Plants Defense-related Cyclic Peptides: Diversity, Structure and Applications

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Accepted: August 15, 2016

Abstract: Plant growth is prone to several unfavorable factors that may compromise or impair development and survival, including abiotic or biotic stressors. Aiming at defending themselves, plants have developed several strategies to survive and adapt to such adversities. Cyclotides are a family of plant-derived proteins that exhibit a diverse range of biological activities including antimicrobial and insecticidal activities that actively participate in plant defense processes. Three main categories of peptides have been described: (i) Cyclotides (ii) Sunflower Trypsin Inhibitor (SFTI) and (iii) peptides MCoTI-I and II, from *Momordica cochinchinensis*. They comprise proteins of approximately 30 amino acids, containing a head-to-tail cyclized backbone, with three disulfide bonds configured in a cystine knot topology, therefore bearing greater peptide stability. Given their features and multifunctionality, cyclotides stand out as promising sources for the discovery of new antimicrobial agents. The present review describes cyclotide occurrence, abundance and action in plants, also their diversity and evolution. Considerations regarding their use in the context of biomedical and agronomical sciences uses are also presented.

Keywords: Cyclotides, MCoTI-I/II, SFTI, disulfide bonds, protease mediated defense.

1. INTRODUCTION

Along their evolutionary processes plants have naturally developed effective and widely diversified mechanisms (including the production of a series of primary and secondary compounds) to defend themselves against the attack from a wide variety of pathogens, as fungi, bacteria and viruses [1, 2]. Usually, the plant protection is accomplished after pathogen recognition, followed by activation of defense mechanisms that help overcoming the deleterious effects caused by the invading microorganism. Even if the plant cells are able to recognize a variety of molecular signatures of microorganisms, the speed and efficiency of this activation are essential for the success of plant defense [3]. Regardless of whether the defense mechanism is constitutive or induced (by pathogenic microorganisms), defense responses in plants involve gene activation and induction of a signal transduction network, as well as responses related to abiotic stress [4], leading to the establishment of physical and chemical barriers.

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Among the main mechanisms used in the course of plant defense some should be highlighted: Hypersensitive Response (HR), Systemic Acquired Resistance (SAR), induction of Pathogenesis-Related proteins (PR-proteins) and synthesis of signaling compounds such as salicylic acid (SA) and hydrogen peroxide (H_2O_2). HR is characterized by a rapid and localized response that occurs at the site of infection by the pathogen. This process leads to a "collapse" of the tissue due to toxic compounds released around the infection site. Such compounds may also act directly on the pathogen, causing its death [5, 6]. SAR mechanism involves a cascade of signaling events (associated to the plant-pathogen interaction) promoting changes in cell metabolism, resulting in reduced disease severity [6, 7].

A wide variety of organisms are also able to synthesize antimicrobial peptides (AMPs) that work in the first line of defense. These peptides belong to the PR-families PR-12, PR-13 and PR-14 [2] and are characterized by the presence of cysteine (Cys) residues (4, 6 and 8) connected by disulfide bridges, providing structural stability to the molecule [8]. Expressed constitutively or induced by biotic stimuli, AMPs

are associated with a wide spectrum of biological roles, with emphasis on their antimicrobial activity [9, 10] in most living organisms, including humans [11].

Reports indicated effectiveness of these molecules as inhibitors of digestive enzymes such as serine protease [12] and α-amylases [13]. Another representatives are known to inhibit protein synthesis [14, 15], besides demonstrating efficacy as inhibitors of viruses and cancer cell growth [16-18], with the advantage of generally presenting a low toxicity to mammalian and plant cells [19, 20]. AMP properties reflect their genetic and protein structure (i.e., small proteins, 12-50 aa; ~50 kDa; encoded by single genes; presenting positive charge, cationic at physiological pH, due to lysine, arginine and histidine excess), commonly rich in a type of amino acid and assigned in several subclasses that share some features responsible for antimicrobial activity, tolerance to acids and organic solvents, thermal stability, and broad biological activity) [21-25]. Even though AMPs present quite similar dimensional structure and physical properties, they generally exhibit low sequence homology, especially when comparing distinct peptide classes [26]. However, members of a given family show a relatively conserved folding pattern. They adopt a three-dimensional structure that involves the formation of secondary structural elements (beta-sheets and alpha helices) stabilized by intramolecular disulfide bridges, forming a rigid structure and giving, therefore, greater stability [2, 27].

Despite the great structural diversity displayed by AMPs, they may be grouped into distinct classes considering their secondary structures [6]. The prevalent structures regard α-helices, formed only when the peptide is in contact with the cell membrane, being the β-sheets stabilized by 2-4 disulfide bonds that occasionally present a small portion of α-helical structure. On the other hand, the less frequent structures are bent, formed by simple disulfide bonds or due to the presence of proline residues [28] or still extended, characterized by the predominance of one or two amino acid residues in the primary sequence. Major plant AMP groups are represented by cyclotides, defensins, lipid transfer proteins (LTPs), snakins, thionins, hevein- and knottin-type of protein [2, 20]. Some of these AMPs play an important role in biocontrol of plant diseases, as verified in transgenic plants expressing partial or total resistance [29].

