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Sequence analysis of gene encoding cyclotides in three species of *Violaceae* and determination of their anti-microbial activities

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Abstract

Cyclotides are plant peptides, which are characterized by their unique cyclic cysteine knot structure. These peptides have a variety of biological activity including anti-HIV, anti-microbial and cytotoxic effects. In the current study, we compared these gene sequences encoding cyclotides from *Viola odorata*, *V. ignobilis* and *V. occulta*. We also extracted total cyclotides content from *V. ignobilis* by fractionation method and semipurified these on a SPE-CI8 (Solid Phase Extraction). Anti-microbial activity of the semi-purified cyclotides was determined by radial diffusion assay (RDA). Three groups of bacteria were studied including human pathogenic bacteria, plant pathogenic bacteria and soil benefit bacteria. Three gene encoding cyclotides were identified in studied species. The most susceptible bacterium in human pathogenic group was *Staphylococcus aureus. Xanthomonas oryzea* was the most susceptible bacterium between the three studied groups.

Keywords: anti-microbial agent; cyclotide; RDA; human and plant pathogenic bacteria; *Violaceae*

Introduction

For many years, medical plants have been used to introduce new drugs. Introduction of new antibacterial agents could be useful due to rapid development of multi-drug-resistant (MDR) bacteria. Anti-microbial peptides discovered recently have potential to offer novel anti-microbial drugs [1].

On the other hand, the control of plants' pests is necessary to provide food for the world's growing population, and it has great effect on all countries' economy. There are various chemical and biological agents to control the pests [2]. However, limited studies have been reported about their environmental effects so far. It is generally accepted that, we should determine the effect of biological agents on the soil before use.

Cyclotides are head-to-tail cyclic peptides containing 28-37 amino acids. They are plant polypeptides, which are characterized by their unique cyclic cystine-knot (CCK) structural motif [3, 4]. The backbone segments between the conserved cysteines, namely loops have various levels of diversity. Loops 1 and 4 are highly conserved among all reported cyclotides. Loop 6 varies in size and sequence among cyclotides, however it has a key role in cyclization and biosynthesis of cyclotides [5]. Although loops 2, 3 and 5 are more diverse, they contain several highly conserved residues [6]. The first cyclotide reported and structurally characterized was kalata B1 [7, 8]. Due to their unique structure, cyclotides are significantly resistant to thermal, chemical and enzymatic agents [9]. This is one reason for their potential as candidates for drug design [10].

According to the presence or absence of a cis-proline residue in loop 5, cyclotides have been classified into two sub-families, called Möbius and bracelet [11].

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Cyclotides are thought to be plant defense peptides against pathogenesis and pests [12]. However, these peptides have got variety of other biological activities such as anti-HIV [13], cytotoxic [14], anti-cancer [14], hemolytic [15], neurotensin antagonism [15], anti-microbial [16], anti-fouling [17] and immunosuppressive [18-20] effects.

More than 300 sequences of cyclotides have so far been discovered from the species of *Violaceae, Cucurbitaceous, Fabaceae, Solonanaceae, Poaceae* and *Rubiaceae,* [21] and have been documented in CyBase (www.cybase.org.au); however, it is expected that more than 50000 cyclotides exist in nature [22].

In the current study, we screened three species of *Violaceae*, including *Viola odorata*, *V. ignobilis* and *V. occulta* for the sequences of cycloides. We also determined the anti-microbial activity of semi-purified cyclotides extracted from *V. ignobilis*; and analyzed the effect of cyclotides on soil bacteria.

In this study, we compared the gene sequences of cyclotides from three different species of *Violaceae*. The semi-purified cyclotides obtained from *V. ignobilis* used to evaluate the anti-micribial activity. Tricine-SDS-PAGE determined the presence of cyclotides in each fraction.

Materials and methods

Plant material and DNA extraction

Aerial parts of species were collected from Mazandaran province (*V. ignobilis* and *V. odorata*), Zanjan province (V. occulta) Iran during spring. Voucher herbarium specimens (MPH-1025, MPH-1026 and MPH-1027) were deposited in the herbarium of Medicinal Plants and Drug Research Institute, Shahid Beheshti University, Tehran, Iran. Total DNA was isolated using C-TAB buffer using a protocol described by Pirtila et al. [23].

