Selivanovskij Rusak (a local spring bread wheat cultivar from the Volga region), and Beloturka (a local durum cultivar from the Volga region).

Resistance also was studied to leaf and stem rust and powdery mildew in spring bread wheat. Lines selected carrying alien genes that would ensure total resistance to leaf and stem rust and powdery mildew were L2166 and L784/03; for resistance to leaf and stem rust was L2075; for resistance to leaf rust and powdery mildew were Мульти 6R, L2505, L1059, L484/03, and L487/03; for resistance to leaf rust were L1078, L2608, and L2870; and for resistance to powdery mildew was L2032. The donors of resistance to these diseases are *Ae. speltoides, S. cereale, Th. intermedium,* and *T. turgidum subsps. durum* and *dicoccoides*.

VAVILOV INSTITUTE OF GENERAL GENETICS, RUSSIAN ACADEMY OF SCIENCES
Gubkin str. 3, 119991 Moscow, Russian Federation.

SHEMYAKIN AND OVCHINNIKOV INSTITUTE OF BIOORGANIC CHEMISTRY, RUSSIAN ACADEMY OF SCIENCES
Ul. Miklukho-Maklaya 16/10, Moscow, Russian Federation.

Novel antimicrobial peptides from seeds of Triticum kiharae and Leymus arenarius.

L.L. Utkina, A.A. Slavokhotova, T.I. Odintsova, T.V. Korostyleva, and V.A. Pukhalskiy (Vavilov Institute of General Genetics) and A.K. Musolyamov and T.A. Egorov (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry).

To protect themselves against pathogens, plants produce a wide array of antimicrobial proteins and peptides (AMPs), some of which are synthesized constitutively, whereas others are induced upon challenge with pathogenic microorganisms (Selitrennikoff 2001; Garcia-Olmedo et al. 2001). Each plant genome encodes hundreds AMPs (Manners 2007). Such biodiversity ensures efficient defense against numerous invading and constantly evolving microorganisms. Most plant AMPs belong to cysteine-rich peptides and contain an even number of cysteine residues, all of which are involved in the formation of intrachain disulphide bridges providing their molecules with high structural stability. Based on cysteine spacing motifs and three-dimensional structures several families of antimicrobial peptides have been discriminated in plants (Broekaert 1997). Hevein-type peptides show structural similarity to the 43-amino-acid residue chitin-binding peptide isolated from the rubber tree *Hevea brasiliensis* L. (Van Parijs et al. 1991) and comprise the single-hevein-domain subfamily in a large group of chitin-binding proteins implicated in plant defense (Raikhel and Lee 1993). Despite sequence similarity, hevein-type AMPs differ in the number of disulphide bonds. Most of them possess 8 cysteine residues forming 4 disulphide bonds and in this respect are close to the chitin-binding domains of class I/IV chitinases (Beintema 1994). Truncated variants with only six cysteine residues also occur (Broekaert et al. 1992). AMPs are regarded as promising agents for plant transformation and production of resistant crops, therefore the search for new, highly potent AMPs is a rapidly developing area of research.

We focused on AMPs from seeds of two Poaceae species, *Leymus arenarius* and *Triticum kiharae*. In contrast to *T. kiharae*, *L. arenarius* grows in a narrow shore region of the White Sea at high soil salinity. We show that both species possess highly homologous hevein-type peptides of unusual structure, which effectively inhibits growth of a wide range of plant pathogens at micromolar concentrations.

Materials and methods. The species used in this study were *T. kiharae* Dorof. et Migush. and *L. arenarius*; the fungi and bacteria *Fusarium solani* VKM F-142, *F. verticillioides* VKM F-670, *F. oxysporum* TSA-4, *Botrytis cinerea* VKM F-85, *Neurospora crassa* VKM F-184, *Pseudomonas syringae* VKM B-1546, *Clavibacter michiganense* subsp. *michiganense* VKM Ac-1144, and *Erwinia carotovora* subsp. *carotovora* VKM B-1247 were obtained from the All-Russian Collection of Microorganisms.