A fascinating plant AMP subgroup includes the cyclic peptides (CPs). They regard proteins ranging from 12 to 80 amino acids, with a “head-to-tail” cyclic backbone, stabilized by one or more disulfide bonds [30]. Such physical and chemical properties have shown advantages over open-chain (linear) peptides [31]. The structural conformation is created due to the lack of free N- and C-termini, creating a continuous, rigid and stable structure, resistant to thermal degradation and cleavage by proteolytic enzymes [32-34]. Moreover, the cyclization of peptides and proteins reduces their flexibility, giving to such compounds a higher binding affinity to receptors [35].

This review will focus on the current knowledge of main cyclic peptides described in plants with a known role in plant-pathogen defense: Cyclotides, SFTI (Sunflower Trypsin Inhibitor) and the MCoTI-I/II (*M. cochinchinensis* Trypsin Inhibitors I and II, also referred to as cyclic knottins).

2. PLANT CPS WITH ANTIMICROBIAL PROPRIETIES

2.1. Structural Aspects and Classification

Over the years, hundreds of linear peptides, including defensins, thionins, glycine-rich proteins, snakins, 2S albumin-type and hevein-like proteins, have been discovered and characterized [36-39]. According to Colan and Anderson [40], the three major classes of plant CPs include (i) small CPs such as Segetalina A (*Vaccaria segetalis*) [41], (ii) a class of trypsin inhibitors named SFTI (Sunflower Trypsin Inhibitor) and (iii) cyclotides. In turn, Thorstholm and Craik [30] grouped the CPs into only two classes: SFTI (Sunflower Trypsin Inhibitor) and cyclotides. The present review will focus on features of CPs considering three groups: (i) cyclotides, (ii) SFTI (considered a homologue of the Bowman-Birk family) and (iii) MCoTI-I / II (both treated as members of the Squash TI family).

2.2. Cyclotides

Cyclotides were originally discovered in extracts of the medicinal plant *Oldenlandia affinis* (Rubiaceae), known as “kalata-kalata” by the indigenous healers in Africa [42] where the plant was used as uterotonic agent (causing uterine contractions) to accelerate child birth [43]. In the early 1970’s studies uncovered the partial sequence of this peptide, identified as “Kalata B1” [44, 45]. Since then, for several years many related peptides were identified [46-50]; part of them, characterized as “macrocyclic peptides”, today known as cyclotides or cyclo-peptides [51].

The cyclotides are one of the largest families of circular proteins. They present 28 to 37 structurally cyclic amino acids (head-tail type), containing six conserved cysteine residues (numbered from I to VI) and linked in a "node" topology by three disulfide bonds, forming six loops [39, 52]. This configuration occurs due to disulfide bonds between cysteine bridges at established positions, i.e., CysI-CysIV and CysII-CysV, whereas the third bridge (CysIII-CysVI) penetrates the ring, promoting the cyclization of the molecule [53]. The shared structural motif between cyclotides regards Cyclic Cystine Knot (CCK), whereas it is evident that the six loops represent topologically key regions for its biological activity (Fig. **1**) [51].

Cyclotides are divided into two subfamilies: Möbius and Bracelet. The subfamily Möbius (assigned in honor to the mathematician August Ferdinand Möbius) is characterized by a cis-proline peptide binding that occurs in the fifth loop, giving a twist in its tertiary structure. In turn, the members of the Bracelet subfamily do not possess this ligation (due to lack of proline in the corresponding position in their primary sequence), and therefore have no twist, giving them the aspect of a "strap", hence the name Bracelet. However, some conserved residues are common to both subfamilies. The most conserved residue is glutamic acid (E) in the loop 1. Loops 1 and 4 show higher residue conservation, exhibiting a consensus (G/A)E(T/S) in the first loop, whereas for loop 4 a single residue is found for all cyclotide sequences, corresponding to a threonine (T), a Serine (S) or a Lysine (K) (Fig. **2**). Most conserved regions regard loops 1, 4 and 6, based on the sequences of Kalata B1 and cycloviolacin O1