Primer designing and PCR

The primers were designed based on the conserved domain of gene sequences in the vbc family (F: 5′-ACC GTC ATC TCA AAT CC-3′, R: 5′-CGA GAG AGT TCC TGT AGC-3′), kalata family (F: 5′-GTG GTC AAT GAW ATC GCT G-3′, R: 5′-GAG TGT TGC AAG TTC CC-3′) and mra family (F: 5′-ATG TGA TCA CCC CTG AAG CTC TTG-3′, R: 5′-AAG AGC ACC CAA TAG CGC TAG TGA-3′) of cyclotides, which were documented in NCBI. These nucleotide sequences located in N-terminal repeat (NTR) domain. The PCR parameters were as below: denaturation at 94°C for 5 min, 30 X (denaturation at 94°C for 1 min, annealing at 57°C – 72°C {based on primer} for 1 min, and elongation at 72°C for 1 min) elongation at 72°C for 20 min. The PCR products were purified by gel electrophoresis and cloned into the A3600 vector using the Promega A3600 kit (USA). Then they were transformed into *E.coli* DH5α cells. Plasmid was extracted using the Bioneer extraction kit (South Korea) and sent for sequencing to Bioneer (South Korea).

Cyclotide extraction

25 g of powdered plant material (*V. ignobilis*) was extracted 5 times by 300 ml CH_2Cl_2. The solution was discarded and the plant residue was dried at room temperature overnight. The dried plant was extracted 3 times with 400 ml 50% EtOH, and then the solution was concentrated to 400 ml and acidified by 2% CH_3COOH. By using a polyamide column, tannins were removed. The tannin free extract was concentrated and then partitioned 3 times with 100 ml C_4H_9OH. The resulting BuOH phases were collected and concentrated to half the initial volume and then lyophilized [24].

Solid phase extraction

Further purification was achieved by solid phase extraction (SPE) using C18 flash cartridges. 25 mg of botanical extract powder which obtained from previous step was dissolved in ammonium acetate buffer (50 mmol l^{-1}, pH=8) and loaded onto C18 SPE (MACHERY-NAGEL, Germany) cartridge. The cartridge was first activated with methanol and equilibrated with the same buffer [24, 25]. For releasing of hydrophobic cyclotides, the column was washed with 4 ml of 20%, 50% and 80% aqueous EtOH. Purity and concentration of the peptides was determined by Tricine-SDS-PAGE and Bradford assay [26].

Tricine- SDS-PAGE

Tricine-SDS-PAGE was performed using the protocol described by Schagger and Jagwa [27, 28] in order to determine the molecular weight of cyclotides. In previous studies, their molecular weight has been reported between 2500 and 3500 Dalton.

Bacterial strains and media

Three groups of bacteria were used in this study. The first group consisted of the human pathogenic bacteria *Escherichia coli* ATCC25922, *Staphylococcus aureus* PTCC1431 and *Pseudomonas aeruginosa* ATCC27853. The second group consisted plant pathogenic bacteria *Xanthomonas oryzea*. The third group contained soil bacteria *Rizobium cicil* and *Bacillus sp* (Table 1). Group 1 bacteria were grown on nutrient agar (MERCK, Germany), and groups 2 and 3 were grown on Trypticase soy agar (MERCK, Germany).

Radial diffusion assay (RDA)

Radial diffusion assay (RDA) was used to examine the anti-microbial potential of the extracted peptides. First, the bacteria were cultured on TSB (Tryptic Soy Broth); after reaching to optimum density, they were plated and washed by cold sodium phosphate buffer. Nearly 4×10^6 cfu were added to the bottom layer of the media containing 0.03% w/v TSB, 1% w/v LE agarose and 0.02% v/v tween 20. The mixture was added to Petri dish (85 mm). Finally, 5 μl or 10 μl of semi-purified cyclotides sample or control (5 μl 10% DMSO) was added to 3 mm gel punch wells. The mixture was incubated at 37°C for 3 hours in order to diffuse the peptides. Then the upper layer of media (6%

Table 1 Strains of bacteria used in the current study.

