungal expression systems. In: A Magyar Mikrobiológiai Társaság 2022. évi Nagygyűlése és a XV. Fermentációs Kollokvium: Absztraktfüzet, Conference place and time: Kecskemét, Hungary 12.10.2022.-14.10.2022., pp. 41-42., 2022.

Based on previous results, first we generated the K4CBP6-producing *P. pastoris* strain. The production of recombinant K4CBP6 was carried out with *P. pastoris* KM71H transformant harbouring the genome inserted pPICZαA_K4CBP6 expression vector. After purification from

the cell-free ferment broth [9], analysis with an electrospray ionization mass spectrometer (**ESI-MS**) identified the K4CBP6 with a monoisotopic molecular mass of 5346 Da. Production with the heterologous expression system was performed several times. Following the third production, a protein with a higher molecular weight of 5746 Da was identified, which corresponds to the monoisotopic molecular weight of the K4CBP6 (**EAEA_K4CBP6**) with four additional amino acids (EAEA) from the N-terminus. In all further production, this K4CBP6 with this glutamine-alanine repeat was identified. The ESI-MS data indicate that in both recombinant defensins, the cysteines are present in the molecule in an oxidized state, which presupposes the formation of disulfide bridges and thus formation of a compact tertiary structure (**Figure 1**). Based on reversed-phase high-performance liquid chromatographic (**RP-HPLC**) and ECD spectroscopic analyses the two recombinant K4CBP6 defensins have the same single disulfide bond-stabilized structure (**Figure 1**). Using this *P. pastoris* expression system, 7.94±1.25 mg of the K4CBP6 can be obtained from one liter of ferment broth. For these reasons, I refer to the defensin as K4CBP6, regardless of the EAEA repeat.

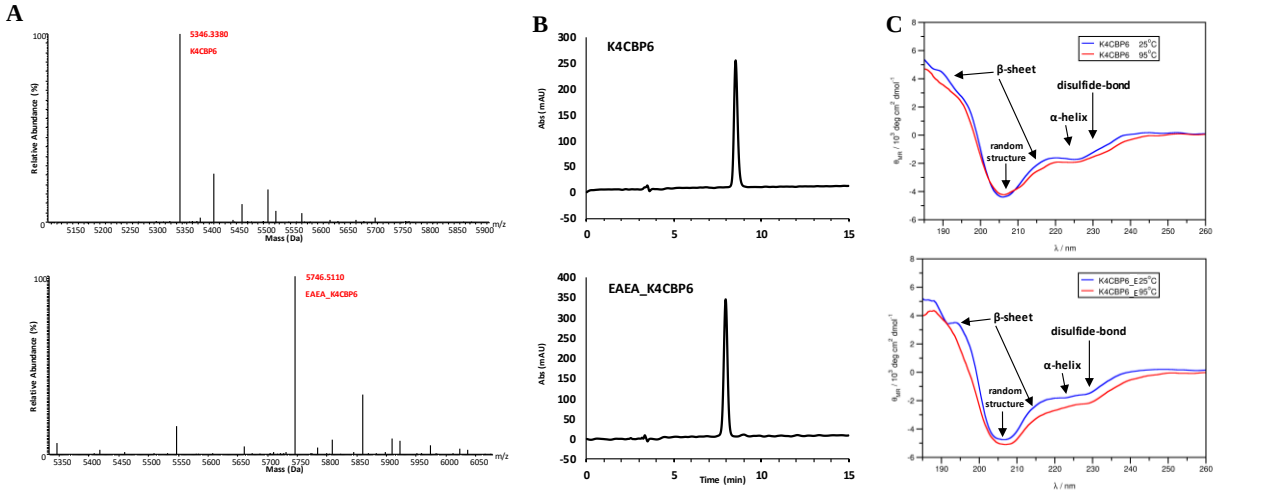

Figure 1. Comparison of the structure of the two recombinant K4CBP6 defensins, as well as their secondary structural elements and the presence of disulfide bridges. ESI-MS spectrum **(A)**, RP-HPLC chromatogram **(B)**, and ECD spectrum **(C)** of the purified K4CBP6 and EAEA_K4CBP6. K4CBP6: recombinant K4CBP6, EAEA_K4CBP6 (K4CBP6_E): recombinant K4CBP6 with Glu-Ala repeat.

These results were partially published in the following conference proceedings.

Tóth L, Papp R, Váradi G, Tóth KG, Papp C, Rákhely G, Poór P, Galgóczy L. Antifungal effect of a novel defensin from *Solanum lycopersicum* L., and it's γ-core peptide derivatives. In: Bánfalvi Zsófia; Gócza Elen; Olasz Ferenc; Pál Magda; Posta Katalin; Várallyay Éva (szerk.) „FIBOK 2022": Fiatal Biotechnológusok V. Országos Konferenciája: Program és angol nyelvű összefoglalók, Conference place and time: Gödöllő, Hungary 11.04.2022.-12.04.2022., Gödöllő, Magyarország: MATE Genetika és Biotechnológia Intézet, pp. 33-33., 2022.

Papp R, Váradi G, Kele Z, Rákhely G, Poór P, Galgóczy L, Tóth L. Production of a novel defensin from *Solanum lycopersicum* L. in fungal expression systems. In: A Magyar Mikrobiológiai Társaság 2022. évi Nagygyűlése és a XV. Fermentációs Kollokvium: Absztraktfüzet, Conference place and time: Kecskemét, Hungary 12.10.2022.-14.10.2022., pp. 41-42., 2022.

Papp R, Váradi G, Kele Z, Rákhely G, Poór P, Tóth KG, Galgóczy L, Tóth L. Heterologous expression and *in vitro* antifungal effect of a novel *Solanum lycopersicum* L. antifungal defensin. In: Renata Teparić; Andreja Leboš Pavunc; Domagoj Kifer (szerk.) Power of Microbes in Industry and Environment 2023: Book of Abstracts, Conference place and time: Porec, Croatia 15.05.2023.-18.05.2023., Zagreb, Horvátország: Croatian Microbiological Society, p. 96., 2023.

Papp R, Borics A, Merber R, Galgóczy L, Tóth L. Heterologous production of a *Solanum lycopersicum* L. antifungal defensin in *Pichia pastoris.* In: Benczúr Kinga; Gócza Elen; Pál Magda; Pusztahelyi Tünde (szerk)

„FIBOK 2024”: 6th National Conference of Young Biotechnologists, Conference place and time: Budapest, Hungary 04.04.2024.-05.04.2024., Budapest, Magyarország: MTA Agrártudományok Osztálya, Mezőgazdasági Biotechnológiai Tudományos Bizottság p. 30., 2024.

Based on the results, the *P. pastoris* system proved to be effective for the production of the K4CBP6, so we also prepared the K4BPB5 and B1N680 defensin-producing expression systems. ESI-MS measurements proved the production of these defensins also. The 49 amino acid-long K4BPB5 identified in the purified sample did not contain the EAEA repeat, however, in the case of the B1N680, a version of the defensin with two amino acids (EA) at the N-terminus was present in the purified sample. Based on all of this, the *P. pastoris*-based expression system proved to be suitable for the production of defensins, so we started the large-scale production and investigation of the proteins.

2) Synthesis of γ-core peptide derivatives of *Solanum lycopersicum* L. defensins

The **γ-core peptide derivatives of K4BPB5**, **B1N680** and **K4CBP6** were rationally designed and chemically synthesised (**Table 2**). These peptides contain three flanking N-terminal amino acids from the protein sequence, and they have N-terminal acetylation, C-terminal amidation and reduced cysteines, therefore they proved to be functional and stable [10].

Table 2. Amino acid sequence and *in silico* predicted physicochemical properties of designed *Solanum lycopersicum* L. antifungal defensins γ-core peptide derivatives (**Table 1**).

Name	Peptid	Number of aa	Mw (kDa)	Cis	Lis/Arg /His	pI	Charge pH 7.0	GRAVY
LpDef1 (K4BPB5): MERKIYGLLFLLVILFTSQMSIRGAEGRVCISQSHGYKGPCVRDHNCALVCRNEYFSGGDCIGFGFNRKCFCTKAC								
K4BPB5γ1	Ac-GYKGPC(-SH)VRDHNC(-SH)K-NH_2	13	1.48	2	2/1/1	8.86	+2.1	-1.308
K4BPB5γ2	Ac-FSGGDC(-SH)IGFGFNRKC(-SH)K-NH_2	16	1.74	2	2/1/0	8.90	+1.8	-0.237
LpDef2 (B1N680): MGNSLRLFATFFLVAMLLLATGPTTSVEARTCESQSHHFKGNCLSDTNCGSVCRTEGFTGGNCRGFRRRCFCTRNC								
B1N680γ1	Ac-HFKGNC(-SH)LSDTNC(-SH)K-NH_2	13	1.47	2	2/0/1	8.06	+1.1	-0.908
B1N680γ2	Ac-KC(-SH)RTEGFTGGNC(-SH)RGFRR-NH_2	17	1.95	2	1/4/0	10.72	+3.8	-1.253
LpDef3 (K4CBP6): MAQSIRFFATLFLLAMLVMATEMGPTRIVEARHCESLSHRFKGPCVSDKNCASVCETERFSGGNCRGFRRRCFCTKPC								
K4CBP6γ1	Ac-RFKGPC(-SH)VSDKNC(-SH)K-NH_2	13	1.48	2	3/1/0	9.39	+2.8	-1.077
K4CBP6γ2	Ac-FSGGNC(-SH)RGFRRRC(-SH)K-NH_2	14	1.64	2	1/4/0	11.54	+4.8	-1.200

GRAVY: grand average of hydropathy value.