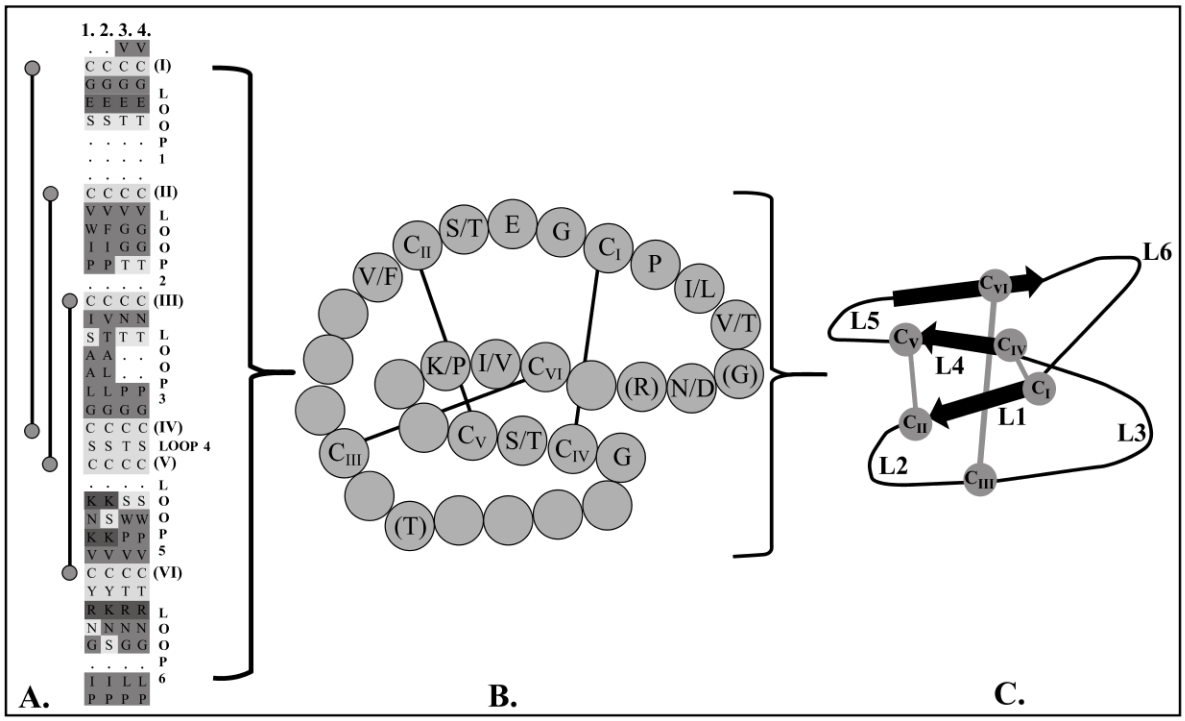

Fig. (1). Schematic illustration of three different cyclotide conformations. (**A**) The arabical numbers above the vertical alignment regard the primary structutre of: (1) Circulin A, (2) Cyclopsychotride A, (3) Kalata B1 and (4) Varv peptide. The conserved cysteine residues are represented by Roman numerals (I to IV), forming three disulfide bonds (vertical black lines). The inter-cysteine loops are numbered from 1 to 6. Other amino acids residues are in grayscale, whereas those chemically similar are showed in same gray intensity; (**B**) Topology of the cyclic cystine knot (CCK) motif and summary of the conserved and variable residues of all known vegetables cyclotides: the disulfide bonds are represented by black lines connecting the cysteine residues (Subscript I-IV); (**C**) Secondary structure of cyclotides, showing beta sheet and disulfide bonds connecting cysteine residues (I-IV Subscript) and the inter-cysteine loops (L1-6).

that represent the families Möbius and Bracelet, respectively. For the loop 6, its conservation is probably related to the ligation or cleavage processing. For example, N and C-terminal connection points occur after the Asn/Asp conserved residue that may play a role in cleavage of the C-terminal tail of the precursor protein, or in the ligation of the extremities or, still, in both [54].

2.3. *Momordica cochinchinensis* Trypsin Inhibitors I and II (MCoTI-I/II)

Other class of knottin (which involves a cystine-knot arrangement of their three conserved disulfide bonds) regards the squash trypsin inhibitor, isolated from squash (*Cucurbita maxima)* seeds [55]. The unique members of this squash family that present a cyclic cystine knot (CCK) are the MCoTI-I and MCoTI-II (*M. cochinchinensis* Trypsin Inhibitors I and II) [56-59]. These peptides were isolated from seeds of both mentioned Curcubitaceae members, which are commonly used in the Chinese traditional medicine against pain and inflammatory processes [60]. Proteins belonging to this family contain 28 to 32 aa residues with six conserved cysteine residues and molecular weight of approximately 3.0 kDa [61]. Although referred to as a member of the cyclotide subfamily due to the presence of a CCK motif [57-63] MCoTI exhibited low sequence homology when compared to other cyclotides and, therefore, was referred by other authors as member of squash trypsin inhibitor group [56, 61, 64], whose reference will also be adopted here. The TI squash family is characterized by containing an inhibitor cystine knot (ICK) [65, 66] composed by small triple-stranded chain of antiparallel β-sheets and a half-turn of a 3_{10} helix, two β turns and the inhibitory loop [61], formed by three disulfide bonds that participate in the stabilization of the molecule. This structural motif comprises a ring formed by disulfide bonds I-IV, II-V and by the third disulfide bond III-VI in the same way as reported by Craik [53] for cyclotides.

MCoTI-I and II present similar sequences to other members of linear squash TI family with the exception of an additional short linker, consisting predominantly of Ser and Gly residues which complete the structure of these CPs (Fig. **3**). The presence of a conserved Gly after the last Cys residue is common to all representatives of the squash TI family (Fig. **3**) and appears to be the processing point of the C-terminus of the molecule. The similarity to CysIV with CysV in Cyclotides and squash TI seems to allow closer positioning of the N and C-termini before cyclization [67].

2.4. Sunflower Trypsin Inhibitor (SFTI-1)

Another small bioactive plant CP was discovered and characterized in the mid-1990s, belonging to the proteinase inhibitor family BBI (Bowman-Birk Inhibitor) [68, 69]. A peptide of this category, the Sunflower Trypsin Inhibitor (SFTI-1) was first isolated from sunflower (*Helianthus annuus*, Asteraceae family) seeds [70, 71], comprising 14-residues with a single disulfide bridge (Fig. **4**) that displays the strongest trypsin inhibitory activity among all Bowman-

