Plant Physiology and Biochemistry 49 (2011) 131–137

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Novel antifungal defensins from *Nigella sativa* L. seeds

Eugene A. Rogozhin [a,*], Yulia I. Oshchepkova [c], Tatyiana I. Odintsova [b], Natalia V. Khadeeva [b], Olga N. Veshkurova [c], Tsezi A. Egorov [a], Eugene V. Grishin [a], Shavkat I. Salikhov [c]

[a] Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya Str. 16/10, 117997 Moscow, Russian Federation
[b] Vavilov Institute of General Genetics, Russian Academy of Sciences, Gubkina Str. 3, 119991 Moscow, Russian Federation
[c] Sadykov Institute of Bioorganic Chemistry, Uzbekistan Academy of Sciences, 700125 Tashkent, Abdullaeva Str. 83, Uzbekistan

ARTICLE INFO

Article history:
Received 22 April 2010
Accepted 23 October 2010
Available online 18 November 2010

Keywords:
Nigella sativa
Antimicrobial peptide
Defensin
Antifungal activity
Antibacterial activity

ABSTRACT

From seeds of *Nigella sativa* L. (Ranunculaceae), an endemic plant of Uzbekistan, two novel defensins named Ns-D1 and Ns-D2, were isolated and sequenced. The peptides differ by a single amino acid residue and show high sequence similarity to *Raphanus sativus* L. defensins Rs-AFP1 and Rs-AFP2. The Ns-D1 and Ns-D2 defensins display strong although divergent antifungal activity towards a number of phytopathogenic fungi. High antifungal activity of *N. sativa* defensins makes them promising candidates for engineering pathogen-resistant plants.

1. Introduction

Antimicrobial peptides (AMPs) are the ancient and widespread players of the defense system in all multicellular organisms [17,61]. Most of them exhibit broad-spectrum antimicrobial activity. Some AMPs act through nonspecific interaction with cytoplasmic membranes leading to disruption of the lipid bilayer and lysis of the pathogen's cells, while others exploit a more sophisticated receptor-mediated mechanism. Intracellular targets for some plant AMPs have also been suggested [18].

Several families of AMPs have been reported in plants on the basis of sequence similarity and the so-called cysteine motifs [10]. Defensins represent a unique AMP family widely spread both in animals and plants. Plant defensins are small (45–54 amino acids) basic peptides containing 4 disulphide bridges with a single exception of flower defensins, which possess 5 disulphide bonds [20]. Plant defensins show structural and functional similarity to defensins of insects [11], mammals [48], and fungi [38].

Defensins have been identified in a vast majority of plant families including Poaceae [7,32,33], Brassicaceae [9], Fabaceae [19,29], Chenopodiaceae [22], Asteraceae [68], Solanaceae [14,35], Liliaceae [16], Hippocastanaceae [43], Ginkgoaceae [50], Rosaceae [64], Amaranthaceae [47], Cucurbitaceae [13], and even in gymnosperms [49]. Most plant defensins were isolated from seeds [65,66], however they were also found in other plant organs, such as leaves [24,56], flowers [21,26], tubers [37], seedpods [4] and fruits [35,41]. Some members of the family are induced upon pathogen attack, while others are constitutively expressed in particular tissues or organs [1,36].

Most plant defensins display antifungal activity [5,37,42,51,54,63], some of they are active against bacteria [12,23,54,55]. Inhibition of protein synthesis in cell-free systems [32,33], inhibition of alpha-amylases [7,27,28,43,45] and proteinases [31], blockage of L-type Ca^{2+} [52] and sodium channels [25], cytotoxic activity on human tumor cell lines [5,44,65], and even on particular plant cells [68] have also been reported. Dual function in defense and development has been recently demonstrated for tomato defensins [53].

The mode of action of plant defensins has been extensively studied [58,59,60]. They are supposed to interact with specific sphingolipids on the fungal membranes [58,59,60]. For some members of the family penetration through the membrane and interaction with intracellular targets have been demonstrated [29,62].

The objective of this work was to isolate and characterize defensins from blackseed, an endemic plant of the Republic of Uzbekistan. To the best of our knowledge, defensins from plants of the Ranunculaceae family have not been studied so far. This work continues our research on AMPs from wild plant species. The genus *Nigella*

Abbreviations: AMP, antimicrobial peptide; TFA, trifluoroacetic acid; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight-mass spectrometry; RP-HPLC, reversed phase high performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DTET, dithioerythritol.

* Corresponding author. Tel.: +7 495 3364022.
E-mail address: rea21@list.ru (E.A. Rogozhin).

0981-9428/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2010.10.008

comprises 20 species growing in the south of Europe, North America and South-West Asia [2]. Seeds of blackseed are traditionally used as spices and in folk medicine [3,67]. Seed extracts of *Nigella sativa* display cytotoxic and anticarcinogenic activity in vitro [30]. Only organic compounds (alkaloids, steroids, carbohydrates, flavonoids, fatty acids etc.) of blackseed have been characterized so far [6,34].

2. Results

2.1. Isolation of Ns-D1 and Ns-D2

Purification of defensins from *N. sativa* seeds included several chromatographic procedures. At each stage the molecular masses of the obtained fractions were measured by MALDI-TOF-MS, and fractions containing peptides in the molecular mass range from 3 to 6 kDa were selected for peptide purification. By affinity chromatography four fractions were obtained (data not shown). Fraction 2, which eluted at 100 mM NaCl concentration, was enriched in 5–6 Da and 8–11-Da peptides. This fraction was further separated by ion-exchange chromatography (Fig. 1A), four fractions were recovered. Fractions 1 and 2 contained mainly proteins (11–12 kDa), fraction 3 was enriched in peptides in the molecular mass range from 5 to 6 kDa, fraction 4 was composed predominantly of peptides of 7–10 kDa. The peptide-containing fraction 3 was further fractionated by RP-HPLC (Fig. 1B). As a result, two peptides named Ns-D1 and Ns-D2, whose molecular masses were 5476.3 Da and 5492.4 Da, respectively, were purified from *N. sativa* seeds.