These results were partially published in the following conference proceedings.

Vass H, Papp R, Galgóczy L, Poór P, Tóth L. Paradicsomnövény (*Solanum lycopersicum* L.)-eredetű defenzinek γ-core régiója alapján tervezett peptidszármazékok *in vitro* antifungális hatása. In: Györgyey János (szerk.) XIII. Magyar Növénybiológiai Kongresszus: Összefoglaló kötet, Conference place and time: Szeged, Hungary 24.08.2021.-27.08.2021., Szeged: Szegedi Biológiai Kutatóközpont, p. 92., 2021.

Papp R, Vass H, Váradi G, Tóth KG, Galgóczy L, Poór P, Tóth L. Gamma (γ)-core peptide derivatives of novel tomato plant defensins effectively inhibit the growth of plant pathogenic filamentous fungi. In: Szabó Dóra; Kocsis Béla; Horváth Andrea (szerk.) Acta Microbiologica et Immunologica Hungarica, Conference place and time: Kecskemét, Hungary 13.10.2021.-15.10.2021., 68(1): pp. 104-104., 2021.

Tóth L, Papp R, Váradi G, Tóth KG, Papp C, Rákhely G, Poór P, Galgóczy L. Antifungal effect of a novel defensin from *Solanum lycopersicum* L., and it's γ-core peptide derivatives. In: Bánfalvi Zsófia; Gócza Elen; Olasz Ferenc; Pál Magda; Posta Katalin; Várallyay Éva (szerk.) „FIBOK 2022”: Fiatal Biotechnológusok V. Országos Konferenciája: Program és angol nyelvű összefoglalók, Conference place and time: Gödöllő, Hungary 11.04.2022.-12.04.2022., Gödöllő, Magyarország: MATE Genetika és Biotechnológia Intézet, pp. 33-33., 2022.

Tóth L, Papp R, Papp C, Váradi G, Tóth KG, Rákhely G, Poór P, Galgóczy L. Antifungal activity of peptides designed on the γ-core of tomato plant defensins. In: FEMS Conference on Microbiology in association with Serbian Society of Microbiology: Abstract Book, Conference place and time: Belgrade, Serbia 30.06.2022.-02.07.2022., Belgrád, Szerbia: Serbian Society of Microbiology, pp. 837-838., 2022.

Vass H, Váradi G, Kele Z, Rákhely G, Poór P, Tóth KG, Galgóczy L, Tóth L. Biocontrol ability of rationally designed peptide derivatives of a novel *Solanum lycopersicum* L. antifungal defensin. In: 16th European Conference on Fungal Genetics: Programme & Abstracts, Conference place and time: 05.03.2023.-08.03.2023., Innsbruck, Ausztria: Universität Innsbruck, pp. 478-479., 2023.

3) Investigating the antifungal effect of *Solanum lycopersicum* L. defensin and γ-core peptide derivatives against plant pathogenic filamentous fungi

A broth microdilution susceptibility testing method [11] was applied to determine the antifungal potential of the produced recombinant **K4CBP6** and the above mentioned **six synthetic γ-core peptide derivatives** (**Table 2**) in 0.1 × PDB (potato dextrose broth) medium against plant pathogens (**Table 3**). The respective minimal inhibitory concentrations (**MIC**s) are summarized in the **Table 3**. The MIC was defined as the lowest protein/peptide concentration that reduces fungal growth to ≤ 5% in comparison with the untreated control which was set to be 100%. The K4CBP6 effectively inhibited the growth of all tested phytopathogenic fungal isolates in the applied concentration range (200-0.195 μg ml^{-1}) except for *Aspergillus* isolates and *Fusarium oxysporum* CBS 123668, and already at a concentration of 25-50 μg ml^{-1} resulted in complete growth inhibition of the fungus. The antifungal effect of synthetic γ-core peptide derivatives was investigated against three important phytopathogens of the tomato plant (*Botrytis cinerea*, *Cladosporium herbarum*, *F. oxysporum*). Among the chemically synthesized peptide derivatives, mainly those with large positive net charge and hydrophilicity (K4CBP6γ2 and B1N680γ2) showed growth inhibitory effect against these fungi in an *in vitro* microdilution susceptibility test. The K4CBP6γ2 effectively inhibited the growth of all investigated isolates and resulted in complete inhibition of the fungal growth at low concentrations (MIC: 12.5-25 μg ml^{-1}). In contrast, B1N680γ2 was only effective against *B. cinerea* (200 μg ml^{-1}) and *C. herbarum* (25 μg ml^{-1}). The peptide derivatives designed according to the first γ-core regions proved to be ineffective against these fungal isolates in the tested concentration range (400-0.39 μg ml^{-1}). Based on the results of the *in vitro* susceptibility tests (**Table 3**), the K4CBP6 and its γ-core peptide derivative (K4CBP6γ2) were considered as effective plant and crop protective biocompounds. Therefore, they were selected for toxicity testing.

Table 3. *In vitro* minimal inhibitory concentrations (**MIC**s) (μg ml^{-1}) of the K4CBP6 and rationally designed γ-core peptide derivatives of tomato defensins against plant pathogens in 0.1 × PDB after incubation for 72 h at 24°C.

Filamentous fungi	rK4CBP6	K4CBP6γ1	K4CBP6γ2	B1N680γ1	B1N680γ2	K4BPB5γ1	K4BPB5γ2
Aspergillus flavus SZMC 20745	>200	-	-	-	-	-	-
Aspergillus niger SZMC 0145	>200	-	-	-	-	-	-
Botrytis cinerea SZMC 21472	25	>400	25	>400	200	>400	>400
Cladosporium herbarum FSU 1148	25	400	12.5	>400	25	400	200
Fusarium avenaceum SZMC 11044	50	-	-	-	-	-	-
Fusarium cerealis SZMC 11045	25	-	-	-	-	-	-
Fusarium culmorum SZMC 11039	25	-	-	-	-	-	-
Fusarium graminearum SZMC 11032	25	-	-	-	-	-	-
Fusarium oxysporum CBS 123668	>200	>400	25	>400	>400	>400	200
Fusarium oxysporum SZMC 6237J	25	>400	25	>400	>400	>400	200
Fusarium proliferatum SZMC 21643	25	-	-	-	-	-	-
Fusarium sporotrichoioides SZMC 11043	25	-	-	-	-	-	-
Fusarium verticillioides SZMC 21642	25	-	-	-	-	-	-
Trichoderma harzianum SZMC 1601	25	-	-	-	-	-	-

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. n.d.: data not available.

These results were partially published in the following conference proceedings.

Vass H, Papp R, Galgóczy L, Poór P, Tóth L. Paradicsomnövény (*Solanum lycopersicum* L.)-eredetű defenzinek γ-core régiója alapján tervezett peptidszármazékok *in vitro* antifungális hatása. In: Györgyey János (szerk.) XIII. Magyar Növénybiológiai Kongresszus: Összefoglaló kötet, Conference place and time: Szeged, Hungary 24.08.2021.-27.08.2021., Szeged: Szegedi Biológiai Kutatóközpont, p. 92., 2021.

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Vass H, Váradi G, Kele Z, Rákhely G, Poór P, Tóth KG, Galgóczy L, Tóth L. Biocontrol ability of rationally designed peptide derivatives of a novel *Solanum lycopersicum* L. antifungal defensin. In: 16th European Conference on Fungal Genetics: Programme & Abstracts, Conference place and time: 05.03.2023.-08.03.2023., Innsbruck, Ausztria: Universität Innsbruck, pp. 478-479., 2023.

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Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

4) Investigating the antifungal mechanism of *Solanum lycopersicum* L. defensin and γ-core peptide derivatives

Fungal cell-killing efficacy

Based on the results of the *in vitro* microdilution tests (**Table 3**), the fungal cell-killing efficacy of the most effective peptide derivatives (**B1N680γ2** and **K4CBP6γ2**) was investigated on the conidia of the most susceptible fungal strain, *C. herbarum* FSU 1148 applying fluorescence-activated cell sorting (**FACS**) coupled with propidium iodide (**PI**) staining. PI, a membrane-impermeable fluorescent dye, enters the cell only through the damaged plasma membrane, where it intercalates with DNA to emit red fluorescence. This red fluorescence indicates cell death. Applying FACS, we determined how quickly and efficiently K4CBP6γ2 and B1N680γ2 peptides are able to kill conidia (**Table 4**). FACS analysis indicated that the antifungal active peptides are able to destruct the plasma membrane integrity of fungi within 16 hours and the peptides used at 2 × MIC had a greater antifungal effect than those with the MIC value, so the fungal cell killing efficacy of both peptides is dose- and time-dependent (**Table 4**).