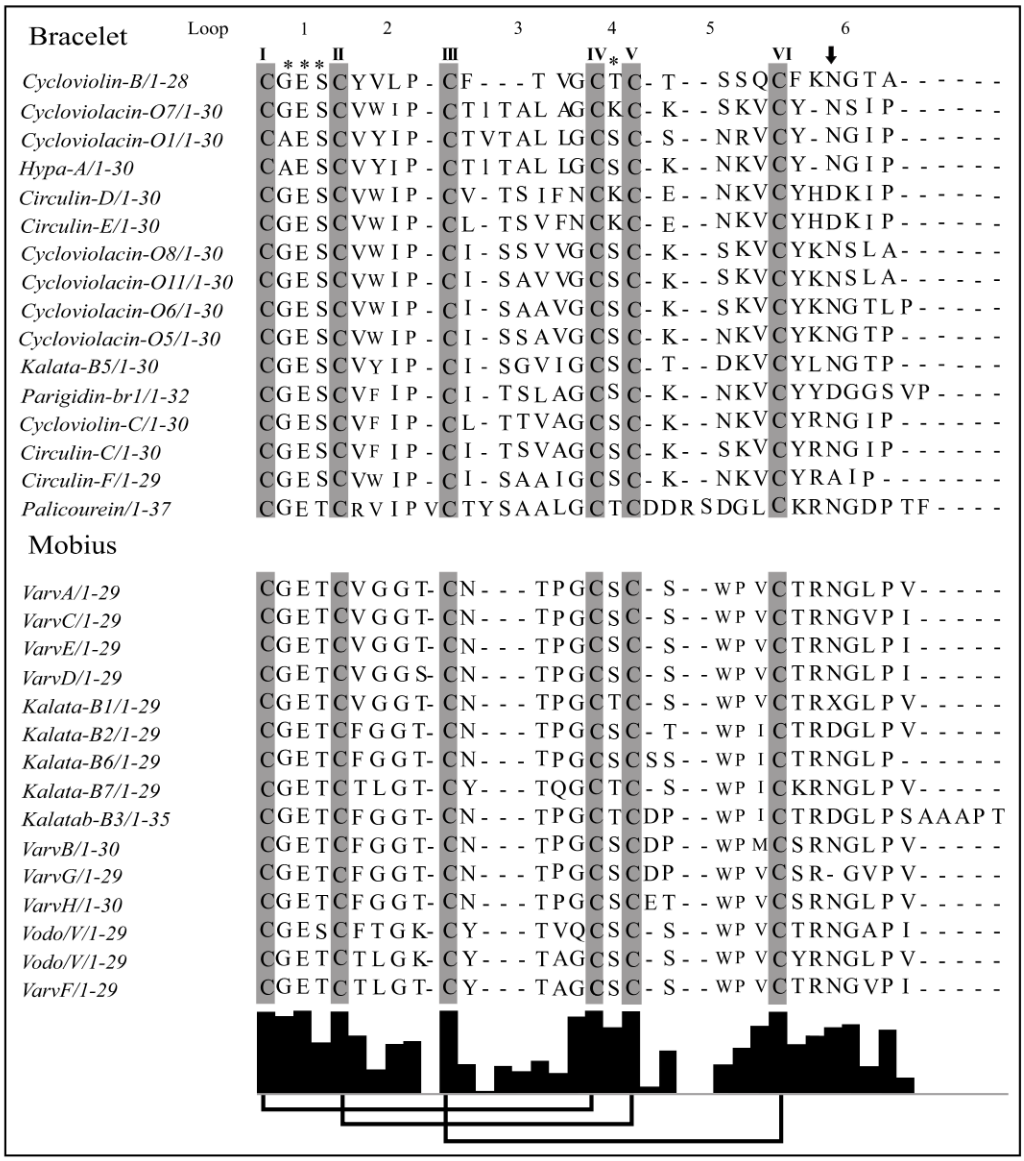

Fig. (2). Alignment of cyclotide sequences available in GenBank (NCBI), showing conserved cysteines residues (gray), represented by Roman numerals (I to VI), forming three disulfide bonds (black lines at the bottom of the Figure). Protein loops are numbered from 1 to 6 at the top of the alignment. Black columns at the bottom indicate conservation. Black arrow indicates residue possibly introduced by mutation that lead to cyclization.

Birk inhibitors [70, 71]. It has been considered that the disulfide bonds and cyclic backbone of SFTI-1 contribute to the activity and stability of this peptide. When the disulfide bond is removed, the trypsin inhibitory activity is considerably reduced [72]. The same occurred when the cyclic structure was opened (between Asp14 and Gly 1) [73] (Fig. **4**).

2.5. Biosynthesis and Cyclization

Plant CPs are encoded and cleaved from a precursor protein prior to cyclization. Its genetic origin, as ribossomal synthesized protein, was firstly reported by Jennings *et al.* [74], with the identification of genes encoding Kalata (B1, B2, B6 and B7) cyclotides from *Oldenlandia affinis* (Rubiaceae), whose precursor protein usually exhibit a basic and highly conserved organization. As illustrated in (Fig. **5A-D**), it includes: (1) a signal addressed to the endoplasmic reticulum (ER), of around 25 aa residues; (2) in the pro-region a N-terminal precursor domain (NTPD) of variable length and with no function currently assigned in regard to maturation of the CP; (3) a small linker region; (4) a mature cyclotide domain; (5) a N-terminal repeat (NTR) region and (6) a C-terminal pro-peptide tail [75, 76]. An exception is the cliotide from *Clitoris ternatea* (Fabaceae), which exhibit an ER signal peptide immediately followed by the cliotide domain (cT7), flanked at the C-terminus by a peptide linker and an albumin a-chain (Ala) (Fig. **5B**) [76]. Also a typical albumin 1 from *Pisum sativum* (pa1) (a plant of the Fabaceae family) presents domains A1a and A1b (Fig. **5A**). The last domain (A1b) encodes a peptide of 37 residues, formed by three disulfide bonds, making them homologous to the cyclotides.