2.2. Determination of Ns-D1 and Ns-D2 amino acid sequences

Estimation of mass differences between the reduced and alkylated peptides and the native peptides showed that Ns-D1 and Ns-D2 contained 8 cysteine residues. Alkylation of native peptides without preliminary reduction demonstrated the absence of free sulfhydryl groups providing evidence of all 8 cysteine residues in Ns-D1 and Ns-D2 being involved in 4 intramolecular disulphide bonds. N-terminal sequencing allowed us to determine 30 amino acids of both peptides, which were identical. Homology search in the UniProt database showed that Ns-D1 and Ns-D2 belong to the defensin family. To determine the complete sequences the peptides were cleaved with the Glu-C proteinase at Glu-4 and Glu-28, and the resultant peptides were separated by RP-HPLC (data not shown). For Ns-D1 the molecular masses of the major peptide fractions were 2999.1 and 2495.8 Da, and for Ns-D2, 2999.3 and 2510.4 Da. Mass analysis allowed us to assign the masses 2999.1 and 2999.3 Da to the N-terminal region of both peptides, while the 2495.8-Da and 2510.4-Da peptides, to their C-terminal regions. Sequencing of the C-terminal regions of Ns-D1 and Ns-D2 enabled us to reconstruct the primary structure of Ns-D1 and Ns-D2 peptides (Fig. 2). They consist of 50 amino acid residues and differ by a single amino acid residue at position 39: in Ns-D2 proline is replaced by leucine. The calculated molecular masses of both peptides (5475.4 Da for Ns-D1 and 5491.4 Da for Ns-D2) agree well with the measured values indicating the absence of other post-translational modifications except for disulphide bonds.

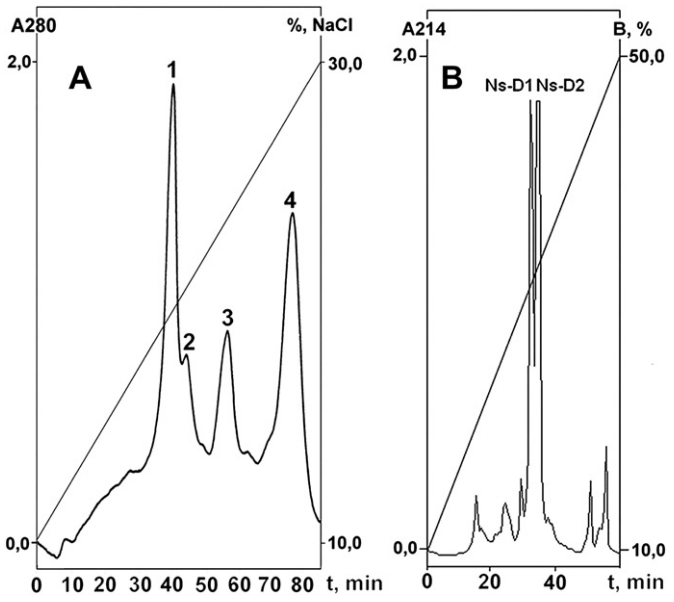

Fig. 1. Isolation of blackseed defensins: (A) ion-exchange chromatography of fraction 2 obtained by affinity chromatography; (B) RP-HPLC of fraction 3 obtained by ion-exchange chromatography.

Fig. 2 shows the alignment of blackseed defensin sequences with those of other defensins. The highest sequence identity was observed with *Raphanus sativus* defensins (43 identical amino acids including conserved substitutions), for which the three-dimensional structure is available [15,46]. This structure was used as a template to model the three-dimensional structure of *N. sativa* defensins. The modeled structure of *N. sativa* consists of an alpha-helix and three beta-strands connected by loops, a structure shared by all plant defensins (Fig. 3). Variable residues are shown in cyan color. The residues assumed to be vital for antifungal activity of *R. sativus* defensins and that differ from *N. sativa* defensins are located in the loop connecting beta2 and beta3: Val-39 in *R. sativus* substituted for Lys-38 in both *N. sativa* defensins, Phe-39 in *R. sativus* replaced for Pro-39/Leu-39 in Ns-D1 and Ns-D2, respectively.

2.3. Antifungal activity of Ns-D1 and Ns-D2

The results of antifungal assays of the Ns-D1 and Ns-D2 defensins are shown in Table 1, indicating high antifungal activity of both *N. sativa* defensins. The IC_{50} values for all fungi tested (except for *Botrytis cinerea*) were below 10 μg/ml (Table 1). The most pronounced effect was observed on growth of *Bipolaris sorokiniana* hyphae (IC_{50} = 1.8 μg/ml). Analysis of conidia germination in the presence of blackseed defensins by light microscopy showed that they had no effect on spore germination, but inhibited hyphal growth. However, we observed spore destruction in the presence of defensins in germinated conidia as illustrated by Fig. 4. Despite minor amino acid sequence variation, *N. sativa* defensins differed in their antifungal activity. Thus, Ns-D2 was more active than Ns-D1 in inhibiting growth of *B. sorokiniana*, *Fusarium oxysporum* and *B. cinerea*. The effect of both defensins on *Aspergillus niger*, *Fusarium graminearum* and *Fusarium culmorum* was similar. The minimum inhibitory concentration producing the maximum effect (MIC) for *Fusarium* species was the same for both defensins and equal to 55 μg/ml (81–86% growth inhibition against control), for *B. sorokiniana* (92% inhibition) – 27.5 (Ns-D1) and 55 (Ns-D2) μg/ml. For other fungi, MIC was also the same for both defensins – 55–82 μg/ml (65–70% inhibition relative to the control). For both defensins an increase in peptide concentration to 110 μg/ml did not lead to enhanced inhibition. In addition to inhibition of hyphae elongation, *N. sativa* defensins induced morphological changes in conidia of *A. niger*, *B. sorokiniana*, *F. oxysporum*, *F. graminearum* and *F. culmorum* with a similar effect on fungi belonging to the same genus, while the effect on fungi of different genera varied. Morphological changes in *B. sorokiniana* conidia after 48 h of incubation with Ns-D2 are shown in Fig. 4. As seen from this figure, the destruction of spores correlated with defensin concentration. The degree of morphological changes in conidia caused by Ns-D1 and Ns-D2 also differed. Ns-D1

