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Properties, Physiological Role and Possible Use in Biotechnology of Proteinase Inhibitor from Buckwheat Seeds

Yakov E. Dunaevsky[1] • Natalya V. Khadeeva[2] • Galina A. Belyakova[3] • Mikhail A. Belozersky[1*]

[1] A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia
[2] N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, 119991, Russia
[3] Faculty of Biology, Moscow State University, Moscow 119899, Russia

Corresponding author: * **mbeloz@belozersky.msu.ru**

ABSTRACT

Isolation and detailed investigation of protein proteinase inhibitor BWI-1a from buckwheat seeds has been carried out. The protein obtained was homogeneous and its physico-chemical, kinetic and biological properties were characterized. The molecular mass of BWI-1a inhibitor was 7.7 kDa and it consisted of 69 amino acid residues. Interaction of the studied inhibitor with a number of proteinases secreted by pathogenic filamentous fungi has been studied. The results obtained made it possible to propose that the inhibitor participated in the defense system of buckwheat seeds. Further complete amino acid sequence of BWI-1a inhibitor was established, the gene of the inhibitor was obtained, and genetic engineered constructions were created for transformation of some plants. Finally, transgenic potato and tobacco plants, containing BWI-1a inhibitor gene, were obtained, which demonstrated increased resistance to 2 bacterial phytopathogens *Pseudomonas syringae* pv. *tomatoe* and *Clavibacter michiganensis* sbsp. *michiganensis*. Biotest consisting in damaging of tobacco plants with white wings butterfly *Trialeurodes vaporariorum* in the greenhouse also demonstrated the existence of defense effect in transgenic tobacco plants. Thus, obtained results confirmed the proposal on the defense role of proteinase inhibitor BWI-1a and point to the possibility of its use for production of transgenic plants with increased resistance to phytopathogens and insect pests.

Keywords: BWI-1a, buckwheat seeds, proteinase inhibitor, plant, proteolytic enzymes, transgenic
Abbreviations: BWI, proteinase inhibitor from buckwheat seeds; ***ISP***, gene of serine proteinase inhibitor BWI-1a from buckwheat seeds; **FPLC**, fast performance liquid chromatography

CONTENTS

INTRODUCTION

Two groups of protein inhibitors of proteolytic enzymes capable of regulation of proteolytic activity of target enzymes are widely distributed in plant seeds and, in the seeds of buckwheat, in particular. The first group, lesser in content, involves inhibitors participating in regulation of intracellular metabolism. Inhibitor of endogenous metalloproteinase, initiating storage protein hydrolysis in buckwheat seeds can be referred to this group of proteinase inhibitors (Voskoboynikova *et al.* 1990). High content of the 2nd group of inhibitors in seeds, most attackable in view of absence of active defense system, their inability to inhibit endogenous proteolytic enzymes of seeds and high activity towards enzymes of animals and microorganisms point to their possible participation in defense of plant against destructive effect of secreted proteinases of the phytopathogens and digestive proteinases of the pest insects. Plants, similar to animals, are permanently subjected to attack of pathogens. However, in contrast to animals, plants do not form immune antibodies. Instead, plants produce antimicrobial proteins capable of destruction or inhibition of the growth of pathogen cells (Sotchenkov and Goldenkova 2003). These proteins may be both constitutive and inducible. Inhibitors of proteolytic enzymes refer to this group of proteins together with hydrolases, enzymes related to biosynthesis of phytoalexins and other antimicrobial proteins (Dunaevsky *et al.* 2005). However, wide qualitative diversity of protein proteinase inhibitors in the same biological object complicates understanding of the functional role of individual inhibitors composing the inhibitory complex.