Homology of cyclotides genes for both A1 genes (Fabaceae) and other cyclotide genes from Rubiaceae and

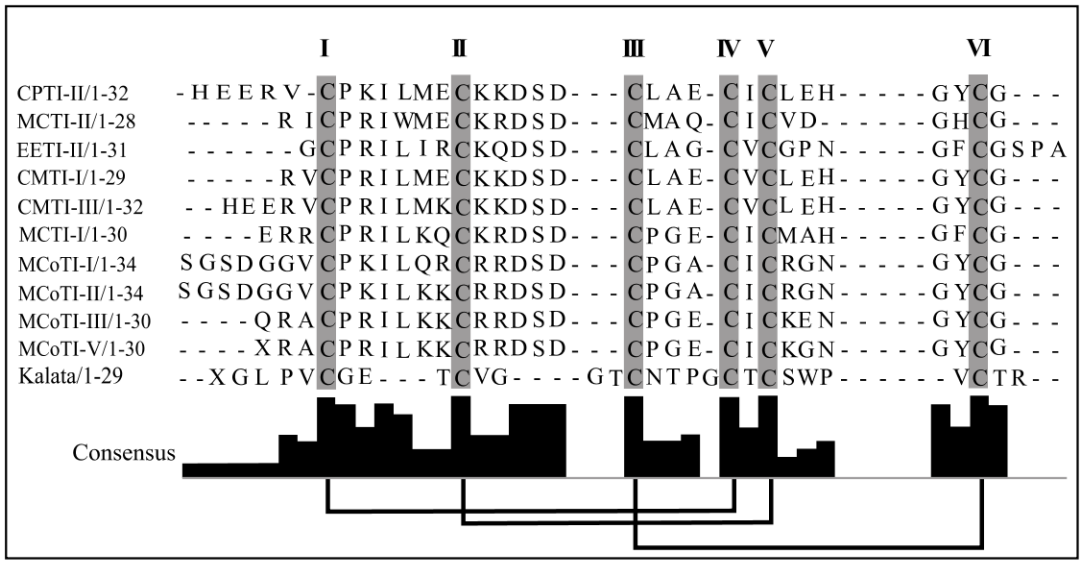

Fig. (3). Alignment of trypsin inhibitors (TI) of the squash family, based on publicaly available sequences (GenBank, NCBI) showing conserved cysteine residues (gray), represented by Roman numerals (I to VI), forming three disulfide bonds (black lines at the bottom of the figure). Legend to species abbreviations: CPTI: *Cucurbita pepo* trypsin inhibitor; MCTI: *Momordica charantia* trypsin inhibitor; EETI: *Ecballium elaterium* trypsin inhibitors; CMTI: *Cucurbita máxima* trypsin inhibitors; MCoTI: *Momordica cochinchinensis* trypsin inhibitors.

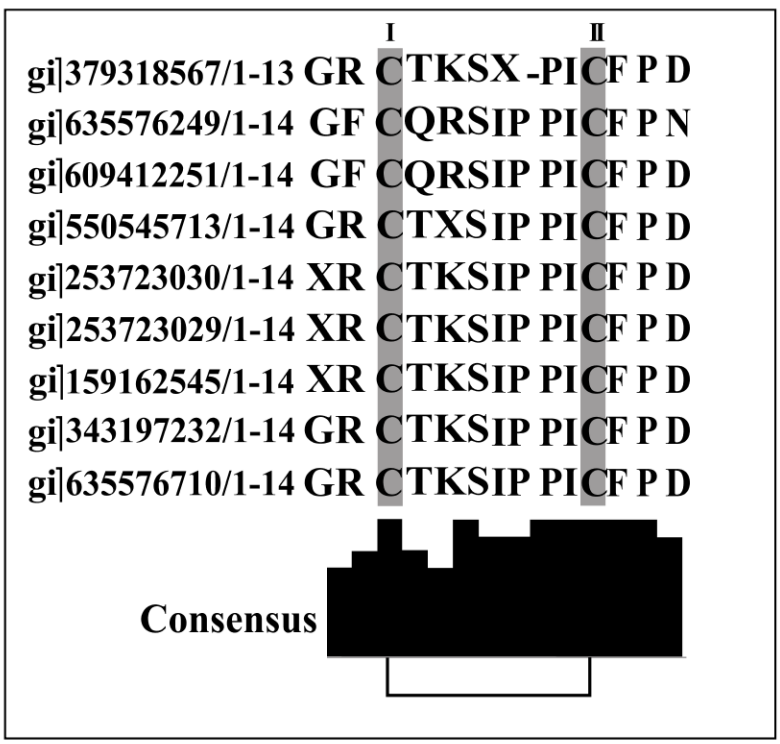

Fig. (4) Alignment of SFTI sequences obtained from GenBank (NCBI), showing conserved residues, cysteines (numbered I-II) and the disulfide bond (gray line at the bottom of the figure). Sequences isolated from *Helianthus annuus* (sunflower).

Violaceae (Fig. **5C** and Fig. **5D**, respectively) reveal its chimeric nature. The absence of NTR region in the structure of cyclotides suggests that NTR is essential for their biosynthesis [76]. Although a wide range of studies on the biosynthesis of these peptides have been carried out [33, 40, 74, 77-81] a lot of aspects remain to be discovered. A common feature is that they all appear to be derived from a precursor protein and are post-translationally processed to produce a cyclic backbone. In general, the mechanism involves a wide variety of amino acids and proteases (from the cysteine or serine class) that catalyze the transpeptidation reactions. In a recent work Craik and Malik [82] clearly described some of the main aspects that underlie the biosynthesis and cyclization of cyclotides. Processing occurs from the transcription of DNA into RNA and translation to prepropeptides.