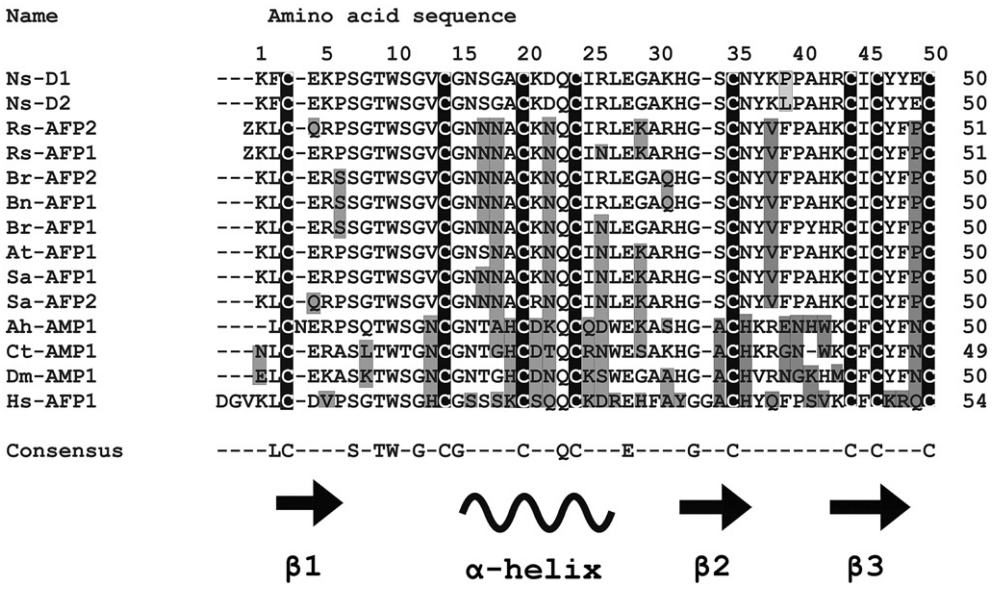

Fig. 2. Multiple sequence alignment of selected plant defensins. Accession numbers of the presented sequences in NCBI and UniProtKB database are as follows: Ns-D1 and Ns-D2 (this work), Rs-AFP1 (AAA69541), Rs-AFP2 (AAA69540), Br-AFP1 (ADA70735), Br-AFP2 (BAH82667), Bn-AFP1 (Q39313), At-AFP1 (NP_565119), Sa-AFP1 (P30231), Sa-AFP2 (P26780), Ah-AMP1 (AAB34970), Ct-AMP1 (AAB34971), Dm-AMP1 (AAB34972), Hs-AMP1 (AAB34974). Amino acid residues in defensins that differ from Ns-D1 and Ns-D2 are shown in dark gray. A single substitution that distinguishes Ns-D1 from Ns-D2 is shown in light gray. Secondary structure elements (α-helix and β-strands) in Rs-AFP1 are given below.

and Ns-D2 caused disruption of cell walls and membranes in 15–17% of *B. sorokiniana* conidia, however, the destructive processes in *B. sorokiniana* conidia started at Ns-D2 concentration of 6.9 μg/ml, and completed at 55 μg/ml. The concentrations of Ns-D1 required for induction of the same morphological changes were 13.6 to 110 μg/ml, respectively. Both defensins provoked similar morphological changes in *A. niger* conidia, however only 5–7% of conidia were affected at defensin concentration of 27.5 μg/ml. *N. sativa* defensins induced vacuolization of germinated macro- and microconidia in *Fusarium* species. The effect was observed in 40% of *F. oxysporum* conidia and 25% of *F. graminearum* and *F. culmorum* conidia at Ns-D1 or Ns-D2 concentration of 13.7 μg/ml.

The results of *Phytophthora infestans* growth inhibition on potato tubers are shown in Table 2. The degree of inhibition was estimated by the size of the infected area of the disc. After 96 h of incubation the development of disease symptoms was below 10% at Ns-D1 concentration of 3.4 μg/ml and Ns-D2 concentration of 13.6 μg/ml. After 120 h of incubation the inhibitory effect was less clear-cut and was estimated 15–17% at defensin concentration of 13.6 μg/ml. Persistent inhibitory effect (less than 10% of infected area) was achieved only at Ns-D2 concentration of 55 μg/ml. After 144 h of incubation the minimum inhibitory effect was observed at Ns-D1 concentration of 27.5 μg/ml and Ns-D2 concentration of 13.6 μg/ml (Table 2). An increase in defensin concentration to 110 μg/ml did not result in enhanced inhibition of *Ph. infestans* growth.

2.4. Antibacterial activity of Ns-D1 and Ns-D2

Antibacterial activity was evaluated by radial diffusion assay after 48 h of incubation. The results showed that blackseed defensins in addition to antifungal activity, inhibited growth of Gram-positive and Gram-negative bacteria (Table 3). The degree of

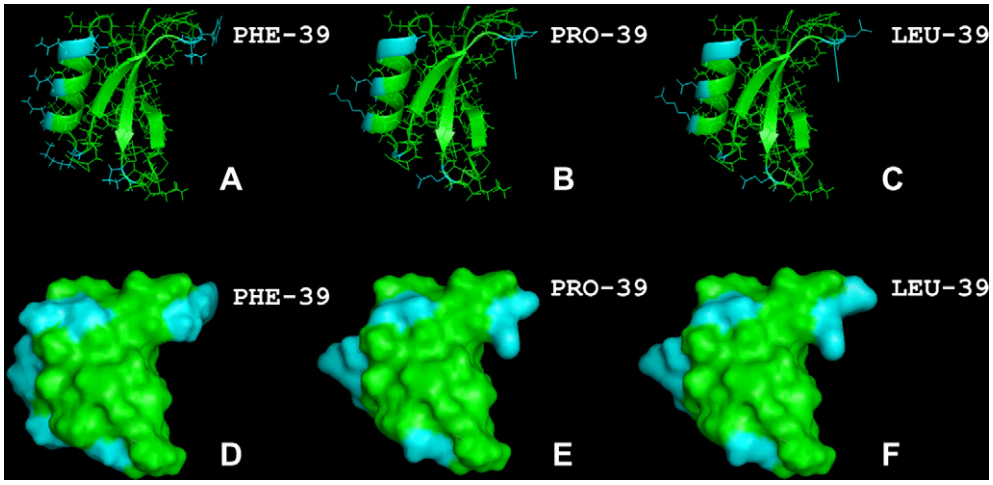

Fig. 3. Three-dimensional models of blackseed defensins. 3D structure of radish defensin Rs-AFP1 is given for comparison [15]. A and D, Rs-AFP1 (accession number in PDB – 1AYJ); B and E, Ns-D1, C and F, Ns-D2. Variable residues between blackseed and radish defensins are shown in cyan color (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Antifungal activity of Ns-D1 и Ns-D2.[a]

Fungus	Defensin	
	Ns-D1	Ns-D2
A. niger	3.5	3.5
B. sorokiniana	3.0	1.8
F. oxysporum	9.5	5.3
F. graminearum	6.9	6.9
F. culmorum	6.9	6.9
B. cinerea	27.4	13.7

[a] IC_{50} values in μg/ml are given.

inhibition depended on the species tested. Of the bacteria examined, *Escherichia coli* was the least affected. The Ns-D2 defensin produced a more pronounced effect on growth of colonies of *Erwinia carotovora* and *Bacillus subtilis* than Ns-D1 (Table 3).

3. Discussion

In this work, we isolated and sequenced two novel highly homologous and biologically active defensins Ns-D1 and Ns-D2 from *N. sativa* seeds. For the first time defensins from the Ranunculaceae family have been analyzed. Blackseed defensins showed the highest sequence similarity (73%) to earlier described defensins Rs-AFP1 and Rs-AFP2 from *R. sativus* seeds belonging to the Brassicaceae family [46,54,55,56,57] providing evidence for the conservation of the defensin structure among evolutionary diverged plant species. Less amino acid sequence homology was revealed with defensins belonging to other plant families, such as Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae (Fig. 2).