Bacteria strain	*Standard number*	*Description*
Escherichia coli	ATCC 2592	G-, human pathogenicbacteria, antibiotic resistance
Staphylococcus aureus	ATCC25923	G+, human pathogenic bacteria, antibiotic resistance
Pseudomonas aeruginosa	ATCC27853	G-, human pathogenic bacteria, antibiotic resistance
Xanthomonas oryzea	(isolated)	G-, plant pathogenic bacteria
Rizobium cicil	(isolated)	G-, soil beneficial bacteria, fixation of N^2.
Bacillus sp.	(isolated)	G-, soil beneficial bacteria, production of cellulose

w/v TSB, 1% w/v agarose in 10 mM SPB) was added to the plates. The plates were incubated at 37°C overnight [1].

Results

Sequences of cyclotides

Total DNA was screened for the presence of vbc, mra and kalata cyclotides in the studied species. The primers were designed based on the conserved sequence of NTR in each family. These regions are preceded by the NTR regions conserved between the plant species. NTRs form a structurally conserved $^{\alpha}$-helix motif. This structural conservation suggests a vital role for the NTR in the *in vivo* folding, processing, or detoxification of cyclotide domains from the precursor protein [29]. The PCR results proved the presence of vbc bands in three species (Figure 1A), but the kalata bands existed just in *V. ignobilis* (Figure 1B), and mra bands did not exist in any of the three species (Figure 1C). Automated sequencing and Blast

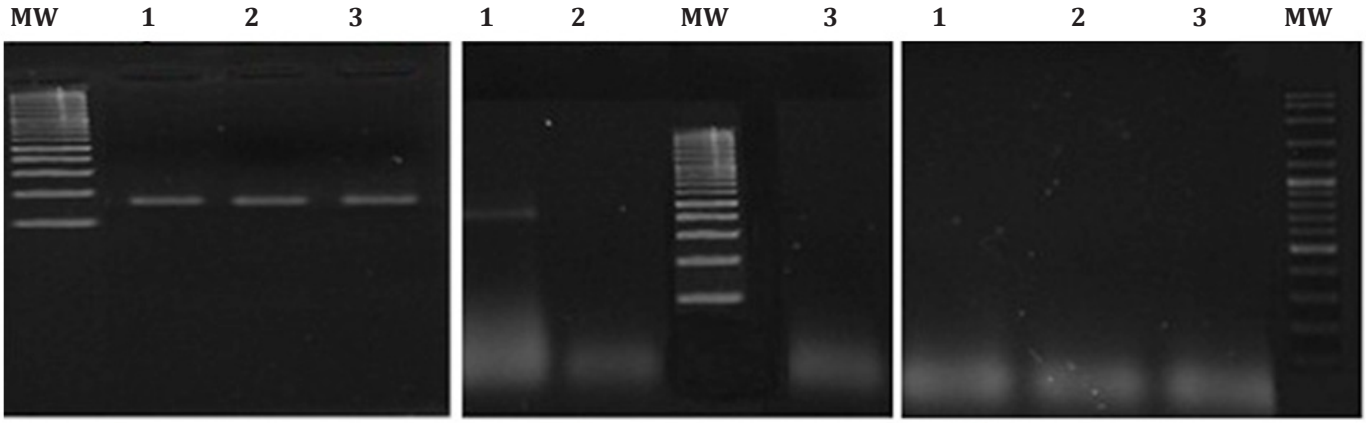

Figure 1 (A) PCR amplification of the vbc primer to the genomic DNA. Lane 1: cyclotide O1 from *V. odorata* lane 2: cyclotide I2 from *V. ignobilis* and lane 3: cyclotide C3 from *V. occulta*. Lanes 1, 2 and 3 have been used for cloning; **(B)** PCR amplification of the kalata primer to the genomic DNA, lane 1: *V. ignobilis*. Lane 2: *V. odorata* and lane 3: *V. occulta*; **(C)** PCR amplification of the mra primer to the genomic DNA, lane 1: *V. ignobilis*. Lane 2: *V. odorata* and lane 3: *V. occulta*.

alignment showed that the most similar cyclotide to the obtained sequences is vbc 6. The identity of cyclotide O1 (novel sequence obtained from *V. odorata*) is 94%, and the identity of cyclotide I2 (sequence obtained from *V. ignobilis*) is 88%; this Figure for cyclotide C3 (sequence obtained from *V. occulta*) is 89%, all of them have been compared to vbc 6 (Figure 2) . While molecular aspect of *V. ignobilis* and *V. occulta* were studied for the first time,