Attempts to increase plant resistance to pathogenic microflora and insect pests by transformation of plants with genes of proteinase inhbitors have been made continually (Marchetti *et al*. 2000; Outchkurov *et al*. 2004; Abdeen *et al*. 2005; Charity *et al*. 2005; Vishnudasan *et al*. 2005). However, it should be taken into account that in the case of combined coexistence, pathogenic microorganisms and plant pests may adapt to life on the hosts containing proteinase inhibitors. In this case they are able either to synthesize new proteinases, insensitive to the exogenous inhibitor, which sometimes belong to a different class or family of proteolytic enzymes (for example, to cysteine instead of serine or chymotrypsin and elastase instead of trypsin)

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Invited Mini-Review

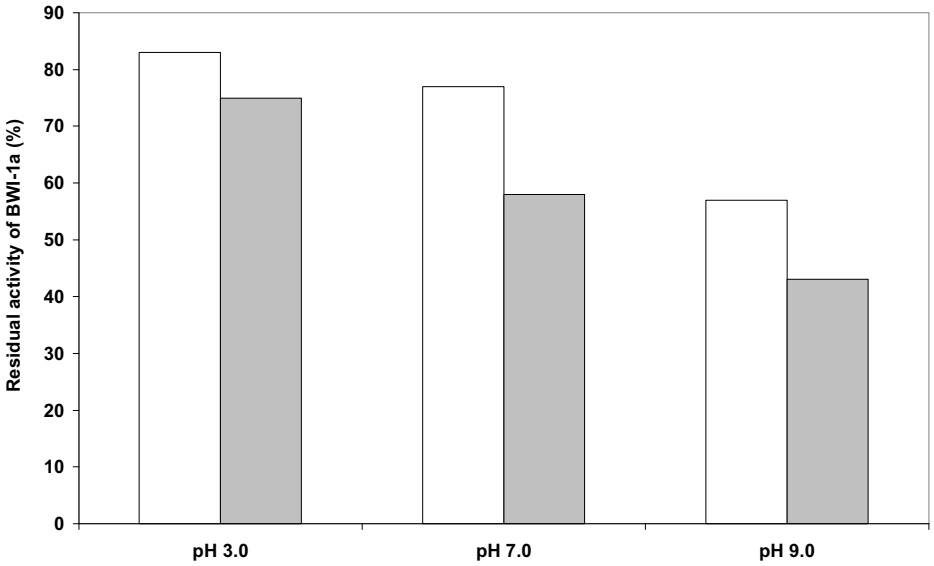

Fig. 1 Stability of buckwheat seed BWI-1a inhibitor at different pHs and 100°C. The inhibitor preparation in appropriate buffer were incubated in boiling water for 15 (□) and 30 (■) min. After that it was quickly cooled and the inhibitor activity measured.

```
          1        10        20        30        40   ↓    50        60
PI-IA     EFECDGKLQWPELIGVPTKLAKGIIEKQNSLISNVHILLNGSPVTMDFRCDRVRLFDDILGSVVEIPRVG
TI-I       LMCEGKQMWPELIGVPTKLAKEIIGKENPSIINIPILLSGSPITLDTLCDRVRLFDNILGFVVQMPVVT
ASI       ARECPGKQEWPELVGEYGYKAAAIIERENPNVRSIVKHER-SGFTKDFRCDRVWVVVDSTGVVVRTPRVT
BWI-1a    LRQCSGKQEWPELVGERGSKAAKIIENENEDVRAIV-LPEGSAVPRDLRCDRVWVFVDERGVVVDTPVVM
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Fig. 2 Amino acid sequences of BWI-1a proteinase inhibitor from buckwheat seeds and of some members of the potato proteinase inhibitor I family. PI-IA – potato tubers (Richardson and Cossins 1974), TI-I – tomato leaves (Graham *et al*. 1985), ASI – *Amaranthus caudatus L*. seeds (Valdes-Rodriguez *et al*. 1993). Conserved amino acid residues are boxed in grey. The arrow indicates position of P_1 amino acid residue at the reactive site.

(Cloutier *et al*. 2000; Bouchard *et al*. 2003) or to cleave inhibitors by other proteinases insensitive to the exogenous inhibitor (Mazumdar-Leighton and Broadway 2001; Zhu-Salzman *et al*. 2003). Because of this, a continuous search for new inhibitors from unrelated plants, capable of opposing such pathogen adaptation, is under way. In this respect, the proteinase inhibitor BWI-1a from buckwheat seeds may be rather promising, because on one side it quite efficiently inhibits activity of serine proteases that are the main proteolytic enzymes in many microorganisms and insect pests, and on the other side it exhibits biological activity inhibiting growth and germination of pathogenic microflora. The present work sums up our multiyear investigations of protein proteinase inhibitor BWI-1a from buckwheat seeds.