Steps regarding prepropeptides cyclization (Fig. **6**) include: (Fig. **6-I**) protein is assigned to the ER when the signal sequence is removed and the folding cyclotide precursor

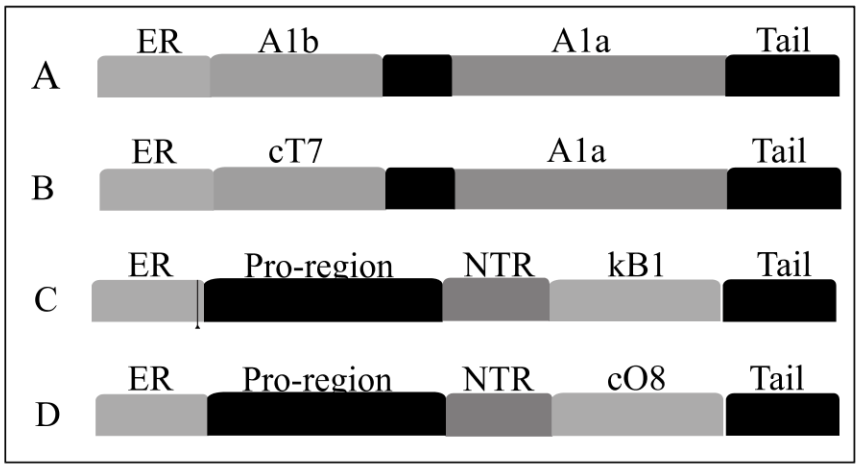

Fig. (5). Schematic representation of chimeric genetic arrangements of cyclotide precursors in different plant families**.** (A) Albumin 1 domain from *Pisum sativum,* Fabaceae (pA1a and pA1b); (B) cyclotide from *Clitoria ternatea,* Fabaceae (cT7)*;* (C) cyclotide from *Oldenlandia affinis*, Rubiaceae (Oak1), including representation of introns (black triangle) and (D) cyclotides from *Viola odorata,* Violaceae (Voc1). ER, endoplasmic reticulum signal; Pro-region NTPD, N-terminal precursor domain; NTR, N-terminal repeat; cyclotide domain and Tail, the C-terminal pro-peptide tail. Note that A1a domain is present only in albumin and cyclotides.

is generated possibly aided by protein disulfide isomerase (PDI); (Fig. **6-II**) shortly after the propeptide is directed to the vacuole where the cyclotide domain is excised and cyclization is presumably promoted by NTR (N-terminal repeat) or NTPP (N-terminal propeptide); (Fig. **6-III)** cleavage occurs at the N-terminus aided by a still unknown enzyme that exposes the amino acid Gly (or Ala); (Fig. **6-IV**) transpeptidation occurs aiming to release the CTR (C-terminal repeat) or CTPP (C-terminal propeptide) and form the CP. This cyclizing is promoted by asparaginyl endopeptidase (AEP) that is specific for asparagine (N) or aspartic acid (D) in the S1 site, followed by a short side chain amino acid (glycine, serine or alanine) and followed by a leucine (L) and a proline (P). The result is shown in Fig. **6-IV** in regard to Kalata B1, presenting in S1', S2' and S3' the following amino acids: glycine (G), leucine (L) and proline (P). After recognition of the sites and their respective amino acids, CTR is cleaved with the formation of an acyl-enzyme intermediate through the AEP attack in the thiol active site of the carbonyl group of the peptide bond. The cleaved CTR leaves the AEP active site, being replaced by an N-terminal region of cyclotide domain whereas the primary amine is a glycine (G) that acts as a nucleophile and attacks the intermediate acyl-enzyme. Finally the enzyme is regenerated and the already cyclized product leaves the active site. The importance of the cyclization model should be highlighted for both the N-terminal tripeptide motif as well as for the C-terminal region flanking the cyclotide domain.

2.6. Mechanisms of Action

A range of studies have confirmed a clear correlation between membrane binding and biological activity of AMPs. In general, this interaction may result in membrane structure alterations, either by forming a discrete pore or by disrupting the bilayer structure, promoting the reorientation of the peptide in the membrane, which may cross the membrane and diffuse into the cytoplasm to reach intracellular targets [83-88].

Regarding the association of AMPs to membranes, several mechanisms of action have been proposed; some of them are represented in Fig. **7**, based on membrane interaction and also pore formation. The most known model regards the "barrel model" (Fig. **7A**), that correspond to the insertion of peptides parallel to the membrane, forming a pore. Another one is represented by the "carpet model" (Fig. **7B**), where insertion portions occurs in the membrane surface, leading to pore formation; or, still, in the "toroidal-pore model" (Fig. **7C**) where peptide helices penetrate the membrane and induce peptide monolayers to bend through the pore, but in this case interspersed with lipids. In addition to these examples, other models have been proposed, such as "disordered-toroidal-pore model" (Fig. **7D**), which occurs with a lower concentration of peptides (compared to the toroidal pore model), arranged in a disorderly manner, forming a pore in the cell membrane; the "membrane thinning model" (Fig. **7E**), where the attraction between the charged phospholipids generate thinner regions and more fragile membranes; the "anions attraction model" (Fig. **7F**) that suggest that AMPs may be able to attract small anions making them cross the membrane so as to cause loss of cell electrostatic potential; while in the "non-lytic depolarization model" (Fig. **7G**) the loss of the electrostatic potential can occur without the attraction of ions. Finally, in the "electroporation model" (Fig. **7H**) peptides affect membrane potential in order to reduce their permeability to various molecules [89].