Flour was extracted with 10% acetic acid for 1 h at room temperature and desalted on an Aquapore RP300 column. Freeze-dried acidic extract was subjected to chromatography on Heparin Sepharose. Proteins and peptides were

eluted with a stepwise NaCl gradient. The 100-mM NaCl fraction was collected, desalted as described above, and separated on a Superdex Peptide HR 10/30 column (Amersham, Pharmacia, Biotech, Uppsala, Sweden). Proteins and peptides were eluted with 0.05% TFA, containing 5% acetonitrile, at a flow rate of 250 µl/min and monitored by absorbance at 214 nm. The peptide fraction was further separated by RP-HPLC on a Vydac C18 column (4.6 x 250 mm, particle size 5 µm) with a linear acetonitrile gradient (10-50%) for 1 h at a flow rate of 1 mL/min and 40°C. Peptides were detected at 214 nm. Mass spectra were acquired on a model Reflex III mass spectrometer (Bruker Daltonics, Bremen, Germany). N-terminal amino acid sequences were determined by automated Edman degradation on a model 492 Procise sequencer (Applied Biosystems) according to the manufacturer's protocol.

The antifungal activity of the peptides was tested against several fungi using microtiter-plate assays. Wells were filled with 10 µl of two-fold serial dilutions of the peptide and mixed with 90 µl half-strength potato-glucose broth containing approximately 10^4 spores/mL. The inhibition of spore germination was evaluated by measuring the absorbance at 620 nm. The antibacterial activity of peptides was assayed against several Gram-positive and Gram-negative bacteria using radial diffusion assay. Petri dishes with LB agar were seeded with test bacteria. The peptide solutions (50 µl) were applied to the wells (5 mm in diameter) punched into the agar, and the Petri dishes were incubated at room temperature for 24-48 h. Antibacterial activity was evaluated by the size of the inhibition zone formed around the wells with the peptide solution. The antibiotic claforan and sterile water were used as controls.

Results and discussion. For the isolation of AMPs from *T. kiharae* and *L. arenarius*, we followed the procedure developed for the isolation of *T. kiharae* defensins (Egorov et al. 2005; Odintsova et al. 2006), which included acidic extraction of flour followed by subsequent separation of the protein-peptide extract by a combination of different types of HPLC (affinity, size-exclusion and reversed-phase). As a result, two novel peptides named WAMP and LAMP were isolated from seeds of *T. kiharae* and *L. arenarius*, respectively. The measured monoisotopic molecular masses of the peptides were 44,31 and 4,444 Da for WAMP and LAMP, respectively. Their amino acid sequences were determined by automated Edman degradation after reduction and alkylation.

Considerable sequence similarity with hevein and homologous peptides was revealed providing evidence that both peptides belong to hevein-type AMPs. However, in contrast to hevein, they possess ten cysteine residues and, thus, may be classified as 10-Cys hevein-like peptides. Only two 10-Cys hevein-like peptides have been described so far, isolated from the bark of the trees *Eucommia ulmoides* Oliv. (Huang et al. 2002) and *Euonymus europaeus* L. (Van den Bergh et al. 2002). Despite similarity in the number of cysteine residues, the cysteine motif in WAMP and LAMP differs remarkably from that of their 10-Cys homologues indicating that isolated peptides belong to a new subfamily of hevein-type peptides. Striking similarity with hevein-type domains of cereal class-I chitinases both in amino acid sequences and cysteine patterns was noticed.

Thiol-specific alkylation of unreduced native WAMP and LAMP peptides did not result in molecular mass changes pointing to the involvement of all 10 SH-groups in the formation of 5 disulphide bridges in each peptide. The measured molecular masses of the peptides were in good agreement with calculated values indicating the absence of post-translational modifications except disulphide bridges. Based on sequence similarity with hevein, for which the cysteine connectivities are known, disulphide bridges in WAMP were predicted as follows: C^4–C^{19}, C^1–C^{25}, C^{18}–C^{32}, C^{37}–C^{41}. An additional fifth disulphide bond is likely to be formed between C^{16} and C^{44}. Because chitin is the main component of fungal cell walls and exoskeleton of insects, chitin-binding activity is assumed to be indicative of the ability of polypeptides to inhibit growth of phytopathogenic fungi or pests. The chitin-binding properties of WAMP and LAMP peptides were assayed *in vitro* . Purified peptides were applied to a chitin column and the bound fraction was eluted with 0.1% TFA. RP-HPLC and mass measurements of unbound and bound fractions showed that both peptides eluted only in the bound fraction thus providing evidence that they bind to chitin. Thus both peptides WAMP and LAMP bind chitin. The inhibitory activity of both peptides towards several pathogens was assayed directly. The results for WAMP are presented in Table 1.

Table 1. Antifungal activity of the WAMP peptide (IC_{50} is the concentration necessary for 50% growth inhibition).