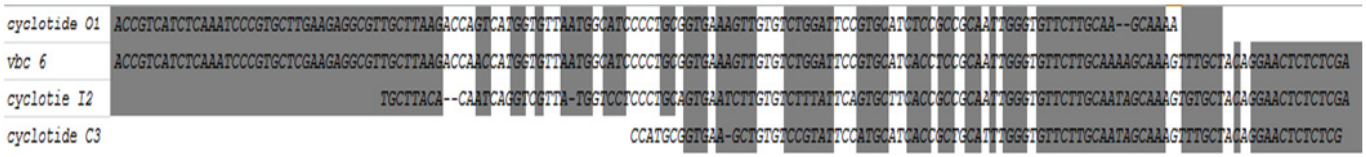

Figure 2 The alignment of nucleotide sequences. Dark grey: identical nucleotides, white: different nucleotides.

V. odorata studied widely and many cyclotides already reported in this species. The amino acid sequences were predicted using Blast X, and the predicted sequences showed that cyclotide 2d and vbc 6 is the most similar peptide to cyclotide I2, cyclotide C3 and cyclotide O1, respectively. Figure 3 shows the alignments. However, there are some differences, which will be discussed.

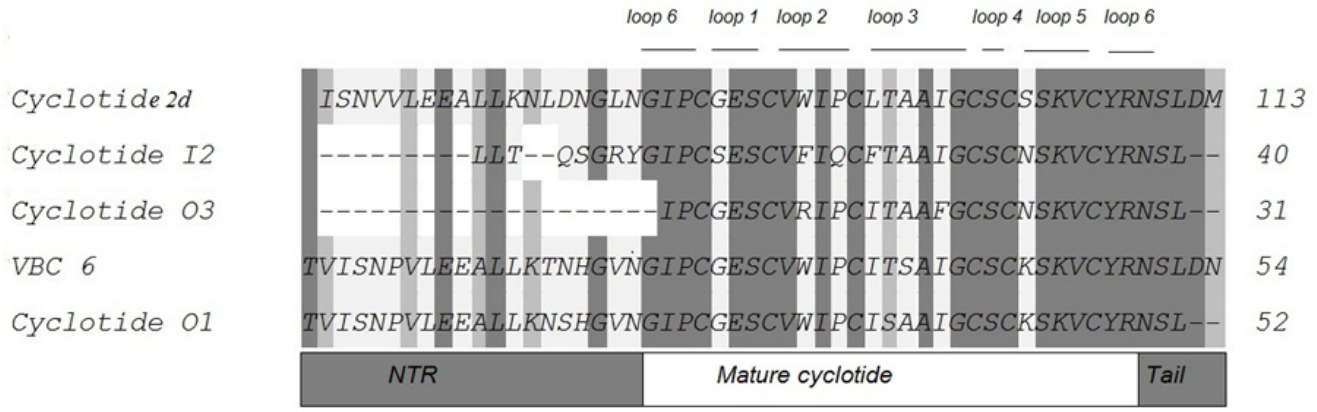

Figure 3 The alignment of predicted sequences with the most similar sequence in the GenBank. White: different amino acid residues, Light grey: similar amino acid residues, and Dark grey: identical amino acid residues.

Cyclotides isolation

To obtain cyclotides from *V. ignobilis* fractionation protocol (FP), solvent-solvent partitioning (SSP) and solid phase extraction (SPE) method were used. Tricine-SDS-PAGE determined the molecular weight of peptides to be about 4800 Da (Figure 4). Molecular weight of cyclotides has been reported in the range of 2500 and 3500Da.

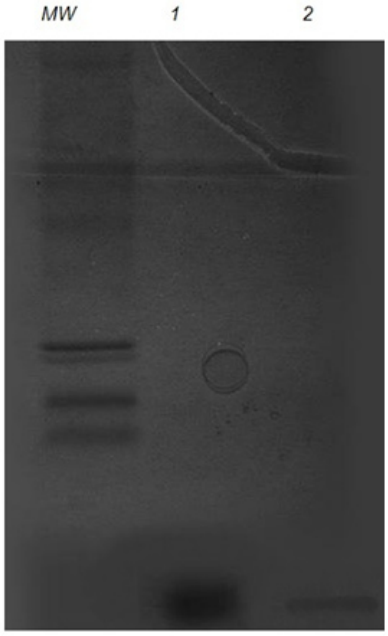

Figure 4 Tricine SDS-PAGE: Lane 1: 50% aq EtOH; Lane 2: 80% aq EtOH.