Physico-chemical and kinetic characteristics of BWI-1inhibitor from buckwheat seeds

BWI-1a inhibitor was extracted from dry buckwheat (*Fagopyrum esculentum* Moench) seeds by 0.1 M phosphate buffer, pH 6.8 (1:4, w/v) for 16 h at 5°C and was purified to homogeneity by a procedure developed in our laboratory including fractionation with ammonium sulfate, affinity chromatography on trypsin-Sepharose 4B, and FPLC procedure on Mono Q column, using a linear NaCl gradient (0-0.1 M, 1 ml/min, 25 min) (Dunaevsky *et al*. 1996).

This purification procedure produced electrophoretically homogeneous preparation of BWI-1a inhibitor. It should be noted that up to 40% of trypsin inhibitory activity present originally in the extract was not adsorbed under any conditions of affinity column and, possibly, belonged to non-protein compounds.

High pH stability is a characteristic property of proteinase inhibitors from higher plants, including buckwheat seed proteinase inhibitor BWI-1a. The stability of this inhibitor was somewhat higher at acidic pH values than at neutral or basic ones. Thus, after 8 h incubation the inhibitor retained 70% of activity at pH 2.0, 60% - at pH 8.0-11.0 and about 40% of trypsin inhibitory activity at pH 13. It was most stable at pH 5.0-6.0 where losses of activity comprised no more than 10% after prolonged storage.

Similar tendency was observed for thermal stability of BWI-1a inhibitor. At acidic pH values BWI-1a inhibitor exhibited highest thermal stability which almost did not depend on the incubation time (**Fig. 1**).

Amino acid analysis of BWI-1a inhibitor revealed the presence of two half-cysteine residues, which is typical for a certain group of seed proteinase inhibitors and the absence of histidine and tyrosine. Besides, studied inhibitor was characteristic of high content of valine and acidic amino acids (Dunaevsky *et al*. 1998).

The inhibitor preparation was further subjected to sequence analysis by automatic Edman degradation and mass spectrometry (Belozersky *et al.* 1995). As the result the complete amino acid sequence of BWI-1a proteinase inhibitor was established (**Fig. 2**). The M_r of the inhibitor (7746.2 Da) determined by mass spectrometry was in good coincidence with calculated value (7743.8 Da).

Analysis of amino acid sequence of the studied buckwheat seed proteinase inhibitor BWI-1a revealed high identity with the trypsin inhibitor from amaranthus seeds (ASI), tomato leaves (TI-I) and potato tubers (PI-IA) (**Fig. 2**) which are the members of the potato proteinase inhibitor I family. These data suggest that BWI-1a inhibitor from buckwheat seeds belonged to the potato proteinase inhibitor I family. Representatives of this family are characteristic of low content of cysteine residues (1 disulphide bond per protomer with molecular mass of 8 kDa). The reactive site of BWI-1a inhibitor proposed to be Arg45-Asp46 (**Fig. 2**) on the basis of its sequence homology to inhibitors of the potato proteinase inhibitor I family. The presence of Arg residue in the active center of BWI-1a was confirmed also by decrease in trypsin inhibitory activity after modification of Arg residues with diacetyl (2,3-butandione) (Smith 1977). Presence of Arg residue in P_1 position of reactive center of the inhibitor differentiated it from majority of representatives of this family containing in P_1 position Leu and Met residues.

Table 1 Effect of the proteinase inhibitor BWI-1a from buckwheat seeds on the activities of various proteolytic enzymes. [I], mg - amount of the inhibitor causing 50% enzyme inhibition.

Proteinases (1 μg)	BWI-1a [I], μg
Trypsin-like enzyme: *A. alternata*	0.15
F. oxysporum	0.29
Bovine trypsin	0.45
Crab trypsin	0.50
alfa-Chymotrypsin	2.30
Bacterial subtilisin	no inhibition
Subtilisin-like enzyme from *Maclura pomifera*	no inhibition
Elastase	no inhibition
Pepsin	no inhibition
Papain	no inhibition

The study of the effect of BWI-1a inhibitor from buckwheat seeds on the activity of various proteolytic enzymes revealed that this inhibitor suppressed not only activities of different trypsins (bovine, crab hepatopancreas) but chymotrypsin too, though less effectively (**Table 1**).