Fungus	IC_{50} (µg/ml)
Fusarium solani	5
Fusarium verticillioides	30
Fusarium oxysporum	15
Botrytis cinerea	20
Neurospora crassa	10

Testing of the biological activity of the recombinant peptide WAMP against several fungi including deiteromycetes and ascomycetes showed marked inhibition of spore germination at micromolar concentrations with an IC_{50} ranging from 5 to 30 µM depending on the fungus (Table 1). The highest inhibitory activity was achieved against *F. solani*; the

IC_{50} for this fungus was 5 µM. The WAMP peptide was also tested for inhibition of bacterial growth against both Gram-positive (*C. michiganense*) and Gram-negative bacteria (*P. syringae* and *E. carotovora*); for the Gram-positive bacterium *C. michiganense* the effect was most pronounced (Table 2). The antifungal activity of WAMP is likely to be associated with its chitin-binding activity, whereas the inhibitory effect on bacteria, which are devoid of chitin, implies the existence of some other mechanism.

Table 2. Antibacterial activity of the WAMP peptide.

Peptide concentration (µg/50 µl)*	Inhibition zone in cm including the size of the peptide application zone**		
	P. syringae	*E. carotovora*	*C. michiganense*
10	1.3 (1.4)	1.5 (3.4)	1.7 (3.6)
5	1.2 (1.2)	1.3 (2.7)	1.5 (3.2)
2.5	0.9 (1.0)	1.1 (1.1)	1.3 (3.0)

* Sample volume was 50 µl.
** Size of the peptide application zone was 0.5 cm. The size of the inhibition zone caused by claforan is shown in parentheses.

In summary, two novel, highly homologous, hevein-type and chitin-binding AMPs, WAMP and LAMP, which share sequence similarity with chitin-binding domains of cereal class-I chitinases, were purified from *T. kiharae* and *L. arenarius* seeds. To the best of our knowledge, this is the first report on the occurrence of 10-Cys hevein-type peptides in plant seeds. The cysteine motif in WAMP and LAMP is new and distinct from those of other previously characterized hevein-type AMPs providing evidence that they belong to a new structural type of AMPs. The peptides showed potent antifungal and antibacterial activities at micromolar concentrations and, thus, may be used in genetic transformation of plants to enhance resistance to pathogenic microorganisms.

References.

Beintema JJ. 1994. Structural features of plant chitinases and chitin-binding proteins. FEBS Lett 350:159-163.

Broekaert WF, Mariën W, Terras FRG, De Bolle MFC, Proost P, Van Damme J, Dillen L, Claeys M, and Rees SB. 1992. Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine-glycine-rich domain of chitin-binding proteins. Biochemistry 31:4308-4314.

Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K, De Samblanx GW, and Osborn RW. 1997. Antimicrobial peptides from plants. Crit Rev Plant Sci 16:297-323.

Egorov TA, Odintsova TI, Pukhalsky VA, and Grishin EV. 2005. Diversity of wheat antimicrobial peptides. Peptides 26:2064-2073.

García-Olmedo F, Rodríguez-Palenzuela P, Molina A, Alamillo JM, López-Solanilla E, Berrocal-Lobo M, and Poza-Carrión C. 2001. Antibiotic activities of peptides, hydrogen peroxide and peroxynitrite in plant defence. FEBS Lett 498:219-222.

Huang R-H, Xiang Y, Liu X-Z, Zhang Y, Hu Z, and Wang D-C. 2002. Two novel antifungal peptides distinct with a five-disulfide motif from the bark of *Eucommia ulmoides* Oliv. FEBS Lett 521:87-90.

Manners JM. 2007. Hidden weapons of microbial destruction in plant genomes. Genome Biol 8:225-228.

Odintsova TI, Egorov TsA, Musolyamov AKh, et al. 2007. Seed defensins from *T. kiharae* and related species: genome localization of defensin-encoding genes. Biochimie 89:605-612.

Raikhel NV and Lee H-I. 1993. Structure and function of chitin-binding proteins. Ann Rev Plant Physiol Plant Mol Biol 44:591-615.

Selitrennikoff PC. 2001. Antifungal proteins. Appl Environ Microbiol 67:2883-2894.

Van Parijs J, Broekaert WF, Goldstein I.J, and Peumans WJ. 1991. Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta 183:258-264.

Van den Bergh KPB, Proost P, Van Damme J, Coosemans J, Van Damme EJM, and Peumans WJ. 2002. Five disulfide bridges stabilize a hevein-type antimicrobial peptide from the bark of spindle tree (*Euonymus europaeus* L.). FEBS Lett 530:181-185.