Although most AMPs interact directly with cell membrane lipids forming transmembrane pores, currently, an increasing amount of studies bring evidence that membrane permeabilization alone appears insufficient to cause cell death and therefore other complementary processes may be involved. More recently it was proposed that AMP driven microbial death can be caused by other mechanisms in addition to membrane disruption, followed by cell lysis. Indeed, evidences indicated that some AMPs can interact with intracellular targets inducing cell damages, by interfering on bac-

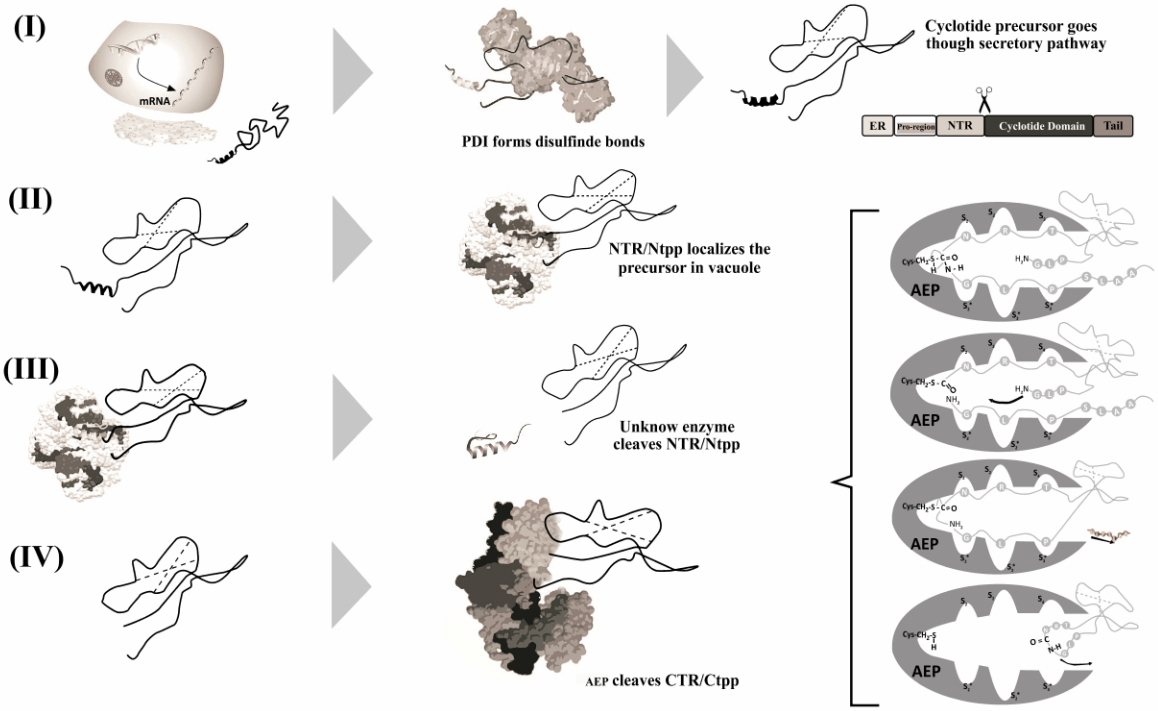

Fig. (6). Schematic representation of cyclotide post transcriptional processing. Hypothetical steps regarding cyclotide intracellular processing (I to IV) and proposed mechanism for AEP mediation in the cyclization reaction expanded in IV. Legend for abbreviations: AEP = Asparaginyl Endopeptidase; NTPP = N-terminal propeptide; NTR = N-Terminal Repeat; PDI = Protein Disulfide Isomerase.

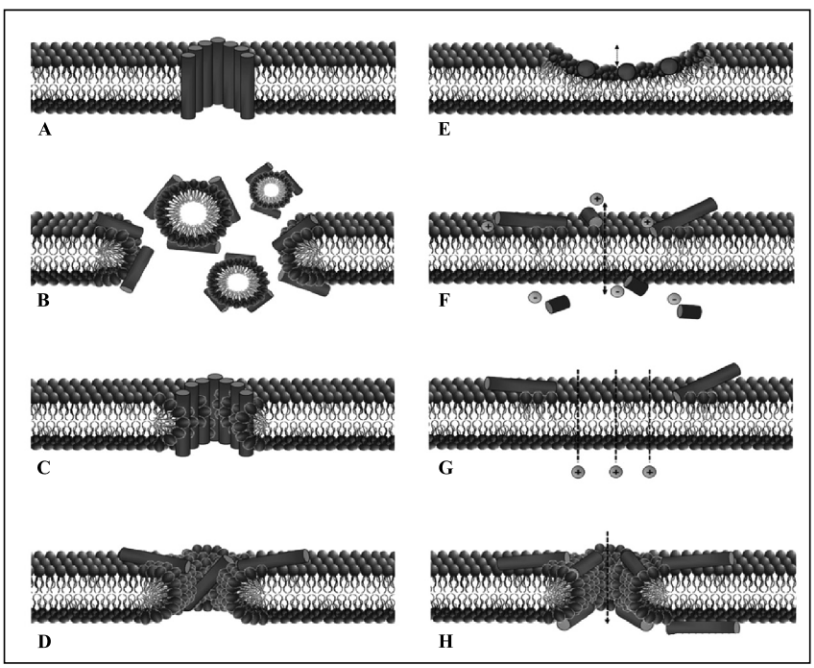

Fig. (7). General outline of the main mechanism of interaction between antimicrobials peptides and pathogen cell membranes. (A) "Barrel" model; (B) "Carpet" model; (C) "Toroidal pore" model; (D) "Toroidal disorderly pore" model; (E) "Membrane thinning" model; (F) "Anion carrier" model; (G) "Non-lytic depolarization" model; (H) Electroporation model.