Kinetic characteristics of BWI-1a inhibitor have been also studied. It was established that inhibition constants of bovine trypsin, chymotyrpsin and cathepsin G from human granulocytes with BWI-1a inhibitor were found to be 1.1, 67 and 200 nM, respectively (**Table 2**). The inhibition constants of BWI-1a inhibitor for serine proteinases were compared with those of typical representatives of potato proteinase inhibitor I family such as potato PI-I, tomato TI-I and leech LIE. As it follows from Table PI-I, TI-I and LIE were 100-1000-fold more effective in binding α–chymotrypsin compared to trypsin, whereas BWI-1a inhibitor inhibited trypsin more strongly than chymotrypsin-like proteinases. The explanation for this fact may be found in the structure of the reactive sites of the inhibitors PI-I, TI-I and LIE, which contain a Leu residue at the P_1 position, making them more specific for α–chymotrypsin. On the other hand BWI-1a has an Arg residue in the P_1 position possessing a higher affinity for trypsin (Gladysheva *et al*. 1995).

There are 2 more representatives of potato proteinase inhibitor I family containing basic residue in the position P_1, though Lys but not Arg. These are trypsin inhibitors from pumpkin (Krishnamoorthi *et al*. 1990) and amaranth (Valdes-Rodriguez *et al*. 1993). However, comparative data on their affinity to trypsin and α-chymotrypsin are lacking.

Biological activity of BWI-1a inhibitor from buckwheat seeds

Further an investigation of the effect of BWI-1a inhibitor on the growth and development of the phytopathogenic fungi *Alternaria alternata* and *Fusarium oxysporum* has been studied. This investigation revealed that the growth of the mycelium of the both fungi was suppressed around the wells containing the inhibitor. Already on the second day of growth of the fungi, light areas were observed around the wells. In these areas the mycelium grew poorly, was scarce and spread over the surface of the medium. The size of the area around the wells where the growth of mycelium was suppressed decreased with decreasing concentration of BWI-1a (**Fig. 3**). In the absence of the inhibitor the growth of mycelium was not suppressed.

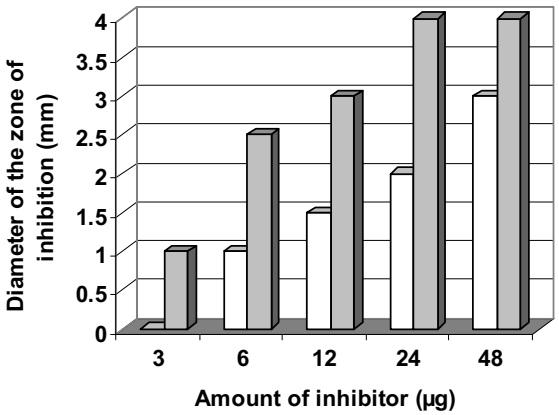

Fig. 3 Effect of BWI-1a inhibitor from buckwheat seeds on the growth of mycelium of the fungi *A. alternata* (□) and *F. oxysporum* (■).

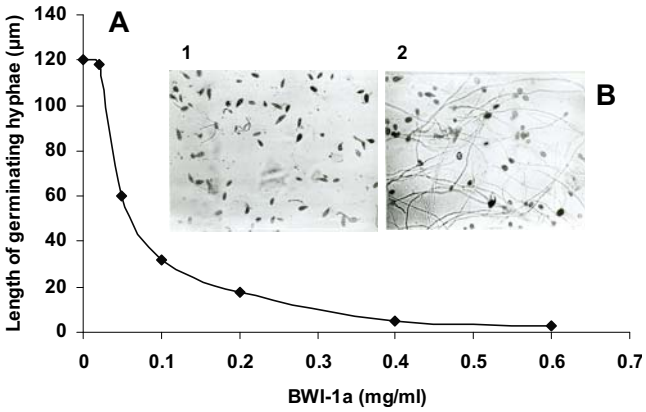

Fig. 4 Effect of BWI-1a inhibitor from buckwheat seeds on germination of spores and growth of hyphae of the fungus *A. alternata.* (**A**) The mean lengths of germinating hyphae of the fungus *A. alternata* as a function of the concentration of BWI-1a inhibitor from buckwheat seeds. (**B**) (1) Germinating hyphae of the fungus *A. alternata* in the presence of BWI-1a inhibitor (1 mg/ml); (2) in the absence of BWI-1a inhibitor. Modified from **Dunaevsky YE, Pavlukova EB, Belyakova GA, Belozersky MA** (1994) Anionic trypsin inhibitors from buckwheat seeds: isolation, specificity of action and effect on growth of micromycetes. *Biochemistry (Moscow)* **59**, 739-743, ©1994, with kind permission from Springer Science + Business Media.