Structure–function relationships in AMPs remain the most intriguing area of research. Earlier it was shown that Rs-AFP2 possesses two sites of antifungal activity. The first site comprises the residues Tyr-38, Phe-40, Pro-41, Ala-42, Lys-44 and Ile-46. The second site is formed by Thr-10, Ser-12, Leu-28 and Phe-49 [46]. Amino acid sequence comparison of *N. sativa* and radish defensins shows that although they differ in 8 nonconserved substitutions, the residues of the second antifungal site are well preserved taking into account a conserved substitution of Phe in Rs-AFP2 for Tyr in Ns-D peptides. As clearly shown for radish defensins, a substitution of a hydrophobic residue Phe-49 for a basic residue Arg in Rs-AFP2 dramatically decreased antifungal activity against *F. culmorum* both under low- and high-salt conditions [46]. However the conserved Phe-49/Tyr substitution in Ns-D defensins seems unlikely to affect antifungal activity. In contrast to the second site, the amino acid residues of the first antifungal site are less conserved in blackseed defensins. The most prominent substitution is the replacement of Phe-40 in Rs-AFP2 for Pro and Leu in Ns-D1 and Ns-D2, respectively. Mutational analysis of Rs-AFP2 showed that substitution of Phe-40 located in the loop connecting beta2 and beta3 for Met decreased antifungal activity of the peptide [46]. This replacement is most likely responsible for a little bit lower antifungal activity of blackseed defensins against *B. cinerea* and *F. culmorum* as compared to exceptionally active radish defensins and supports the role of this residue in antifungal activity. The significance of Val/Lys substitution at position 39 is unclear. As shown for radish defensins, the effect of Val/Arg substitution at low salt conditions depends on the fungus tested. [46]. Our results demonstrate that Ns-D2 is more potent in inhibiting hyphal growth of a number of fungi (*B. sorokiniana*, *F. oxysporum*, *B. cinerea*), this peptide is also a more potent inhibitor of *Ph. infestans* infection (Table 2). Higher biological activity of Ns-D2 most likely arises from the substitution of Pro for a more hydrophobic residue Leu-39, since it is a single amino acid that discriminates two peptides, thus providing another convincing evidence for the significant role of this residue in the antifungal activity of the peptide.

In addition to antifungal activity of blackseed defensins, they also inhibited growth of Gram-positive and Gram-negative bacteria (Table 3). The effect on Gram-negative bacteria strongly depended on the species tested, with nonpathogenic *E. coli* being the least sensitive bacterium and *Pseudomonas syringae*, the most sensitive one. Highly homologous radish defensins failed to inhibit growth of the bacteria tested (*B. subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Streptococcus faecalis*) at concentrations below 200 μg/ml [43]. We suppose that the effect on bacteria of *N. sativa* defensins may be associated with the substitution of Val-39 for a positively charged Lys contributing to a higher positive charge of the active site of the molecule interacting with the negatively charged phospholipids of bacterial membranes.

In summary, two novel highly homologous defensins Ns-D1 and Ns-D2 have been isolated from seeds of backseed *N. sativa*. This is

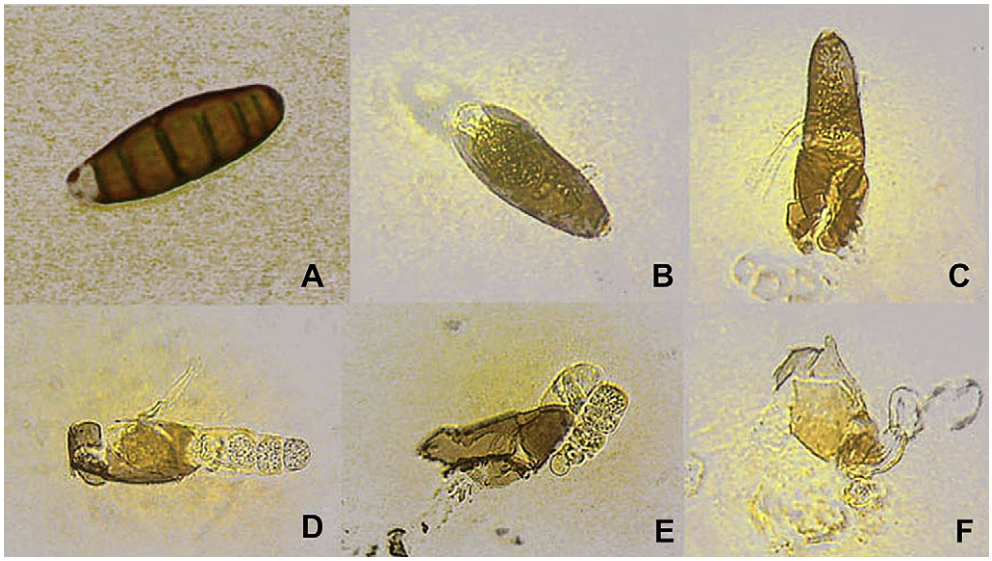

Fig. 4. Morphological changes in *B. sorokiniana* conidia after 48 h incubation with Ns-D2. (A) Control conidia; (B–F) Conidia at different Ns-D2 concentrations: 6.9 μg/ml (B), 13.8 μg/ml (C), 7.5 μg/ml (D), 55 μg/ml (E), 110 μg/ml (F).

Table 2
Inhibitory activity of blackseed defensins on disease development caused by *Ph. infestans*.[a]

Concentration, μg/ml	Incubation time, h		
	96	120	144
55.0	+++/+++	++/+++	+/++
27.5	+++/+++	++/++	+/+
13.6	+++/+++	++/++	−/+
6.8	++/+++	+/+	−/−
3.4	++/+++	+/+	−/−

[a] "+++" − disease development below 10% of infected area; "++" − below 20% of infected area; "+" − below 40% of infected area; "−" − disease development above 40%. For details see "Material and methods, Section 4.10". The first number in the column refers to Ns-D1 and the second to Ns-D2.

the first report on defensins from plants of the Ranunculaceae family. Minor variation in amino acid sequence between the two defensins (a single amino acid substitution) results in changes in biological activity, with the Ns-D2 defensin being more active than Ns-D1 indicating the importance of the residue at position 39 for the antifungal activity of blackseed defensins. *N. sativa* defensins provide another spectacular example that minor variation in amino acid sequences can alter the biological activity of defensins. Similar observations were made for two radish defensins Rs-AFP1 and Rs-AFP2 [43] and *Echinochloa crusgalli* defensins [39]. Blackseed defensins exhibit high antifungal activity inhibiting growth of hyphae and causing spore destruction in a number of fungal pathogens. In contrast to earlier described *R. sativus* defensins, they show antibacterial activity as well.