Table 4. Percentage of propidium iodide-positive *Cladosporium herbarum* FSU 1148 conidia after MIC (B1N680γ2: 25 μg ml^{-1}, K4CBP6γ2: 12.5 μg ml^{-1}), and 2 × MIC (B1N680γ2: 50 μg ml^{-1}, K4CBP6γ2: 25 μg ml^{-1}) peptide treatments for 10 minutes and 16 hours at 25°C, 160 rpm.

Concentration of peptides	Untreated	10 minutes	16 hours	50% EtOH
		K4CBP6γ2		
MIC	8.3±0.1%	6.6±0.1%	15,1±3,6%	83.4±2.3%
2 × MIC		8.2±1.2%	**24.8±0.1%***	
		B1N680γ2		
MIC	8.3±0.1%	6.8±2.6%	22.8±7.2%	83.4±2.3%
2 × MIC		14.7±6.0%	21.7±6.1%	

Significant differences (p values from unpaired t-test) were determined based on the comparison with the untreated control; *p ≤ 0.05.

These results were partially published in the following conference proceedings.

Tóth L, Papp R, Váradi G, Tóth KG, Papp C, Rákhely G, Poór P, Galgóczy L. Antifungal effect of a novel defensin from *Solanum lycopersicum* L., and it's γ-core peptide derivatives. In: Bánfalvi Zsófia; Gócza Elen; Olasz Ferenc; Pál Magda; Posta Katalin; Várallyay Éva (szerk.) „FIBOK 2022": Fiatal Biotechnológusok V. Országos Konferenciája: Program és angol nyelvű összefoglalók, Conference place and time: Gödöllő, Hungary 11.04.2022.-12.04.2022., Gödöllő, Magyarország: MATE Genetika és Biotechnológia Intézet, pp. 33-33., 2022.

Tóth L, Papp R, Papp C, Váradi G, Tóth KG, Rákhely G, Poór P, Galgóczy L. Antifungal activity of peptides designed on the γ-core of tomato plant defensins. In: FEMS Conference on Microbiology in association with Serbian Society of Microbiology: Abstract Book, Conference place and time: Belgrade, Serbia 30.06.2022.-02.07.2022., Belgrád, Szerbia: Serbian Society of Microbiology, pp. 837-838., 2022.

Effect on the growth of fungi

The effect of the recombinant **K4CBP6** on the growth of phytopathogenic fungi was investigated using lactophenol cotton blue (R-8) staining. Based on microscopic examination the morphology of the hyphae of the fungal species treated with the MIC defensin is the same as the hyphae observed in the control case (**Figure 2**). Therefore, the rK4CBP6 may belong to the group of non-morphogenic defensins and exerts its antifungal effect by damaging the cell wall/membrane [12].

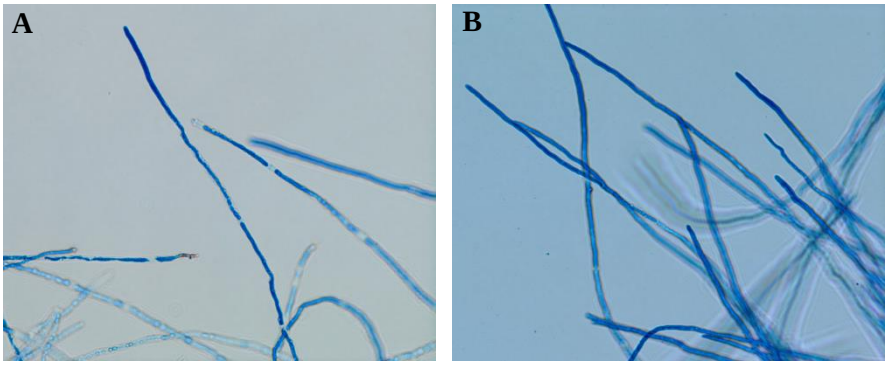


Figure 2. Examination of the morphology of the K4CBP6 treated *Fusarium culmorum* SZMC 11039 hyphae. Microscopic view of *F. culmorum* SZMC 11039 hyphae (10^5 con ml^{-1}) untreated (**A**) and treated with the K4CBP6 defensin (12.5 μg ml^{-1}) (**B**) after 72 h incubation at 25°C in 0.1 × PDB.

These results were partially published in the following conference proceedings.

Papp R, Borics A, Merber R, Galgóczy L, Tóth L. Heterologous production of a *Solanum lycopersicum* L. antifungal defensin in *Pichia pastoris*. In: Benczúr Kinga; Gócza Elen; Pál Magda; Pusztahelyi Tünde (szerk) „FIBOK 2024": 6th National Conference of Young Biotechnologists, Conference place and time: Budapest,

Hungary 04.04.2024.-05.04.2024., Budapest, Magyarország: MTA Agrártudományok Osztálya, Mezőgazdasági Biotechnológiai Tudományos Bizottság p. 30., 2024.

Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

5) Investigating the cytotoxic potential of *Solanum lycopersicum* L. defensin and their γ-core peptide derivative

Due to the antifungal effect of the **K4CBP6** and its γ-core peptide derivative (**K4CBP6γ2**) were considered as effective plant and crop protective biocompounds, although the application in agriculture of antifungal proteins and peptides can be limited if they have haemolytic activity, toxicity, and other side effects. For all these reasons, we investigated the toxicity and haemolytic activity of the K4CBP6 and K4CBP6γ2 for their use as potential biopesticides.

Toxicity effect on *Galleria mellonella* larvae

A well-established and described acute toxicity test in *Galleria mellonella* (greater wax moth) larvae was conducted to reveal the potential *in vivo* harmful effects of the K4CBP6 and its γ-core peptide derivative (K4CBP6γ2) in animals [13]. During the experiment, the larvae were treated with defensin, peptide, and then their survival was monitored (**Figure 3**). According to the survival analysis of the larvae injected with K4CBP6 and K4CBP6γ2, the treatments did not cause significant differences compared to the survival rates of the untreated and insect physiological saline (**IPS**) control groups, so neither the defensin nor the peptide proved to be toxic.

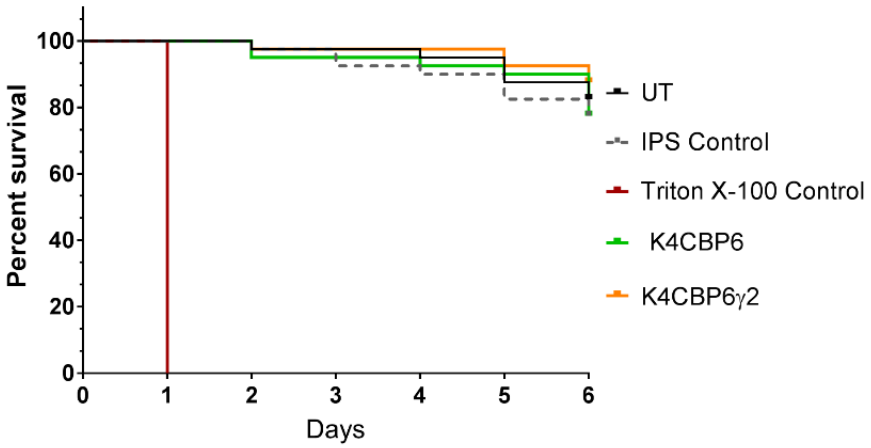


Figure 3. Survival of *Galleria mellonella* larvae after injection with the K4CBP6 and its γ-core peptide derivative (K4CBP6γ2) (20 µl from 200 µg ml^{-1} solution) in comparison with the untreated control. UT: untreated control, IPS: insect physiological saline - treated control. *: p≤ 0.05 from both Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.

These results were partially published in the following conference proceeding.

Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

Haemolytic potential on blood agar plates

The potential human cell membrane disruption ability of the K4CBP6 and its γ-core peptide derivative (K4CBP6γ2) was investigated on sheep blood agar plates [10]. Based on the results of the haemolysis test, neither the K4CBP6 nor K4CBP6γ2 peptide cause haemolysis after incubation for 24 hours at 37°C, in contrast to Triton X-100 used as a positive control (**Figure 4**).

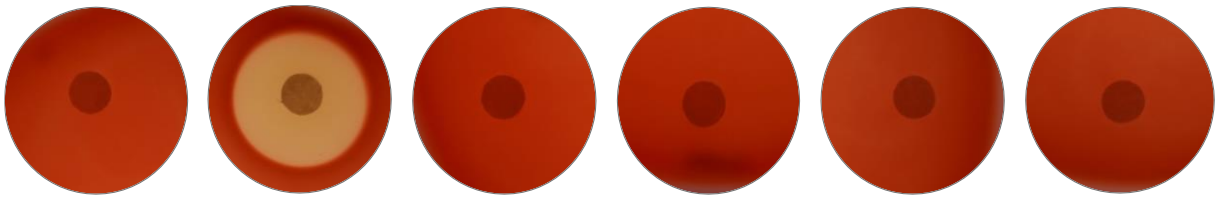


Figure 4. Haemolytic activity of K4CBP6 and its γ-core peptide derivative (K4CBP6γ2) in aqueous solution on Columbia blood agar plates after incubation for 24 h at 37°C. Triton X-100 (20% (v/v)) and distilled water (**dd. H_2O**) were used as the positive and negative controls, respectively.