Dependence of the germination of *A. alternata* spores on the concentration of BWI-1a inhibitor is demonstrated in **Fig. 4A, 4B**. At 1 mg/ml concentration of BWI-1a the ger-

Table 2 Inhibition constants of serine proteinases with inhibitors of potato proteinase inhibitor I family. BWI-1a – proteinase inhibitor from buckwheat seeds; TI-I – inhibitor I from tomato leaves; PI-I – inhibitor I from potato tubers; LIE – serine proteinase inhibitor from leech.

Inhibitor	Reactive site	Proteinase	Ki, M	Reference
BWI-1a	Arg-Asp	Trypsin	1.1×10^{-9}	Gladysheva *et al*. 1995
		alfa-Chymotrypsin	6.7×10^{-7}	
		HLE	no inhibition	
		Cathepsin G	2.0×10^{-7}	
TI-I	Leu-Asp	Trypsin	2.0×10^{-7}	Plunkett *et al*. 1982
		alfa-Chymotrypsin	1.0×10^{-9}	
PI-I	Leu-Asp	Trypsin	$>10^{-7}$	Kiyohara *et al*. 1973
		alfa-Chymotrypsin	5.0×10^{-10}	
LIE	Leu-Asp	Trypsin	10^{-9}-10^{-6}	Ascenzi *et al*. 1988
		alfa-Chymotrypsin	5.0×10^{-9}	Frigerio *et al*. 1992
		HLE	1.0×10^{-10}	Okada *et al*. 1989
		Cathepsin G	1.5×10^{-11}	

From **Dunaevsky YE, Pavlukova EB, Belyakova GA, Belozersky MA** (1994) Anionic trypsin inhibitors from buckwheat seeds: isolation, specificity of action and effect on growth of micromycetes. *Biochemistry (Moscow)* **59**, 739-743, ©1994, with kind permission from Springer Science + Business Media.

mination of fungi spores was completely suppressed (**Fig. 4B**1). Decrease in the concentration of BWI-1a caused an increase in the lengths of the growing hyphae. At 0.07 mg/ml concentration of the inhibitor these lengths were found to be 50% of the controls (**Fig. 4A**). Thus, the performed experiments demonstrated the ability of the BWI-1a inhibitor from buckwheat seeds to suppress the growth and development of mycelium and germination of spores of two pathogenic filamentous fungi (Dunaevsky *et al.* 1994).

Transformation of higher plants with the gene of BWI-1a inhibitor from buckwheat seeds

In order to examine the possibility of use the studied protein proteinase inhibitors for defense of plants against phytopathogens and insect pests transformation of plants with the gene of buckwheat seed anionic proteinase inhibitor BWI-1a has been performed (Khadeeva *et al*. 2009).

Analysis of presence and expression of the functional *ISP* gene in the cells of transformed potato and tobacco lines indicated that all transformed lines contained a common DNA fragment of $0.6.10^3$ bp corresponding to the fragment of gene construction. On the whole, predominance of single insertions was observed what corresponds with literature data on insertion of gene constructions in the course of T-DNA-mediated agrobacterial transformation.

Further Nothern blot analysis has been carried out for detection of mRNA transcript of the gene of proteinase inhibitor in obtained transgenic tobacco and potato lines as well as for determination of the level of gene expression. Fragment of vector construction of $1.2.10^3$ bp, containing the sequence of *ISP* gene, was used as the sound. DNA-sound of 1.8. 10^3 bp, containing α-tubulin TUA3 gene from arabidopsis was used as control in the course of Northern hybridization. mRNA transcript of expected value (213 bp) was detected in transgenic potato lines Res-24/1, Res-17/2, Res-18/12 and potato C22 and C20. **Fig. 5** demonstrates that expression of *ISP* gene was different for various potato and tobacco lines. mRNA amount was the highest in potato line Ре3-18/12, whereas potato lines Res-17/2, Res-24/1 and tobacco line C20 showed a lesser expression level of *ISP* gene. Almost no expression of ISP gene was found in tobacco C12 line, which lost tolerance to kanamycin. This may point either to elimination of the *ISP* gene or to gene silence.

Analysis of obtained transformed plants indicated that transformation frequency was increased when mixed cultivation and regeneration were performed on the cysteine containing medium (Olhoft and Somers 2001). It is noteworthy that the used cysteine concentration of 400 μg/ml inhibited growth of agrobacteria what increased the duration of sub-cultivation and decreased their number on media containing antibiotics. Possibly, it stimulated also formation of more regenerated plants.