The results obtained demonstrate the potential of wild plants as valuable donors of potent AMPs expanding the list of candidate genes for engineering pathogen-resistant crops.

4. Materials and methods

4.1. Plant material

Seeds of *N. sativa* L. (Ranunculaceae) collected in the Republic of Uzbekistan in 2008 were used in this study.

4.2. Microorganisms

The pathogens used were as follows *F. oxysporum* strain 16/10, *B. cinerea* strain SGR-1 isolated from infected plants in the Timiryazev Agricultural Academy, *F. graminearum* strain VKM F-1668, *F. culmorum* strain VKM F-2303, *B. sorokiniana* strain VKM F-1446, *A. niger* strain VKM F-33 from the All-Russian Collection of Microorganisms, *Ph. infestans* strain OSV 12, obtained from the Institute of Plant Protection of the Republic of Belarus', *P. syringae* strain VKM B-1546 and *E. carotovora* strain VKM B-1247 from the All-Russian Collection of Microorganisms, *B. subtilis*, *Clavibacter michiganensis*, and *E. coli* were obtained from the Collection of the Institute of General Genetics of the Russian Academy of Sciences.

4.3. Defensin isolation

Seeds of *N. sativa* (100 g) were ground to a fine powder in a coffee mill. Seed flour was homogenized in 10% acetic acid (1:7, w/v) in the presence of the inhibitor cocktail (Sigma) for 2 h at 20 °C with constant stirring. The homogenate was centrifuged at 4500 rpm for 20 min at 4 °C. The pellet was discarded, the supernatant was filtered through the paper filter (Whatman, USA) and concentrated four-fold in a rotor concentrator (IKA, Germany). Proteins and peptides were precipitated with six volumes of ice-cold acetone overnight at 4 °C. The pellet was collected by centrifugation at 4500 rpm for 15 min and air-dried at room temperature. The precipitate was dissolved in 48 ml 0.1% TFA (solvent A) and desalted by RP-HPLC on an Aquapore C8 column (10 × 100 mm) (Applied Biosystems, USA) at a flow rate of 1.2 ml at 38 °C. After washing salts and other unadsorbed components proteins and peptides were eluted with 75% solvent B (solvent B: 80% acetonitrile in 0.1% TFA). The obtained fraction was concentrated using a Speedvac concentrator (Savant, USA) and freeze-dried on a Labconco liophilyzer (USA). The pellet was dissolved in 48 ml of 10 mM tris–HCl, pH 7.2 and loaded onto a Heparin HiTrap-Sepharose column (5 ml, GE Healthcare, USA). After elution of unbound fraction, proteins and peptides were eluted with a step-wise NaCl gradient (100 mM, 500 mM and 1 M) in 10 mM tris–HCl buffer, pH 7.2 at a flow rate of 1 ml/min and detected at 280 nm. The fraction eluted at 100 mM NaCl concentration was further separated by cation-exchange chromatography on a CM-52 column (16 × 100 mm, Whatman, USA) in 10 mM tris–HCl buffer, pH 7.2 containing 100 mM NaCl,. Proteins and peptides were eluted with a linear NaCl gradient (100–300 mM) in 10 mM tris–HCl buffer, pH 7.2 for 90 min at a flow rate of 1 ml/min and room temperature and detected at 280 nm. Final purification of peptides was performed by RP-HPLC on a ReproSil-Pur 300 ODS-3 (4.6 × 250 mm, particle size 5 micron, "Dr. A. Marsch Ammerbuch", Germany) in a linear acetonitrile gradient (10–50% B) (solvent B as above) for 1 h at a flow rate of 0.7 ml/min and 38 °C. Elution of peptides was monitored at 214 nm.

4.4. MALDI-TOF-MS analysis

Molecular masses of proteins and peptides were measured on an Ultraflex MALDI mass spectrometer (Bruker Daltonics, Germany) in a positive ion mode. 2,5 Dihydroxybenzoic acid was used as a matrix. Mass spectra were analyzed with Bruker DataAnalysis for TOF software. The accuracy of mass determination was 0.015%.

4.5. Determination of peptide concentration

Peptide concentration was determined by RP-HPLC chromatography on a C_{18} Luna column (4.6 × 150 mm, Phenomenex, USA) calibrated with bovine insulin. The peptides were eluted with an acetonitrile gradient (10–50% B) for 30 min at a flow rate of 0.75 ml/min at 38 °C and detected at 214 nm.

Table 3
Antibacterial activity of blackseed defensins.[a]

Defensin concentration (μg in 50 μl)	Inhibition zone including the sample application zone (in cm)[b]				
	Clavibacter michiganensis	*Pseudomonas syringae*	*Erwinia carotovora*	*Escherichia coli*	*Bacillus subtilis*
11.0	1.4/1.5	1.3/1.3	0.9/1.3	0.7/0.7	1.2/1.4
5.5	1.2/1.2	1.2/1.3	0.6/1.2	0.6/0.7	1.2/1.3
2.25	0.9/1.0	1.0/1.1	0.6/0.8	0.6/0.6	1.0/1.0

[a] Sample volume 50 μl.
[b] Size of the peptide application zone 0.5 cm, the first number refers to Ns-D1 and the second to Ns-D2.

4.6. Reduction and alkylation

Peptides (2 nm) were dissolved in 35 μl of 6 M guanidine hydrochloride containing 3 mM EDTA in 0.5 M tris–HCl, pH 7.8. Four microliters of 1 M dithioerythrol (DTET) in 2-propanol were added to the solution. The reaction proceeded under argon for 4 h at 40 °C. For alkylation 4 μl of 50% (v/v) 4-vinylpyridine in 2-propanol were added to the reaction mixture and incubated 20 min in the dark. The mixture was then diluted with 40 μl of 0.1% TFA, and the products of the reaction were separated by RP-HPLC on a Luna C_{18} column (10 × 250 mm, Phenomenex, USA) in a linear acetonitrile gradient (10–50%B) for 40 min at a flow rate of 0.75 ml/min. Peptides were detected at 214 nm. To determine the presence of free thiol groups peptides were alkylated without preliminary reduction.

4.7. Amino acid sequencing

Reduced and alkylated peptides were sequenced by automated Edman degradation on a model 492 Procise sequencer (Applied Biosystems, USA) according to the manufacturer's protocol. Homology search was performed using Swiss-Prot and TrEMBL databases with a BLAST algorithm.