RDA assay for the human pathogenic bacteria

Previous studies have demonstrated that RDA is a useful method to examine the anti-microbial activity of various substances [30]. We have thus used this method to assay the antimicrobial activity of 20%, 50% and 80% aqueous EtOH peptide extracts. Activity against *E. coli, Staphylococcus aureus, Pseudomonas aeroginosa* and the diameter of clear zones is shown in Table 2. The concentration of the extracted peptides was 70, 40 and 25 μg/ml for 50%, 80% and 20% aqueous EtOH respectively. Semi-purified peptides inhibited the growth of *S. aureus* as gram positive bacteria, resulting in the zone sizes of 0, 10 and 4 mm for 20%, 50% and 80% aqueous EtOH, respectively. However the inhibition zones for *E. coli* and *P. aeroginosa* as gram negative bacteria are not completely clear. Furthermore, 50% aqueous EtOH was revealed to be the most potent buffer to inhibit the growth of bacteria. The results showed that *S. aureus* is more susceptible to the extracted cyclotides than *E. coli* and *P. aeroginosa.*

Table 2 Diameter of inhibition zone (mm) for studied species.

Species	*80% aqueous EtOH*	*50% aqueous EtOH*	*20% aqueous EtOH*
Staphylococcus aureus	4	10	0
Escherichia coli	0	0	0
Pseudomonas aeroginosa	0	0	0
Xanthomonas oryzea	9	13	12
Bacillus sp.	5	8	0
R. cicil	6	9	5

RDA assay for plant pathogenic bacteria

The relatively high concentration of cyclotides in plant tissue, the presence of several isoforms within one plant as well as geographical and seasonal variations in expresson suggest that cyclotides may have a defensive role in their host plant [2]. So these peptides should be more potent against plant pathogens.

In order to verify this theory, we used *Xanthomonas oryzea*. The results are shown in Table 2. As it can be seen, in the same concentration, *X. oryzea* is more susceptible to the extracted peptides than all of the other bacteria examined in this study. The diameter of the clear zone for 50% elution buffer is 13 mm. In addition this bacterium is susceptible to 20% aqueous EtOH with the lowest concentration of cyclotides.

RDA assay for the soil beneficial bacteria

Various biological activities have been reported for cyclotides, but there are limited studies about their environmental effects. In this study, we determined

the activity of the extracted cyclotides against the soil beneficial bacteria such as Bacillus sp. and Rizobium cicil which contribute to O_2 fixation and cellulose production, respectively. The results are shown in Table 2. As it can be seen, the *Bacillus* sp. and *R. cicil* are less susceptible to the extracted peptides than plant pathogenic bacteria. The diameter of clear zone for 50% aqueous EtOH is 8 and 9 mm against *Bacillus* sp. and *R. cicil*, respectively.

Discussion

Cyclotides are peptides from the gene-coded plants. These mini-proteins have different biological properties, including anti-HIV, anti-microbial and cytotoxic effects. Because of their unique scaffold, cyclotides have exceptional stability against protease, thermal and chemical agents, so they have attracted much research interest for drug design and protein engineering.

Sequence analysis

In the current study, we screened *V. odorata, V. ignobilis* [31] and *V. occulta* for tracing the cyclotides. Recent studies have proven that cyclotides expression varies in different tissues, and that within the same tissues, their expression also varies with seasonal and environmental changes [32]. In this study, we compared the three sequences of cyclotides in studied species. In these species, there was a sequence with high similarity to vbc 6. Since cyclotides play defensive role in their host, depending on the environmental conditions, as the results of this study showed, their content is different. Cyclotids' gene in *V. ignobilis* and *V. occulta* has not been investigated before. The results showed there are some differences in their nucleotide and amino acid sequences, which can affect the activity of peptides. All of three reported peptides in this study, based on the absence of cis-Pro in loop 5, are categorized in the bracelet sub-family. Previous studies have illustrated the predicted orientations of bracelet and Möbius cyclotides in a lipid bilayer [10]. While Möbius cyclotides interact with the membrane by loops 5 and 6, bracelet cyclotides interact by loops 2 and 3. Since peptides interact with membrane via hydrophobic residues, so the number of hydrophobic residues in the connected loops has a key role in the cyclotides' activities including anti-HIV and anti-microbial effects. Therefore, differences within these two loops were checked with the most similar peptides documented in the GenBank.