These results were partially published in the following conference proceeding.

Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

Toxicity Tests with Plants Seedling

Germinating *Medicago truncatula* A-17 and *Solanum lycopersicum* L. cv. Ailsa Craig seeds were used to investigate the potential toxic effect of the K4CBP6 and K4CBP6γ2 [14, 15]. *M. truncatula* is a small legume, while *S. lycopersicum* is a relatively tall plant with thicker stems. Despite their different phenotypes, they have in common that they grow fast and easily cultivated on water agar in small to large Petri dishes, thus allowing reliable testing of the potential toxic effects of pesticide candidates, such as plant defensins and their peptide derivatives, on intact plants [14, 15]. For application of the K4CBP6 and K4CBP6γ2 as a biopesticide, it is essential that they do not have any adverse effects on the plant seedlings and do not cause a delay in the plant growth and development. These effects were investigated by treatment of the apical root regions of germinating *M. truncatula* and S. *lycopersicum* L. seeds with 400 μg ml^{-1} of K4CBP6 and K4CBP6γ2 solution for 10 and 6 days, respectively. As a result of the treatment with the K4CBP6γ2, there were no changes on the plants after the incubation period in the case of any of the model plants. The germinated seeds developed into healthy, mature seedlings with no significant difference in primary root length or in the number of lateral roots compared to untreated controls (**Figure 5**). In the case of *M. truncatula* seedlings treated with K4CBP6, no changes were also observed. The seedlings grew to healthy mature plants without showing any significant differences in primary root length or number of lateral roots compared to the water-treated control (**Figure 5**). In contrast, K4CBP6-treated *S. lycopersicum* seedlings showed a significant difference in primary root length. Despite reduced primary root growth, there was no difference in the number of lateral roots (**Figure 5**).

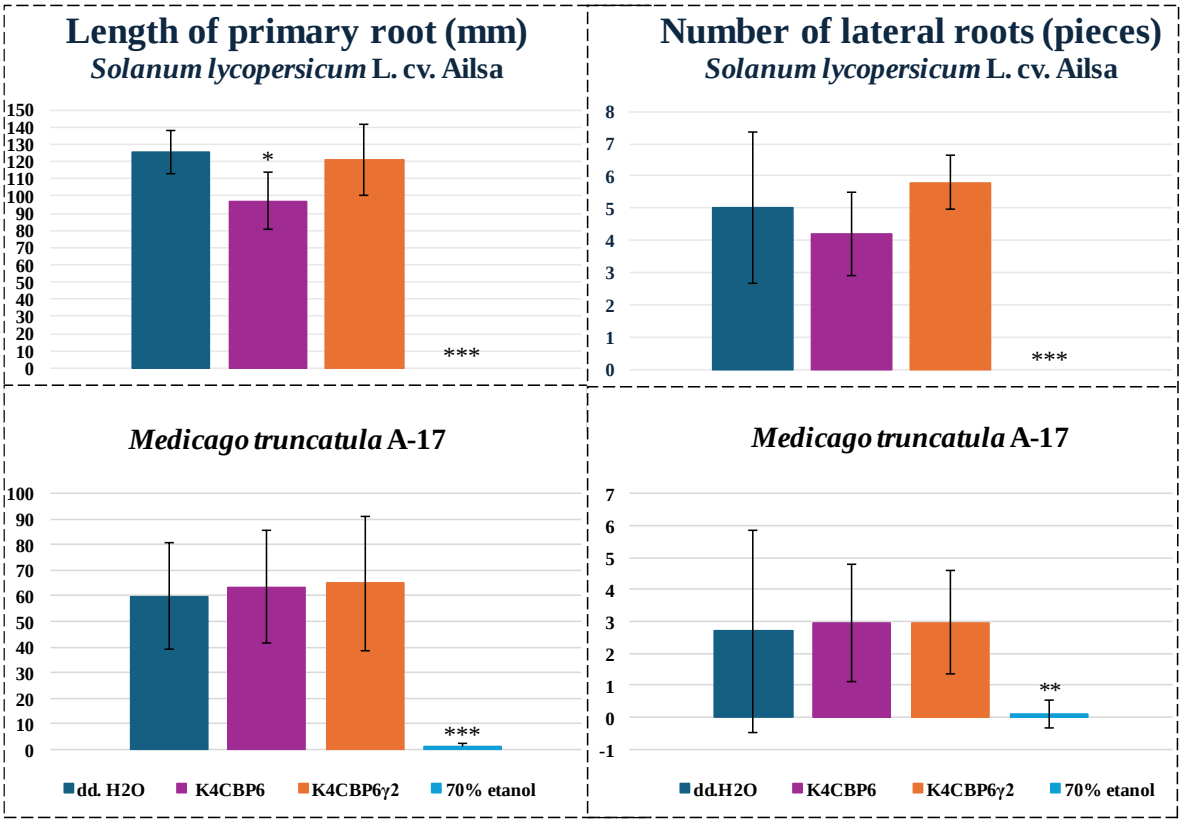


Figure 5. The length of evolved primary roots and the number of lateral roots of *Medicago truncatula* A-17 and *Solanum lycopersicum* L. cv. Ailsa after treatment with 400 µg ml^{-1} **K4CBP6** and **K4CBP6γ2** for 10 or 6 days at 23°C, 60% humidity under continuous illumination (1200 lux) in comparison with **dd.H_2O**- and **70% (v/v) ethanol**-treated controls. 10 (*S. lycopersicum*), and 20 (*M. truncatula*) seedlings were involved in the experiment. Significant differences (p values from unpaired t-test) were determined based on the comparison with the untreated control; *p≤0.05, **P≤0.005, ***P≤0.0001 in comparison with the dd.H_2O-treated sample.

These results were partially published in the following conference proceeding.

Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

6) Investigation of the applicability of *Solanum lycopersicum* L. defensin and γ-core peptide derivative as biopesticides

Considering the promising results of the *in vitro* microdilution and toxicity tests, we investigated the plant and crop protection ability of **K4CBP6** and its γ-core peptide derivative (**K4CBP6γ2**) to use them as biopesticides. After testing the toxicity of the defensin and the peptide, we investigated their antifungal effect against *B. cinerea* infection of tomato leaves and *C. herbarum* infection of tomato fruits [16, 17, 18].

Plant toxicity assay

The possible toxic effect of K4CBP6 and K4CBP6γ2 was investigated on the leaves of two *S. lycopersicum* L. plant species (*S. lycopersicum* L. cv Ailsa, *S. lycopersicum* L. cv Moneymaker)

[16, 17]. In doing so a reliable cell viability assay applying Evan's blue staining [19] 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 plasma membrane due to a microbial infection or suffer from membrane disruption by the activity of protein or peptide. To determine the toxic effect of the K4CBP6 and K4CBP6γ2, the uninfected intact leaves were treated with 10 µl of the defensin or peptide at a concentration of 400 µg ml^{-1} and 25 µg ml^{-1} on five points between the lateral veins of the abaxial leaf epidermis from fully expanded leaves of the third leaf level of tomato plants (**Figure 6**). The applied Evan's blue staining did not indicate any plant cell-killing ability of K4CBP6 (**Figure 6**). However, the area where K4CBP6γ2 was applied stained blue indicating toxicity on plant cells (**Figure 6**). The results were the same for the two investigated tomato species. Results of experiments with *S. lycopersicum* L. cv Moneymaker are shown in **Figure 6**.