4.8. Enzymatic digestion with Glu-C proteinase

Approximately 1 nm of reduced and alkylated peptide was dissolved in 20 μl of 100 mM NH4HCO3 buffer, pH 8.0, and 1 μl of the enzyme in water (0.1 mg/ml) was added to the reaction mixture. Enzymatic hydrolysis was carried out for 4 h at 37 °C. The peptides produced were purified by RP-HPLC.

4.9. 3D structure modeling

Modeling of defensin spatial structure was accomplished using PyMol v. 0.9.3 software.

4.10. Antifungal assays

The antifungal activity of the peptides was tested against several fungi using microtiter-plate assays essentially as described previously [8]. Wells were filled with 10 μl of twofold serial dilutions of the peptide and mixed with 90 μl half-strength potato–glucose broth containing 10^4 spores/ml. The inhibition of spore germination was evaluated by measuring the absorbance at 620 nm in microtiterplates. IC_{50} values showing protein concentration required for 50% growth inhibition were calculated. Inhibition of hyphae elongation and morphological changes in the fungi were examined by light microscopy. Experiments were performed at least in three replicates.

The activity of peptides against *Ph. infestans* was estimated as given in [40]. Shortly, protein samples were mixed with 50 μl of zoosporangium suspension and incubated at 20 °C for 2 h, whereupon the mixture was applied to the center of two potato discs in a Petri dish. Infected potato discs were incubated at 20 °C for 120 h. Disease symptoms were recorded 96, 120 and 144 h after inoculation by measuring the infected area (in %) and scored from "–" to "++++", with "++++" denoting complete inhibition of disease symptoms, "+++" disease development less than 10%, "++" disease development less than 20%, "+" disease development less than 40% and "–" denotes symptom development more than 40% (the absence of inhibition). In control discs disease symptoms were above 40%. A total of 10 discs were assayed in each of three independent experiments.

4.11. Antibacterial assays

The antibacterial activity of peptides was assayed against several Gram-positive and Gram-negative bacteria using radial diffusion assay [40]. Petri dishes with Luria–Bertani 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 48 h. The antibacterial activity was evaluated by the size of the inhibition zone formed around the wells with the peptide solution.

Acknowledgments

This work was supported in part by the Russian Foundation for Basic Research (Grants No 08-04-90257-Uzb_a, 08-04-00783a and 09-04-00250a) and the Programs "Molecular and Cell Biology" and "Biodiversity" of the Russian Academy of Sciences.

References

[1] I.P. Ahn, K. Park, C.Y. Kim, Rhizobacteria-induced resistance perturbs viral disease progress and triggers defense-related gene expression, Mol. Cells 13 (2002) 302–308.
[2] M.S. Al-Ghamdi, The anti-inflammatory, analgesic and antipyretic activity of *Nigella sativa*, J. Ethnopharmacol. 76 (2001) 45–48.
[3] B.H. Ali, G. Blunden, Pharmacological and toxicological properties of *Nigella sativa*, Phytother. Res. 17 (2003) 299–305.
[4] M.S. Almeida, K.M. Cabral, R.B. Zingali, E. Kurtenbach, Characterization of two novel defense peptides from pea (*Pisum sativum*) seeds, Arch. Biochem. Biophys. 378 (2000) 278–286.
[5] J.L. Anaya-Lopez, J.E. Lopez-Meza, V.M. Baizabal-Aguirre, H. Cano-Camacho, A. Ochoa-Zarzosa, Fungicidal and cytotoxic activity of *Capsicum chinense* defensin expressed by endothelial cells, Biotechnol. Lett. 28 (2006) 1101–1108.
[6] A.A. Ansari, S. Hassan, L. Kene, A. Ur-Rahman, T. Wohler, Structural studies on a saponin isolated from *Nigella sativa*, Phytochemistry 27 (1988) 3977–3979.
[7] C.J. Bloch, M. Richardson, A new family of small (5 kDa) protein inhibitors of insect α-amylases from seeds of sorghum (*Sorghum bicolor* (L.) Moebch.) have sequence homologies with wheat γ-purothionins, FEBS J. 279 (1991) 101–104.
[8] W.F. Broekaert, F.R.G. Terras, B.P.A. Cammue, J. Vanderleyden, An automated quantitative assay for fungal growth inhibition, FEMS Microbiol. Lett. 69 (1990) 55–59.
[9] W.F. Broekaert, F.R.G. Terras, B.P.A. Cammue, R.W. Osborn, Plant defensins: novel antimicrobial peptides as components of the host defense system, Plant Physiol. 108 (1995) 1353–1358.
[10] W.F. Broekaert, B.P.A. Cammue, M.F.C. De Bolle, K. Thevissen, G.W. De Samblanx, R.W. Osborn, Antimicrobial peptides from plants, Crit. Rev. Plant Sci. 16 (1997) 297–323.
[11] P. Bulet, R. Stöcklin, Insect antimicrobial peptides: structures, properties and gene regulation, Protein Pept. Lett. 12 (2005) 3–11.
[12] G.-H. Chen, M.-P. Hsu, C.-H. Tan, H.-Y. Sung, C.G. Kuo, M.-J. Fan, Cloning and characterization of a plant defensin VaD1 from azuki bean, J. Agric. Food Chem. 53 (2005) 982–988.
[13] L. Da-Hui, J. Gui-Liang, Z. Ying-Tao, A. Tie-Min, Bacterial expression of a *Trichosanthes lirilowii* defensin (TDEF1) and its antifungal activity on *Fusarium oxysporum*, Appl. Microbiol. Biotechnol. 28 (2007) 62–75.
[14] H.M. Do, S.C. Lee, H.W. Jung, K.H. Sohn, B.K. Hwang, Differential expression and *in situ* localization of a pepper defensin (CADEF1) gene in response to pathogen infection, abiotic elicitors and environmental stresses in *Capsicum anuum*, Plant Sci. 166 (2004) 1297–1305.
[15] F. Fant, W. Vranken, W. Broekaert, F. Borremans, Determination of the three-dimensional solution structure of *Raphanus sativus* antifungal protein 1 by ^{1}H NMR, J. Mol. Biol. 279 (1998) 257–270.
[16] M. Fujiumura, M. Ideguchi, Y. Minami, K. Watanabe, K. Tadera, Purification, characterization and sequencing of novel antimicrobial peptides Tu-AMP1 and Tu-AMP2 from bulbs of tulip (*Tulipa gesneriana* L.), Biosci. Biotechnol. Biochem. 63 (2004) 571–577.
[17] T. Ganz, Defensins: antimicrobial peptides of innative immunity, Nat. Rev. Immunol. 3 (2003) 710–720.
[18] F. Garcia-Olmedo, A. Molina, J.M. Alamillo, P. Rodriguez-Palenzuela, Plant defense peptides, Biopolymers (Peptide Science) 47 (1998) 479–491.
[19] M.A. Graham, K.A. Silverstein, S.B. Cannon, K.A. VandenBosch, Computational identification and characterization of novel genes from legumes, Plant Physiol. 135 (2004) 1179–1197.
[20] B.J.C. Janssen, H.J. Schirra, F.T. Lay, M.A. Anderson, D.J. Craik, Structure of *Petunia hybrida* defensin 1, a novel plant defensin with five disulfide bonds, Biochemistry 42 (2003) 8214–8222.