In cyclotide O1 within loop 3 we observed Ala 16 while in the same position in vbc 6 which is the most similar peptide to the cyclotide O1 Ser was reported. In addition, Thr 15 was replaced with Ser. Because of hydrophobic property of Ala, cyclotide O1 could bind to the membrane more effectively than vbc 6. Since the side chain's property of Thr and Ser is the same, this replacement did not affect the binding ability of peptides. Cyclotide 2d is the most similar peptide to cyclotide C3 and cyclotide I2. About cyclotide C3, in compare with cyclotide 2d, Trp was replaced with Arg within loop 2. Hydrophobic property of Arg is less than Trp. In addition, Ile was replaced with Phe within loop 3 both of them are hydrophobic residues. The intermolecular reaction in loops 2 and 3 determined the interaction of peptide with the bilayer membrane. In cyclotide I2, within loop 2 we observed Phe 10 while in the same position in cyclotide 2d Trp was reported. The side chain's property of these residues is the same. Also Leu was replaced with Phe within loop 3 to compare with cyclotide 2d; both of them are hydrophobic residues. These replacements may change the interaction of peptide and membrane; the anti-microbial effect of peptide could also change.

Anti-microbial assay

Cyclotides are remarkable for having multitude biological activities, and their anti-microbial activity has been examined in many studies. In the current study, we assayed this activity of cyclotides (peptides extracted from *V. ignobilis*) against human and plant pathogenic and soil beneficial bacteria. Fifty percentage of aqueous EtOH was the most potent fraction. The inhibition zone for *E. coli* and *Pseudomonas* aeroginosa was not clear, though in the same concentration (70μg/ml), the diameter of the inhibition zone for *Staphylococcus aureus* as gram positive bacterium was 10 mm. The antibacterial properties of cyclotides have been reported in previous studies, though with contradictory results. Pranting et al. examined the activity of cyO2 and kalata B1 against some gram positive and gram negative bacteria. They reported the most susceptible bacterium was *E. coli*, and none of the above cyclotides had high activity against *S. aureus* [1]. Gran et al. reported that kalata B1 which had no effect against *S. aureus* while it was active against gram negative bacteria [33]. There is some reason for these contradictory results. It is possible that the susceptibility between the studied strains was different. Other explanation could be the synergism effect of the total cyclotides used in the present study. Since, the environmental conditions and location could determine the cyclotides' content as defensive peptides; the content of cyclotides used in current study might be totally different in sequences and effects with that of the cyclotides that were used in printings' study. As shown in the sequence result in the previous section, it could be the other explanation for these contradictory results.

In the study by Tam et al. [16], antibacterial activities of four synthetic cyclotides were determined against different bacteria (four gram-negatives and two gram-positives); these synthetic cyclotides were active against gram positive bacteria such as *S. aureus*. However, they were almost not active against gram negative bacteria. The synthetic cyclotides used by Tam were likely to have different properties comparing with the cyclotides isolated from the plants; for instance, their structure or disulfide bonds.

Zarrabi et al. examined the anti-bacterial activity of total cyclotides extracted from *V. odorata* against *E.coli, P.*

aeroginosa and *S. aureus.* The most susceptible bacterium was *S. aureus* [29]. The sampling locations of current study and Zarrabi's study were very close to each other and this is likely to be the reason for similar results.

In current study the most susceptible bacterium to the extracted peptides was *X. oryzea*, plant pathogenic bacteria (the diameter of clear zone was 13 mm). It is another evidence for defensive role of cyclotides in their host.

There are many applications for cyclotides in agriculture provided that they have no bad effects on the soil bacteria. As we showed, the susceptibility of soil benefit bacteria was less than that of plant pathogenic bacteria. This suggests that cyclotides are potent to use in agriculture in concentrations that have no negative effect on the soil bacteria, or using protein engineering to minimize the undesirable effects.

The results of this study indicated that obtained sequences in studied species have high similarity to vbc, but there are some differences in their sequence, and it is the most likely cause of their different activity against gram positive bacteria. Environmental differences can cause these dissimilarities. Because of less susceptibility of soil bacteria against semi-purified peptides compared to plant pathogenic bacteria, we suggest cyclotides as proper candidates for control pest.

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Conflict of interest

The authors wish to express that they have no conflict of interest.

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