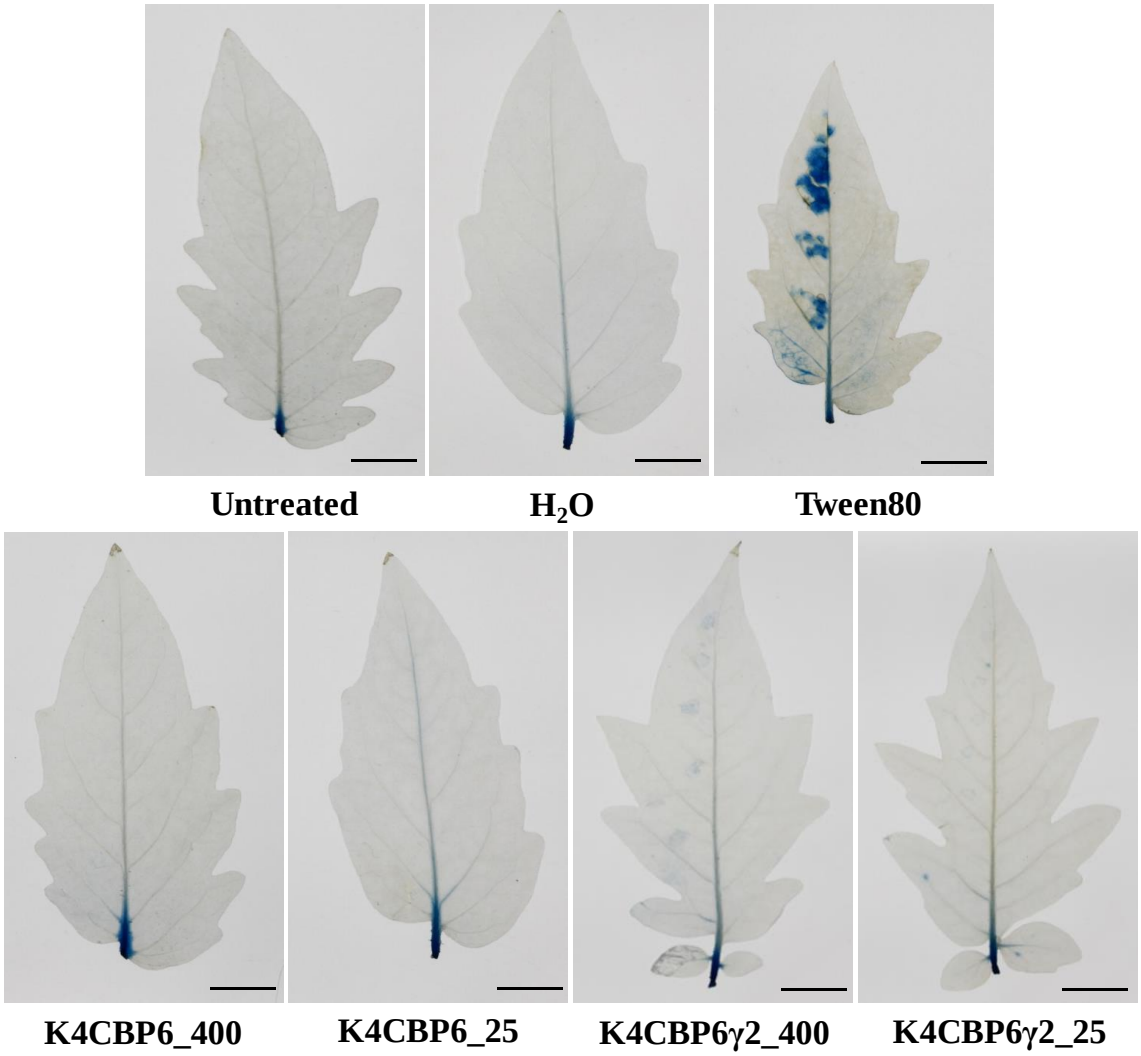


Figure 6. Evan's blue staining of *Solanum lycopersicum* L. cv. Moneymaker leaves to monitor cytotoxic effects of K4CBP6 and γ-core peptide derivative (K4CBP6γ2). The leaves were treated with 400 µg ml^{-1} and 25 µg ml^{-1} K4CBP6 (**K4CBP6_400** and **K4CBP6_25**), 400 µg ml^{-1} and 25 µg ml^{-1} K4CBP6γ2 (**K4CBP6γ2_400** and **K4CBP6γ2_25**) and the appearance of necrotic areas was compared with that of control leaves left **untreated** or treated with **H_2O** (negative control) or 10% **Tween80** (positive control). Blue-coloured zones indicated cell death at the treatment points. Scale bars represent 1 cm.

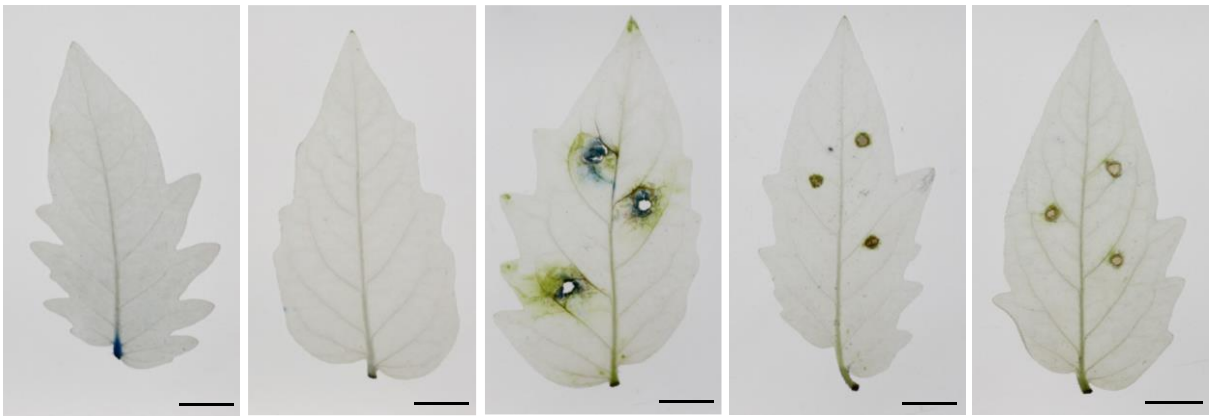
These results were partially published in the following conference proceedings.

Vass H, Váradi G, Kele Z, Rákhely G, Poór P, Tóth KG, Galgóczy L, Tóth L. Biocontrol ability of rationally designed peptide derivatives of a novel *Solanum lycopersicum* L. antifungal defensin. In: 16th European Conference on Fungal Genetics: Programme & Abstracts, Conference place and time: 05.03.2023.-08.03.2023., Innsbruck, Ausztria: Universität Innsbruck, pp. 478-479., 2023.

Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

Plant Protection Experiment

The plant protective ability of K4CBP6 and its γ-core peptide derivative was investigated against *B. cinerea* SZMC 21472 infection of the detached of the two tomato plant species (*S. lycopersicum* L. cv Ailsa, *S. lycopersicum* L. cv Moneymaker) leaves used in the plant toxicity test (**Figure 7**) [16, 17]. *B. cinerea* is known as necrotrophic pathogen of tomato plant. During the experiments 10-10 µl of control solutions, fungal suspension or mixture of protein and fungal suspension was dropped onto abaxial leaf epidermis in three points between the lateral veins. The untreated leaves infected with *B. cinerea* (10^7 conidia ml^{-1}) showed extensive necrotic lesions and blue-coloured zones around the infection points, which indicated an established and extensive fungal infection and plant cell death (**Figure 7**). In contrast, the absence of intense blue-coloured zones and necrotic lesions around the inoculation points on the tomato leaves infected with *B. cinerea* and treated with K4CBP6 or K4CBP6γ2 at MIC (25 µg ml^{-1}) indicated that the tested defensin and peptide protected the leaves from fungal infection and the invasion of the fungus into the leaf tissue (**Figure 7**). The results were the same for the two investigated tomato species. Results of experiments with *S. lycopersicum* L. cv Moneymaker are shown in **Figure 7**.



Untreated **0.1 × PDB** **Bcin** **Bcin + K4CBP6** **Bcin + K4CBP6γ2**

Figure 7. Evan's blue staining of necrotic plant tissue on *Solanum lycopersicum* L. cv Moneymaker leaves after *Botrytis cinerea* SZMC 21472 infection in comparison with the **untreated** control. Leaves were treated with 10 µl **0.1 × PDB**, *B. cinerea* (10^7 conidia ml^{-1}) (**Bcin**), *B. cinerea* (10^7 conidia ml^{-1}) + K4CBP6 (25 µg ml^{-1}) (**Bcin + K4CBP6**), *B. cinerea* (10^7 conidia ml^{-1}) + K4CBP6γ2 (25 µg ml^{-1}) (**Bcin + K4CBP6γ2**). Blue-colored zones indicate cell death at the treatment points. Scale bars represent 1 cm.

These results were partially published in the following conference proceedings.

Vass H, Váradi G, Kele Z, Rákhely G, Poór P, Tóth KG, Galgóczy L, Tóth L. Biocontrol ability of rationally designed peptide derivatives of a novel *Solanum lycopersicum* L. antifungal defensin. In: 16th European

Conference on Fungal Genetics: Programme & Abstracts, Conference place and time: 05.03.2023.-08.03.2023., Innsbruck, Ausztria: Universität Innsbruck, pp. 478-479., 2023.

Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

Crop Protection Experiment

The crop protection ability of K4CBP6 and K4CBP6γ2 was studied on tomato fruits against *C. herbarum* FSU 1148, known as a postharvest spoilage of fresh fruits and vegetables [18] (**Figure 8**). Tomato fruits were stung in 3 mm depth at three points near the stalk. Then 10 µl of control solutions, fungal suspension or mixture of protein and fungal suspension were pipetted into the holes. The control treatments of K4CBP6 (25 µg ml^{-1}) and K4CBP6γ2 (12.5 µg ml^{-1}) did not cause any decay on the surface of the tomato fruits, similarly to treatment with 0.1 × PDB (**Figure 8**). In contrast, the spread of the fungus was observed on the surface at the sting points and deeper tissues of fruits when they were only infected with *C. herbarum* (10^7 conindia ml^{-1} diluted in 0.1 × PDB). In case of the fruits treated with mixture of protein and fungal suspension the application of K4CBP6 (25 µg ml^{-1}) and K4CBP6γ2 (12.5 µg ml^{-1}) inhibited the development of infection (**Figure 8**). No intensive fungal growth was observed on the surface or in deeper tissues of the tomato fruits (**Figure 8**). During the experiment, we used unwounded tomato fruits without infection and treatment as a natural decay controls (**Figure 8**).