[21] B. Karunanandaa, A. Singh, T.H. Kao, Characterization of a predominantly pistil-expressed gene encoding a gamma-thionin-like protein of *Petunia inflala*, Plant Mol. Biol. 26 (1994) 459–464.
[22] A.K. Kristensen, J. Brunstedt, J.E. Nielsen, J.D. Mikkelsen, P. Roepstorff, K.K. Nielsen, Processing, disulfide pattern, and biological activity of a sugar beet defensin, AX2, expressed in *Pichia pastoris*, Protein Expr. Purif. 16 (1999) 377–387.
[23] M. Koike, T. Okamoto, S. Tsuda, R. Imai, A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation, Biochem. Biophys. Res. Commun. 298 (2002) 46–53.
[24] K.M. Kragh, J.E. Nielsen, K.K. Nielsen, S. Dreboldt, J.D. Mikkelsen, Characterization and localization of a new antifungal cysteine-rich proteins from *Beta vulgaris*, Mol. Plant Microbe Interact. 8 (1995) 424–434.
[25] C. Kushmerick, M. de Souza Castro, J. Santos Cruz, C. Bloch, P.S. Beirao, Functional and structural features of gamma-zeathionins, a new class of sodium channel blockers, FEBS Lett. 440 (1998) 302–306.
[26] F.T. Lay, F. Brugliera, M.A. Anderson, Isolation and properties of floral defensins from ornamental tobacco and petunia, Plant Physiol. 131 (2003) 1283–1293.
[27] K.F. Lin, T.R. Lee, P.H. Tsai, M.P. Hsu, C.S. Chen, P.C. Lyu, Structure-based protein engineering for alpha-amylase inhibitory activity of plant defensin, Proteins 68 (2007) 530–540.
[28] Y.I. Liu, C.S. Cheng, S.M. Lai, M.P. Hsu, C. Chen, P.C. Lyu, Solution structure of the plant defensin VrD1 from mung bean and its possible role in insecticidal activity against bruchids, Proteins 63 (2006) 777–786.
[29] D.S. Lobo, I.B. Pereira, L. Fragel-Madeira, L.N. Medeiros, L.M. Cabral, J. Faria, M. Bellio, R.C. Campos, R. Linden, E. Kurtenbach, Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle, Biochemistry 46 (2007) 987–996.
[30] L.A. Mbarek, H.A. Mouse, N. Elabbadi, M. Bensalah, A. Gamouh, R. Aboufatima, A. Benharref, A. Chait, M. Kamal, A. Dalal, A. Zyad, Anti-tumor properties of blackseed (*Nigella sativa* L.) extracts, Braz. J. Med. Biol. Res. 40 (2007) 839–847.
[31] F.R. Melo, D.J. Rigden, O.L. Franco, L.V. Mello, M.B. Ary, M.F. Grossi-de-Sa, C. Bloch, Inhibition of trypsin by cowpea thionin: characterization, molecular modeling and docking, Proteins 48 (2002) 311–319.
[32] E. Mendez, A. Moreno, F. Colilla, F. Pelaez, G.G. Limas, R. Mendez, Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, γ-hordothionin, from barley endosperm, Eur. J. Biochem. 194 (1990) 533–539.
[33] E. Mendez, A. Rocher, M. Calero, T. Girbes, L. Citores, F. Soriano, Primary structure of ω-hordothionin, a member of a novel family of thionins from barley endosperm, and its inhibition of protein synthesis in eukaryotic and prokaryotic cell-free systems, Eur. J. Biochem. 239 (1996) 67–73.
[34] I. Merfort, V. Wary, H.H. Barakat, S.A.M. Hussein, M.A.M. Nawwar, G. Willuhn, Flavonol triglycosides from seeds of *Nigella sativa*, Phytochemistry 46 (1997) 359–363.
[35] B. Meyer, G. Houlne, J. Pozueta-Romero, M.L. Schantz, R. Schantz, Fruit-specific expression of a defensine-type gene family in bell pepper, Upregulation during ripering and upon wounding, Plant Physiol. 112 (1996) 615–622.
[36] M. Mirouze, J. Sels, O. Richard, P. Czernic, S. Loubet, A. Jacquier, I.E.J.A. Francois, B.P.A. Cammue, M. Lebrun, P. Berthomieu, L. Marques, A putative novel role for plant defensins: a defensin from zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance, Plant J. 47 (2006) 329–342.
[37] M. Moreno, A. Segura, F. Garcia-Olmedo, Pseudothionin-St1, a potato peptide active against potato pathogens, Eur. J. Biochem. 223 (1994) 135–139.
[38] P.H. Mygind, R.L. Fischer, K.M. Schnorr, M.T. Hansen, C.P. Sönksen, S. Ludvigsen, D. Raventós, S. Buskov, B. Christensen, L. De Maria, O. Taboureau, D. Yaver, S.G. Elvig-Jørgensen, M.V. Sørensen, B.E. Christensen, S. Kjærulff, N. Frimodt-Moller, R.I. Lehrer, M. Zasloff, H.-H. Kristensen, Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus, Nature 437 (2005) 975–980.
[39] T.I. Odintsova, E.A. Rogozhin, Yu Baranov, A.Kh. Musolyamov, N. Yalpani, Ts.A. Egorov, E.V. Grishin, Seed defensins of barnyard grass *Echinochloa crusgalli* (L.) Beauv, Biochimie 90 (2008) 1667–1673.
[40] T.I. Odintsova, A.A. Vassilevski, A.A. Slavokhotova, A.K. Musolyamov, E.I. Finkina, N.V. Khadeeva, E.A. Rogozhin, T.V. Korostyleva, V.A. Pukhalsky, E.V. Grishin, T.A. Egorov, A novel antifungal hevein-type peptide from *Triticum kiharae* seeds with a unique 10-cysteine motif, FEBS J. 276 (2009) 4266–4275.
[41] B.J. Oh, M.K. Ko, I. Kostenyuk, B. Shin, K.S. Kim, Coexpression of a defensin gene and thionin-like via different signal transduction pathways in pepper and *Colletotrichum gloeosporioides* interactions, Plant Mol. Biol. 41 (1999) 313–319.
[42] S. Olli, P.B. Kirti, Cloning, characterization and antifungal activity of defensin Tfgd1 from *Trigonella foenum-graecum* L. J. Biochem. Mol. Biol. 39 (2006) 278–283.
[43] R.W. Osborn, G.W. De Samblanx, K. Thevissen, I. Goderis, S. Torrekens, F. Van Leuven, Isolation and characterization of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae, FEBS Lett. 368 (1995) 257–262.