Antibacterial activity of transgenic lines was determined using dish tests. Tissues of non-transformed control tobacco plant (C_{nt}) and that transformed by the *ISP* gene-free vector (C_o) practically did not inhibit growth of any used bacterium, whereas tissues of all analyzed transgenic plants exhibited antibacterial activity (**Table 3**).

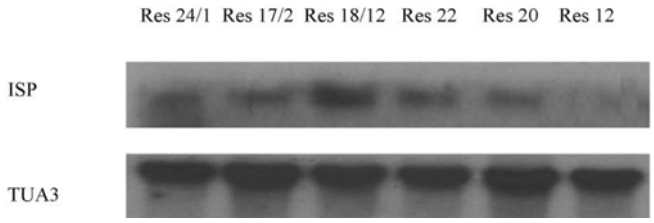

Fig. 5 Results of Nothern blot hybridization of transgenic potato lines (Res-24/1, Res-17/2 and Res-18/12) and tobacco lines (C20, C22 and C12). *ISP* – gene of serine proteinase inhibitor from buckwheat seeds, *TUA3* – gene of α-tubulin from arabidopsis, used as standard. From **Khadeeva NV, Kochieva EZ, Tcherednitchenko MY, Yakovleva EY, Sydoruk KV, Bogush VG, Dunaevsky YE, Belozersky MA** (2009) Use of buckwheat seed protease inhibitor gene for improvement of tobacco and potato plant resistance to biotic stress. *Biochemistry (Moscow)* **74**, 320-329, ©2009, with kind permission from Pleiades Publishing Ltd.

Table 3 Antibacterial activity of tissues of primary tobacco transformants.

Line	Mean diameter of the zone of bacteria growth inhibition including diameter of the well (cm)		
	Escherichia coli	*Pseudomonas syringae*	*Clavibacter michiganensis*
C_{nt}	0.5*	0.5*	0.5*
C_o	0.5*	0.5*	0.5*
C1	1.10±0.08	2.27±0.24	1.28±0.08
C2	0.71±0.04	1.63±0.13	1.23±0.11
C7	0.62±0.02	1.82±0.21	1.20±0.07
C8	0.80±0.03	2.09±0.18	1.25±0.13
C10	1.30±0.24	1.92±0.17	1.26±0.11
C11	0.85±0.11	1.98±0.19	1.24±0.09
C12	0.5*	0.5*	0.5*
C15	1.00±0.15	1.81±0.22	1.32±0.13
C16	1.35±0.22	1.84±0.12	1.21±0.07
C18	1.02±0.41	1.77±0.16	1.56±0.21
C19	0.71±0.11	1.78±0.26	1.18±0.08
C20	1.11±0.27	1.92±0.15	1.43±0.16
C22	0.63±0.08	2.18±0.19	1.63±0.11
C37	1.00±0.09	1.80±0.18	1.15±0.08
C41	1.21±0.14	1.76±0.14	1.31±0.14
C44	0.92±0.10	1.92±0.23	1.52±0.13
C47	1.31±0.15	2.01±0.19	1.42±0.09

* diameter of the well - 0.5 cm

From **Khadeeva NV, Kochieva EZ, Tcherednitchenko MY, Yakovleva EY, Sydoruk KV, Bogush VG, Dunaevsky YE, Belozersky MA** (2009) Use of buckwheat seed protease inhibitor gene for improvement of tobacco and potato plant resistance to biotic stress. *Biochemistry (Moscow)* **74**, 320-329, ©2009, with kind permission from Pleiades Publishing Ltd.

Evidently, transformants synthesized a target functional protein exhibiting inhibitory effect on bacterial growth. However, significant differences were detected in the extent of inhibition of different bacterial species. It should be noted that selection of bacterial strains of the tomato bacterial mottling pathogen *Pseudomonas syringae* pv. *tomato*, and the tomato bacterial cancer pathogen *Clavibacter michiganensis* subsp. *Michiganensis* is explained by the fact that they secrete serine proteinases during their vital activities (Jang *et al*. 1996; Burger *et al*. 2005). Transgenic clones inhibited growth of *P. syringae* and to somewhat lower extent growth of *C. michiganensis*. Besides, secondary growth of separate *C. michiganensis* colonies was always observed in the inhibition zone. To the least extent transgenic clones inhibited growth of *E. coli*; in this case even not all transgenic lines exhibited inhibitory effect. It should be noted that tissues of C12 line, that lost their resistance to kanamycin after passage for 2 years, also lost the ability to inhibit bacterial growth (**Fig. 6, Table 3**).