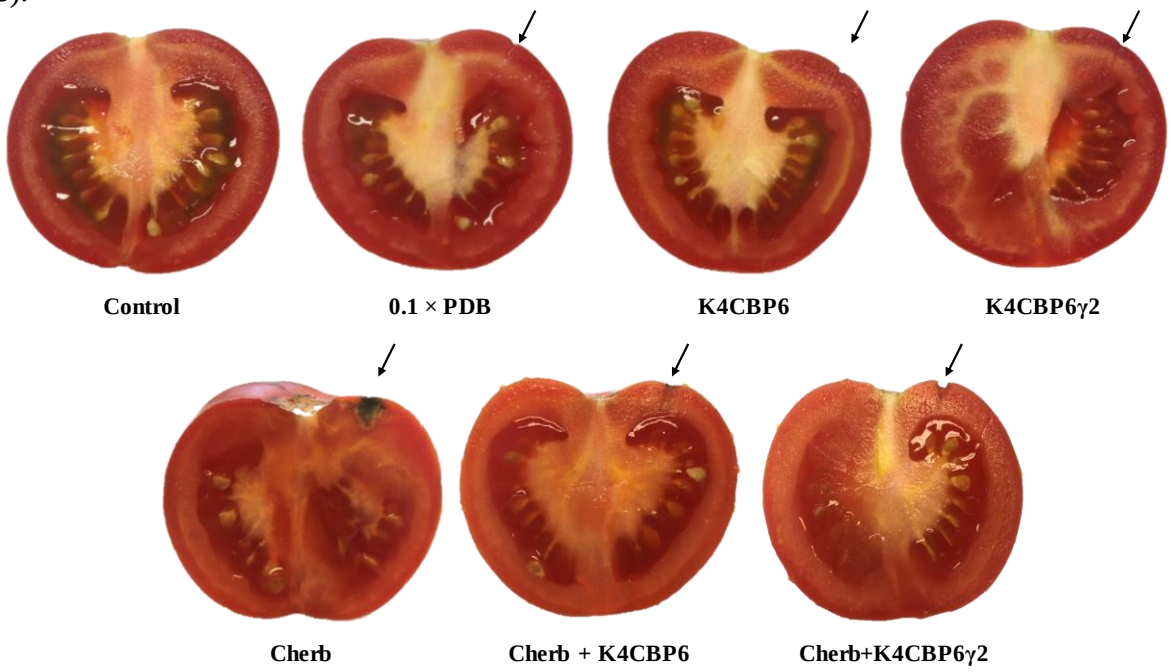


Figure 8. Investigation of the cytotoxicity and antifungal effect of K4CBP6 and K4CBP6γ2 of postharvest tomato fruits infected with *Cladosporium herbarum* FSU 1148 after incubation at 24°C for 7 days. The controls were uninfected but treated with **0.1 × PDB**, **K4CBP6**, and **K4CBP6γ2** or infected with *C. herbarum* FSU 1148 (10^7 conidia ml^{-1}) (**Cherb**) but without defensin or peptide treatment. Infected tomatoes were treated with 10 µl of mixture: *C. herbarum* FSU 1148 (10^7 conidia ml^{-1}) + 25 µg ml^{-1} K4CBP6 (**Cherb +K4CBP6**) or 12.5 µg ml^{-1} K4CBP6γ2 (**Cherb + K4CBPγ6**). Unwounded tomato fruits without infection and treatment were used as natural decay **control**s. The sites of the infections and treatments are indicated with black arrow.

These results were partially published in the following conference proceeding.

Tóth L, Papp R, Bende G, Poór P, Czékus Z, Váradi G, Kele Z, Tóth KG, Galgóczy L. A *Solanum lycopersicum* eredetű K4CBP6 defenzin antifungális hatásának és növényvédelmi alkalmazhatóságának vizsgálata / Investigation of the antifungal effect and plant protection applicability of the K4CBP6 defensin from *Solanum lycopersicum*. In: Dima Bálint; Papp Viktor (szerk.) VII. Magyar Mikológiai Konferencia. Conference place and time: Budapest, Hungary 05.06.2024.-07.06.2024., Budapest: Magyar Agrár- és Élettudományi Egyetem, Magyar Mikológiai Társaság, pp. 34-35., 2024.

7) Investigation of the environmental stability of *Solanum lycopersicum* L. defensin

For efficient agricultural application of antifungal proteins, it is essential that their structure and activity are not affected by environmental influences. Therefore, we investigated the antifungal efficacy of K4CBP6 after exposure to ultraviolet (UV) irradiation, different pH and temperatures [20, 21] (**Table 5**, **Figure 9**). To investigate the pH- and thermal tolerance of K4CBP6, the antifungal susceptibility tests were repeated against *B. cinerea* SZMC 21472, *C. herbarum* FSU 1148 and *F. oxysporum* SZMC 6237J in the 100-6.25 μg ml^{-1} concentration range of the defensin, but the 0.1 × PDB was prepared in sodium phosphate buffer (50 mM, pH 6.0-8.0), or the defensin solution was exposed to different temperatures (25°C, 50°C, 100°C) for 60 min [21]. To investigate the effect of UV irradiation on K4CBP6, the plates containing only protein dilutions were exposed to UV irradiation for 20 min with 25 cm under the germicide lamp of a laminar flow box [21]. Based on our results, the K4CBP6 maintained its antifungal activity after heat treatment at 100°C and UV irradiation, and its ability to inhibit the growth of applied filamentous fungi was not decreased significantly in comparison with the respective samples treated at 25°C (**Table 5, Figure 9 A, B**). In contrast, the 0.1 × PDB at pH 6-8 resulted in an increase in the MIC concentration of K4CBP6 against *B. cinerea* and *F. oxysporum* in comparison with the untreated growth control (**Table 5, Figure 9 C**), however it maintained their antifungal activity within pH 6–8 against *C. herbarum* without any significant loss of efficacy (**Table 5, Figure 9 C**). Based on all this, K4CBP6 maintains its antifungal effect at pH 6, while it shows reduced activity at pH 7-8.

These results were partially published in the following conference proceeding.

Papp R, Borics A, Merber R, Galgóczy L, Tóth L. Heterologous production of a *Solanum lycopersicum* L. antifungal defensin in *Pichia pastoris*. In: Benczúr Kinga; Gócza Elen; Pál Magda; Pusztahelyi Tünde (szerk) „FIBOK 2024": 6th National Conference of Young Biotechnologists, Conference place and time: Budapest, Hungary 04.04.2024.-05.04.2024., Budapest, Magyarország: MTA Agrártudományok Osztálya, Mezőgazdasági Biotechnológiai Tudományos Bizottság p. 30., 2024.

Experiments and results not included in the proposal but related to the project

During our experiments, we established that the **K4CBP6γ2** peptide derived from the γ-core region of K4CBP6 has antifungal effect against the investigated three important phytopathogens of the tomato plant (*B. cinerea*, *C. herbarum*, *F. oxysporum*), however, the antifungal activity of the aqueous peptide solutions decreased over time after several thaw-freeze cycles. This is because the cysteines in these peptides have free sulfhydryl groups, that allow cyclization and dimerization under oxidative conditions, thereby impairing antifungal efficacy. To overcome this problem, cysteine residues were substituted by serines or S-*tert*-butyl was applied as a cysteine-protecting group [22]. First, the two cysteines in the K4CBP6γ2 sequence were substituted with structural very similar serines (**K4CBP6γ2_Ser**), but do not contain a sulfhydryl group [22]. This modification helped maintain the structural integrity of K4CBP6γ2, but it slightly decreased the antifungal effect against *F. oxysporum* **Table 6**. Therefore, we used the other method to inhibit the cyclization and dimerization of the peptide by forming disulfide bonds between the cysteine residues [22]. Similar to the previous method, the S-*tert*-butylated K4CBP6γ2 (**K4CBP6γ2_StBu**) maintained its integrity as well as its antifungal efficiency (**Table 6**).

Table 5. *In vitro* minimal inhibitory concentrations of the K4CBP6 against three important phytopathogens of the tomato plant (*Botrytis cinerea*, *Cladosporium herbarum*, *Fusarium oxysporum*) after exposure to UV irradiation (25 min), different temperatures (25°C, 50°C, 100°C for 60 min) and pH (50 mM sodium phosphate buffer, pH 6.0-8.0) after incubation for 72 h at 24°C.