[44] P.B. Pellegrini, O.L. Franco, Plant gamma-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins, Int. J. Biochem. Cell. 37 (2005) 2239–2253.
[45] P.B. Pelegrini, F.T. Lay, A.M. Murad, M.A. Anderson, O.L. Franco, Novel insights on the mechanism of action of alpha-amylase inhibitors from the plant defensin family, Proteins 73 (2008) 719–729.
[46] G.W. De Samblanx, I.J. Goderis, K. Thevissen, R. Raemaekers, F. Fant, F. Borremans, D.P. Acland, R.W. Osborn, S. Patel, W.F. Broekaert, Mutational analysis of a plant defensin from radish (*Raphanus sativus* L.) reveals two adjacent sites important for antifungal activity, J. Biol. Chem. 272 (1997) 1171–1179.
[47] A. Segura, M. Moreno, A. Molina, F. Garcia-Olmedo, Novel defensin subfamily from spinach (*Spinacea oleracea*), FEBS Lett. 435 (1998) 159–162.
[48] M.E. Selsted, A.J. Ouellette, Mammalian defensins in the antimicrobial immune response, Nat. Immunol. 6 (2005) 551–557.
[49] P. Sharma, A. Lönneborg, Isolation and characterization of a cDNA encoding a plant defensin-like protein from roots of Norway spruce, Plant Mol. Biol. 31 (1996) 707–712.
[50] G. Shen, Y. Pang, W. Wu, Z. Miao, H. Qian, L. Zhao, Molecular cloning, characterization and expression of a novel jasmonat-dependent defensin gene from *Ginkgo biloba*, J. Plant Physiol. 162 (2005) 1160–1168.
[51] J. Solis, G. Medrano, M. Ghislain, Inhibitory effect of a defensin gene from the Andan crop maca (*Lepidium meyenii*) against *Phytophthora infestans*, J. Plant Physiol. 164 (2007) 1071–1082.
[52] R.G. Spelbrink, N. Dilmac, A. Allen, T.J. Smith, D.M. Shah, G.H. Hockerman, Differential antifungal and calcium channel-blocking activity among structurally related plant defensins, Plant Physiol. 135 (2004) 2055–2067.
[53] H.U. Stotz, B. Spence, Y. Wang, A defensin from tomato with dual function in defense and development, Plant Mol. Biol. 71 (2009) 131–143.
[54] F.R.G. Terras, H.M.E. Schoofs, M.F.C. de Bolle, F. Van Leuven, S.B. Rees, *In vitro* antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer proteins, Plant Physiol. 100 (1992a) 1055–1058.
[55] F.R.G. Terras, H.M.E. Schoofs, M.F.C. de Bolle, F. Van Leuven, S.B. Rees, J. Vanderleyden, B.P.A. Cammue, W.F. Broekaert, Analysis of two novel classes of antifungal proteins from radish (*Raphanus sativus* L.), J. Biol. Chem. 267 (1992b) 15301–15309.
[56] F.R.G. Terras, S. Torrekens, F. Van Leuven, R.W. Osborn, J. Vanderleyden, B.P.A. Cammue, W.F. Broekaert, A new family of basic cysteine-rich plant antifungal proteins from Brasicaceae species, FEBS Lett. 316 (1993) 233–240.
[57] F.R.G. Terras, K. Eggermont, V. Kovakva, N.V. Raikhel, R.W. Osborn, A. Kester, S.B. Rees, S. Torrekens, F.V. Leuven, J. Vanderleyden, B.P.A. Cammue, W.F. Broekaert, Small cysteine-rich antifungal proteins from radish: their role in host defense, Plant Cell 7 (1995) 573–588.
[58] K. Thevissen, I.E.J.A. Francois, J.Y. Tokemoto, K.K.A. Ferket, E.M.K. Meert, B.P.A. Cammue, $DmAMP_1$, an antifungal plant defensin from Dahlia (*Dahlia merckii*), interacts with sphingolipids from *Saccharomyces cerevisiae*, FEMS Microbiol. Lett. 226 (2003a) 169–173.
[59] K. Thevissen, K.K.A. Ferket, I.E.J.A. Francois, B.P.A. Cammue, Interactions of antifungal plant defensins with fungal membrane components, Peptides 24 (2003b) 1705–1712.
[60] K. Thevissen, D.C. Warnecke, I.E.J.A. Francois, M. Leipelt, E. Heinz, C. Ott, Defensins from insects and plants interact with fungal glucosylceramides, J. Biol. Chem. 279 (2004) 3900–3905.
[61] B.P.H.J. Thomma, B.P.A. Cammue, K. Thevissen, Plant defensins, Planta 216 (2002) 193–202.
[62] N.L. Van der Weerden, F.T. Lay, M.A. Anderson, The plant defensin, NaD1, enters the cytoplasm of *Fusarium oxysporum* hyphae, J. Biol. Chem. 283 (2008) 14445–14452.
[63] H.X. Wang, T.B. Ng, An antifungal peptide from baby lima bean, Appl. Microbiol. Biotechnol. 73 (2006) 576–581.
[64] M.E. Wisniewski, C.L. Bassett, T.S. Artlip, R.P. Webb, W.J. Janisiewicz, J.L. Norelli, Characterization of a defensin from bark and fruit tissues of peach and antimicrobial activity of a recombinant defensin in the yeast, *Pichia pastoris*, Physiol. Plant 119 (2003) 563–572.
[65] J.H. Wong, T.B. Ng, Gymnin, a potent defensin-like antifungal peptide from the Yunnan bean (*Gymnocladus chinensis* Baill), Pepides 24 (2003) 963–968.
[66] J.H. Wong, T.B. Ng, Sesquin, a potent defensin-like antimicrobial peptide from ground beans with inhibitory activities toward tumor cells and HIV-1 reverse transcriptase, Peptides 25 (2005) 1120–1126.
[67] D.R. Worthen, O.A. Ghosheh, P.A. Crooks, The *in vitro* anti-tumour activity of some crude and purified components of black seeds, *Nigella sativa* L. Anticancer Res. 18 (1998) 1527–1532.
[68] A. de Zélicourt, P. Letousey, S. Thoiron, C. Campion, P. Simoneau, K. Elmorjani, D. Marion, P. Simier, P. Delavault, Ha-DEF1, a sunflower defensin, induces cell death in *Orobanche* parasitic plants, Planta 226 (2007) 591–600.