Tissues of transgenic lines of different species highly differed in the extent of antibacterial activity. Thus, wells containing tissue homogenates of transgenic tobacco plants on the lawn of *E. coli* model culture were surrounded by a halo of growth inhibition of 1.5-1.7 cm in diameter (including the well diameter). In potato, protective effect was much more pronounced in the case of application of the same amount of homogenized tissue, and the halo diameter reached 3-5 cm (**Fig. 7**). Probably, so pronounced difference in the growth inhibition zone dimensions is caused by individual biological peculiarities of each plant, and possibly by different copy numbers of vector constructs inserted into their genomes.

Exposure of tobacco plants by the glasshouse whiteflies (*Trialeurodes vaporariorum*) observed in the greenhouse has shown that transgenic plants were resistant to the effect of these insects, whereas non-transformed ones were strongly affected by them. Numerous eggs were laid by the whitefly on the control plant leaves and full-value insects

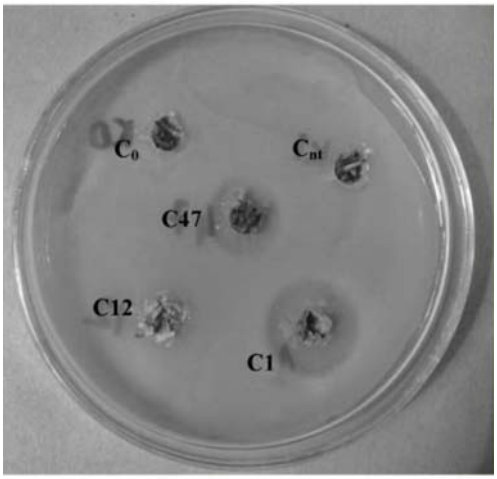

Fig. 6 Antimicrobial activity of tobacco plant tissue homogenates towards *Pseudomonas syringae*. C_{nt}, non-transformed tobacco plant; C_0, a plant transformed by a vector free of *ISP* gene; C1, C12, and C47, transgenic lines (C12 lost kanamycin resistance and antibacterial activity). From **Khadeeva NV, Kochieva EZ, Tcherednitchenko MY, Yakovleva EY, Sydoruk KV, Bogush VG, Dunaevsky YE, Belozersky MA** (2009) Use of buckwheat seed protease inhibitor gene for improvement of tobacco and potato plant resistance to biotic stress. *Biochemistry (Moscow)* **74**, 320-329, ©2009, with kind permission from Pleiades Publishing Ltd.

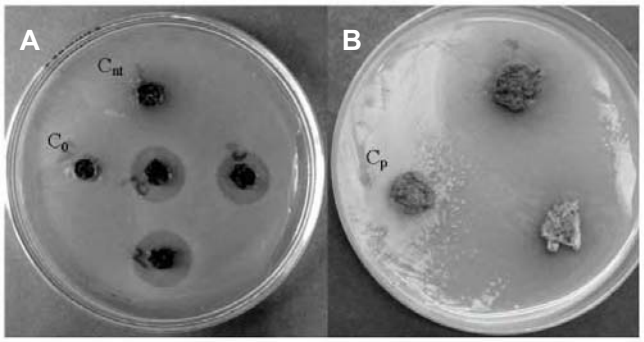

Fig. 7 Antibacterial activity of tobacco (A) and potato (B) tissues towards *E. coli*. C_t and C_p, -non-transformed tobacco and potato plants, respectively. From **Khadeeva NV, Kochieva EZ, Tcherednitchenko MY, Yakovleva EY, Sydoruk KV, Bogush VG, Dunaevsky YE, Belozersky MA** (2009) Use of buckwheat seed protease inhibitor gene for improvement of tobacco and potato plant resistance to biotic stress. *Biochemistry (Moscow)* **74**, 320-329, ©2009, with kind permission from Pleiades Publishing Ltd.