Sample/Isolate	*Botrytis cinerea* SZMC 21472	*Cladosporium herbarum* FSU 1148	*Fusarium oxysporum* SZMC 6237J
K4CBP6	25 µg ml^{-1}	25 µg ml^{-1}	25 µg ml^{-1}
K4CBP6_UV	25 µg ml^{-1}	25 µg ml^{-1}	25 µg ml^{-1}
K4CBP6_25°C	25 µg ml^{-1}	25 µg ml^{-1}	25 µg ml^{-1}
K4CBP6_50°C	25 µg ml^{-1}	25 µg ml^{-1}	25 µg ml^{-1}
K4CBP6_100°C	25 µg ml^{-1}	25 µg ml^{-1}	25 µg ml^{-1}
K4CBP6_pH=6	25 µg ml^{-1}	25 µg ml^{-1}	50 µg ml^{-1}
K4CBP6_pH=7	50 µg ml^{-1}	25 µg ml^{-1}	50 µg ml^{-1}
K4CBP6_pH=8	100 µg ml^{-1}	25 µg ml^{-1}	100 µg ml^{-1}

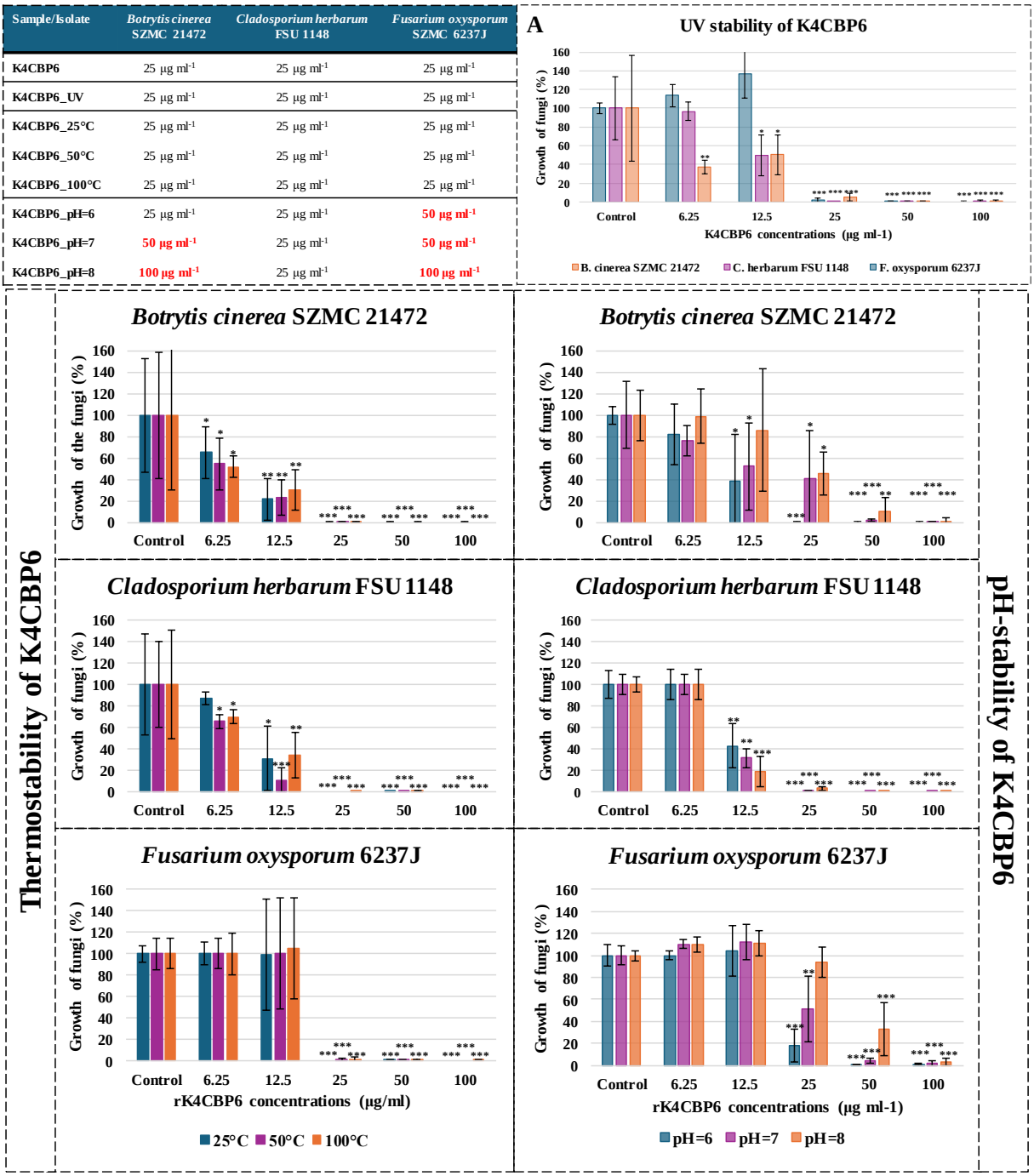


Figure 9. Antifungal activity of K4CBP6 against three important phytopathogens of the tomato plant (*Botrytis cinerea*, *Cladosporium herbarum*, *Fusarium oxysporum*) in broth microdilution test after exposure to UV radiation (25 min) (**A**), different temperatures (25°C, 50°C, 100°C for 60 min) (**B**) and pH (50 mM sodium phosphate buffer, pH 6.0-8.0) (**C**). The untreated control culture was referred to 100% growth. Significant differences (p values from unpaired t-test) were determined based on the comparison with the untreated control; *p≤0.05, **P≤0.005, ***P≤0.0001 in comparison with the dd.H_2O-treated sample.

Table 6. *In vitro* minimal inhibitory concentrations (**MIC**s) (µg ml^{-1}) of the **K4CBP6γ2**, the cysteine-serine substituted (**K4CBP6γ2_Ser**) and S-*tert*-butylated K4CBP6γ2 (**K4CBP6γ2_StBu**) before (**KM**) and after (**4°C**) oxidative exposure against the investigated three important phytopathogens of the tomato plantin 0.1 × PDB after incubation for 72 h at 24°C.

Peptides/Isolates	MIC		
	Botrytis cinerea SZMC 21472	*Cladosporium herbarum* FSU 1148	*Fusarium oxysporum* CBS 123668
K4CBP6γ2 KM	25 µg ml^{-1}	12.5 µg ml^{-1}	25 µg ml^{-1}
K4CBP6γ2 4°C	25 µg ml^{-1}	12.5 µg ml^{-1}	25 µg ml^{-1}
K4CBP6γ2_Ser KM	50 µg ml^{-1}	12.5 µg ml^{-1}	200 µg ml^{-1}
K4CBP6γ2_Ser 4°C	50 µgml^{-1}	12.5 µg ml^{-1}	200 µg ml^{-1}
K4CBP6γ2_StBu KM	50 µg ml^{-1}	12.5 µg ml^{-1}	12.5 µg ml^{-1}
K4CBP6γ2_StBu 4°C	50 µg ml^{-1}	12.5 µg ml^{-1}	12.5 µg ml^{-1}

Then, this peptide was also included in the toxicity and applicability experiments. We found that K4CBP6γ2_StBu does not have membrane disruption effect (**Figure 10A**) and does not cause the death of *G.mellonella* larvae (**Figure 10B**), however, as a result of the modification, the effect of the K4CBP6γ2 on germinating plants changes.

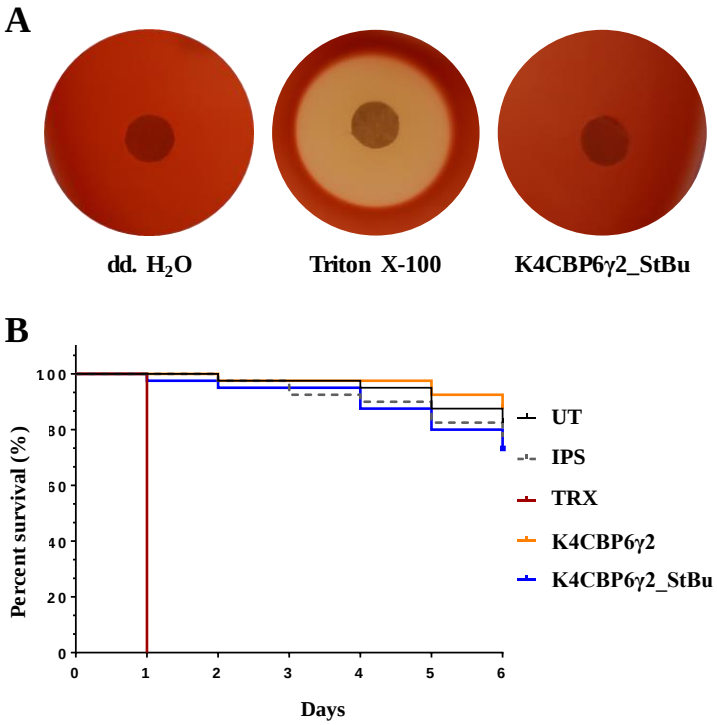


Figure 10. Investigation of haemolytic activity (**A**) and cytotoxicity (**B**) of the S-*tert*-butylated K4CBP6γ2 (**K4CBP6γ2_StBu**) (**A**) Haemolytic activity of K4CBP6γ2 in aqueous solution on Columbia blood agar plates after incubation for 24 h at 37°C. **Triton X-100** (20% (v/v)) and distilled water (**dd. H_2O**) were used as the positive and negative controls, respectively. (**B**) Survival of *Galleria mellonella* larvae after injection with the **K4CBP6γ2_StBu** (20 µl from 200 µg ml^{-1} solution) in comparison with the untreated control. UT: untreated control, IPS: insect physiological saline - treated control. *: p≤ 0.05 from both Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.

The peptide stimulates the growth of the primary root of *M. truncatula*, while reduced them of *S. lycopersicum* L. We also observed that K4CBP6γ2_StBu, applied at MIC, does not cause tissue damage on tomato plant leaves and does not have a cytotoxic effect on the fruit (**Figure 11**), but it inhibits the growth and spread of *B. cinerea* and *C. herbarum* in the infected areas (**Figure 11**). Based on these results, the synthesis of peptides with S-*tert*-butyl protected cysteine residues is a more suitable method to maintain the structural integrity, widen the antifungal spectrum and make them more effective in the short term.