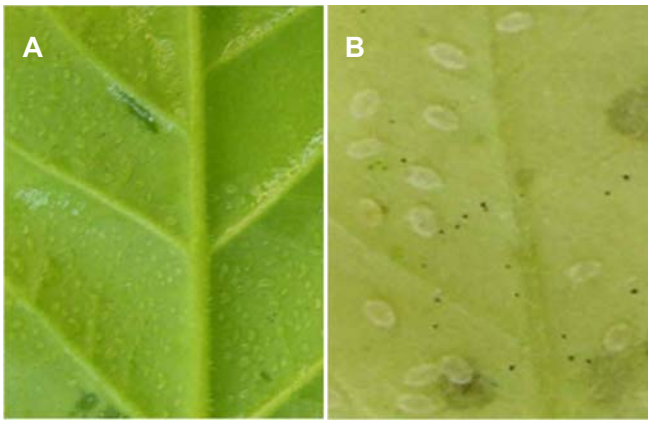

Fig. 8 The absence of egg laying by the whitefly on transgenic tobacco plant (A), and affection of the control tobacco plant (B) in the greenhouse. From **Khadeeva NV, Kochieva EZ, Tcherednitchenko MY, Yakovleva EY, Sydoruk KV, Bogush VG, Dunaevsky YE, Belozersky MA** (2009) Use of buckwheat seed protease inhibitor gene for improvement of tobacco and potato plant resistance to biotic stress. *Biochemistry (Moscow)* **74**, 320-329, ©2009, with kind permission from Pleiades Publishing Ltd.

emerged from these eggs (**Fig. 8B**). Only single oviposition of 1-2 eggs were observed on transgenic plants, and no progeny appeared in this case (**Fig. 8A**).

It should be noted that unlike a number of different insects, serine proteinases are prevalent in the whitefly alimentary canal (Terra and Ferreira 1994). Thus, introduction of just a single gene of serine proteinase inhibitor into the plants of heterologous group has shown the possibility of obtaining protective effects against insects and phytopathogenic bacteria.

The results indicate that, unlike control plants, those grown by us are able to synthesize certain functional proteins that exhibit protective effect and inhibit development of bacteria. It appeared that the presence of just a single gene of serine proteinase inhibitor provides for sufficient protection against at least two phytopathogenic bacteria. It is quite important to note that to a higher extent transgenic clones inhibited growth of phytopathogenic bacteria *P. syringae* and *C. michiganensis* and to a significantly lower extent growth of model culture *E. coli*, which is probably due to the specificity of proteolytic enzymes of different bacterial species. In addition, significant genotypic distinctions in protection efficiency were observed between members of different genera of the same family (potato and tobacco), which requires further investigation for final clarification of this question. The use of tissue homogenates of the same transgenic tobacco plants for inhibition of growth of phytopathogenic fungi *A. alternata* and *F. culmorum*, secreting serine proteinases into the culture medium (Dunaevsky *et al.* 2008), did not give a significant effect. which might be due to insufficient amount of this gene product for inhibition of these fungal proteinase activities. At the same time, experiments on potato transgenic lines carrying the *ISP* gene of buckwheat proteinase inhibitor revealed fungicidal effect on *Fusarium sp*., resulted in cessation of fungus growth at a distance >5 mm from the transgenic line explants whereas control (non-transformed) variants were completely overgrown (Tcherednitchenko 2004). Note that the purified preparation of this proteinase inhibitor at concentrations of 0.05-0.5 μg/ml inhibited by 50-100% growth of hyphae of the mycelial fungus *A. alternata* (Dunaevsky *et al.* 1998).

Thus, the possibility of involvement of the recombinant plant inhibitor of proteolytic enzymes in protection of various plants against pathogenic microorganisms and insects is shown in this work. In connection with recent achievements of biotechnology in creation of genetically modified plants characterized by increased resistance to different unfavorable effects, such approach becomes more and more actual because it not only enables the increase in productivity of cultured plants but contributes to improvement of ecological conditions due to reduced usage of highly toxic protective chemicals.

The observed differences in the extent of growth inhibition of different bacterial species are also of undoubted interest for further investigation of possible mechanisms of antibacterial activity of transgenic lines expressing this inhibitor.

FUTURE PERSPECTIVES

In the near future we plan to use the buckwheat BWI-1a inhibitor gene for transformation of *Arabidopsis thaliana* plants in order to check the change in their resistance to pathogens after transformation. We also plan to use the BWI-1a inhibitor gene in combination with genes of other buckwheat seed proteinase inhibitors as well as with genes of some antifungal proteins and peptides for improving plant resistance to pathogens and insects.

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