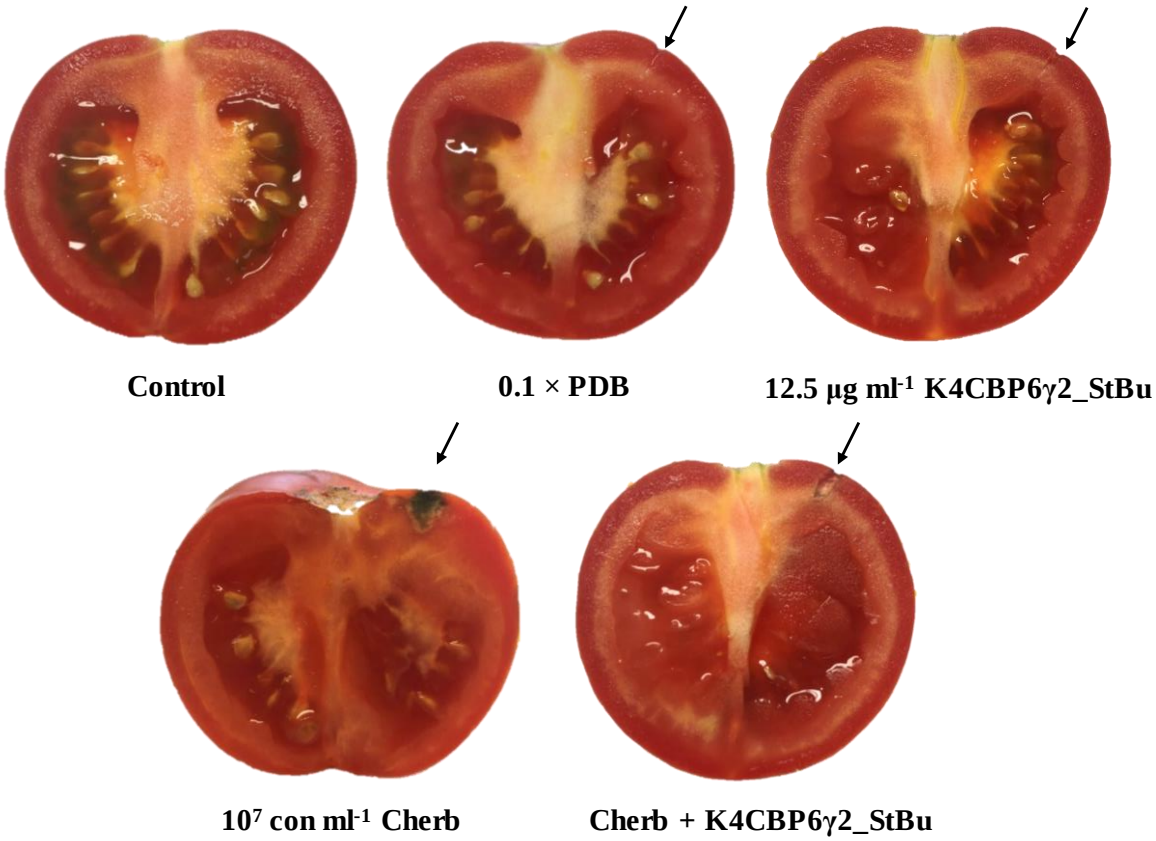


Figure 11. Investigation of the cytotoxicity and antifungal effect of the S-*tert*-butylated K4CBP6γ2 (**K4CBP6γ2_StBu**) of postharvest tomato fruits infected with *Cladosporium herbarum* FSU 1148 after incubation at 24°C for 7 days. The controls were uninfected but treated with **0.1 × PDB**, **K4CBP6γ2_StBu** or infected with *C. herbarum* FSU 1148 (10^7 conidia ml^{-1}) (**Cherb**) but without peptides treatment. Infected tomatoes were treated with 10 µl of mixture: *C. herbarum* FSU 1148 (10^7 conidia ml^{-1}) + 12.5 µg ml^{-1} K4CBP6γ2_StBu (**Cherb + K4CBPγ6_StBu**). Unwounded tomato fruits without infection and treatment were used as natural decay **control**s. The sites of the infections and treatments are indicated with black arrow.

Conclusions

Taken together, our results demonstrated that K4CBP6 and synthetic γ-core peptide derivatives (K4CBP6γ2) are promising plant protective and crop preservative biofungicides:

1) **They effectively inhibit the growth of several plant pathogenic filamentous fungi.**
2) **They do not have membrane disruption effect and not show cytotoxicity on *G. mellonella* larvae, seedlings and intact plants.**
3) **They are able to inhibit the spread of fungal infection on plant, and to protect stored crop against decay caused by postharvest moulds.**
4) **K4CBP6 retains its stability and antifungal activity at high temperatures, over a wide pH range and after UV irradiation.**

However, for their applicability, further experiments are necessary, which primarily focus on the investigation of their effects on the environment and the human body.

PhD student working on the project

Rebeka Papp (2023-2027) Expression, identification and characterization of *Solanum lycopersicum* L.-derived novel defensins University of Szeged, PhD School of Biology

MSc theses based on the project results

Rebeka Papp (2023) Expression of *Solanum lycopersicum* L.-derived novel defensin in *Pichia pastoris*-based expression system. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

Hilda Vass (2024) Applicability of a peptide designed on the second γ-core region of the K4CBP6 defensin from *Solanum lycopersicum* L. in plant protection. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

Zsófia Emese Oláh (2024) Investigation of S-tert-butyl modified γ-core peptide derivatives of tomato plant-derived K4CBP6 defensin. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

BSc theses based on the project result

Rebeka Papp (2021) Expression of *Solanum lycopersicum* L.-derived novel defensins in *Penicillium chrysogenum.* University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

Hilda Vass (2021) Antifungal activity of peptide derivatives designed on the γ-core region of Solanum lycopersicum L.-derived defensins. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

Zsófia Emese Oláh (2022) Effect of cysteine-serine substitution on the stability and antifungal efficacy of γ-core peptide derivatives of K4CBP6 defensin from tomato plant. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

Scientific Conference for Students thesis based on the project result

Rebeka Papp (2022, 2023) Production of a new defensin from *Solanum lycopersicum* L. in fungal-based expression systems. University of Szeged, Faculty of Science and Informatics, Department of Biotechnology

The local Scientific Conference for Students (TDK): 2022 - first place

The national TDK (OTDK): 2023 – second place

Project collaborators

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 – Györgyi Váradi and Zoltán Kele** (Department of Medical Chemistry, Albert Szent-Györgyi Medical School, University of Szeged, Szeged, Hungary): Electrospray ionization mass spectrometry, reversed-phase high performance liquid chromatography, solid-phase peptide synthesis. **Attila Borics** (Institute of Biochemistry, Biological Research Centre, Eötvös Loránd Research Network; Szeged, Hungary): Electronic circular dichroism spectroscopy. **Csaba Papp** (Department of Microbiology, Faculty of Science and Informatics, University of Szeged): fluorescence-activated cell sorting analysis.

Péter Poór and Zalán Czékus (Department of Plant Biology, Faculty of Science and Informatics, University of Szeged): They provided the tomato plants needed for the experiments.

The peer-reviewed paper published during the project:

In the following paper were published the bio-protective potential of antifungal proteins and their γ-core peptide derivatives was published. The publication presents the plant toxicity and protection experiments optimized for this project.

Tóth L, Poór P, Ördög A, Váradi G, Farkas A, Papp C, Bende G, Tóth KG, Rákhely G, Marx F, Galgóczy L. The combination of *Neosartorya* (*Aspergillus*) *fischeri* antifungal proteins with rationally designed γ-core peptide derivatives is effective for plant and crop protection. BIOCONTROL. 67 (2): pp. 249-262. (2022). IF2021: 2,581 (Q2 Agronomy and crop science)

Manuscript pending submission:

Papp R, Poór P, Czékus Z, Váradi G, Kele Z, Borics A, Bende G, Tóth KG, Galgóczy L, Tóth L. Heterologous expression and characterization of a novel antifungal defensin from *Solanum lycopersicum* L. Molecular plant pathology. IF2021: 5.520 (Q1) 2024.

The manuscript includes the following experiments:

1) Identification and chacterization of a novel defensin-like proteins from *Solanum lycopersicum* L, the K4CBP6; 2) Produce of K4CBP6 in *Pichia pastoris* heterologous expression system; 3) Investigation of structure and stability of K4CBP6 with RP-HPLC and ECD; 4) Investigating their antifungal effect against plant pathogenic filamentous fungi; 5) Investigating their toxicity (Toxicity test with *Galleria mellonella*, haemolytic potential on blood agar, toxicity tests with plants seedling); 6) Investigating their applicability as biofungicides; 7) Investigating their environmental stability

Further planned publication in the following period:

-Investigation of γ-core peptide derivatives of *Solanum lycopersicum* L. defensins

-Heterologous expression and characterization of B1N680 and K4BPB5 from *Solanum lycopersicum* L. (PhD projekt and paper)

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Liliána Tóth
Principal investigator