terial or fungal biosynthesis and biding abilities, also making important lipid precursors of peptidoglycan synthesis unavailable. Suppression of protein synthesis has been also proposed by inhibiting nucleic acid synthesis as well as transcription and, therefore, affecting the translation process [90, 91].

The relationship between the cyclotide structures and their actions with the membrane is increasingly evident [92-98], as summarized in Table **1**. According to Wang *et al.* [92] cyclotides (as monomers or oligomers) bind to the membrane surface and insert deeply into lipid bilayers forming a "barrel-stave" or "toroidal" pore, resulting in mem-

Table 1. Selected examples of antimicrobial peptides and putative modes of membrane interaction.

Groups	AMPs*	Mode of action	Refs
Plants	Cyclotides	Barrel-stave, toroidal pore, membrane thinning	[92, 143]
	Defensins	Barrel-stave	[144]
Mammal	Protegrin I	Toroidal pore	[145]
	Indolicidin	Anion carrier	[146]
	Bovine Lactoferricin	Non-lytic membrane depolarization	[147]
	NK-lysin	Electroporation	[148]
Insect	Melittin	Disordered toroidal pore	[149, 150]
	Cecropin	Detergent micellization	[24]
Fungi	Alamethicin	Barrel-stave pore	[24, 151]

*The peptides listed in this table are not limited to their modes of action and related herein may employ multiple mechanisms simultaneously.

brane disruption. The formation of a structural and defined pore has also been suggested by Pinto *et al.* [93]. In the mentioned study, a bracelet cyclotide from *Palicourea rigida* (Rubiaceae), identified as "parigidin-br1", exhibited a potent *in vivo* and *in vitro* insecticidal activity, reinforcing the role of cyclotide as potential target for bioinsecticide development. Interactions, via membrane thinning and pore-formation have been evidenced in members of the bracelet family (cycloviolacin O2) whereas in Möbius (i.e., kalata B1/B2) pore-formation occurs [94].

Simulations conducted with biophysical characterization and molecular-dynamics confirmed this relationship, suggesting that cationic charges and amphipathicity, as well as variety in the secondary structure, rigidity, and size of the AMPs are essential features for the complexity and diversity of the pore structure and activity of peptide on the integrity of target membrane [99, 100]. Membrane peculiarities between the bodies (i.e., composition of different phospholipids, presence or absence of sterol, presence of fillers) also confer specificities in regard to interactions with peptides [101]. Several studies reported on selectivity of cyclotides for certain subtypes of biological membranes. Regarding Kalata B1, for example, there is considerable affinity for 'disordered' membranes, i.e., more rigid membranes rich in cholesterol and sphingomyelin, being able to break the membranes by formation of multimeric pores [101-103].

Another group of the cyclic peptides of microbial origin (Gram-positive bacteria, phylum Firmicutes) are the bacteriocins. These proteins are synthesized in the ribosomes and act by forming pores in the target cell membrane (i.e., Carnocyclin A). Unlike plant cyclic peptides, bacteriocins present higher molecular weight (approximately 5.6 to 7.2 kDa) [67, 104]. In addition, the antimicrobial peptide RTD-1 (rhesus theta defensin-1, isolated from leukocytes of rhesus monkeys) is also a cyclic peptide. Similarly to SFTI, RDT-1 is a small peptide, composed by 18 amino acid residues, whose six Cys form three disulfide bonds (like cyclotides and MCoTI), but characterized by a Cyclic Cystine Ladder (CCL) motif and presenting more flexibility than plant cyclic peptides here described [67].

2.7. Evolution and Occurrence Among Plants

According to Gruber *et al.* [105] the most parsimonious interpretation for the observed cyclotide distribution within the plant kingdom is convergent evolution, starting from linear precursors of the cyclotide-like type with at least four independent sources within Rubiaceae. Among the plant families with more cyclotide representatives Rubiaceae, and Violaceae stand out, whereas the lack of direct phylogenetic relationships between these groups may indicate that these peptides evolved independently in far related families.

According to Gruber *et al.* [105] differences in structure of precursor cyclotide genes, including the presence of introns in some representatives remove the possibility of lateral transfer, as shown, for example, in (Fig. **5C**) where a representative of Rubiaceae family (Oak1) presents one intron (black triangle) while a member of the Violaceae family (Voc1) presents no intron. Such differences between families of plants evidence the lack of a common ancestor, and therefore turn the possibility of divergent evolution to be unlikely. On the other hand, the discovery of linear sequences in monocots (Poaceae) with similarity to cyclotides [35, 106] have strengthened the hypothesis that an Asn/Asp residue would have been introduced by mutation (Fig. **2**, arrow) in a fundamental region for the cyclization of the linear ancestor near the C-terminus ancestor. It has also been proposed that the cyclization mechanism successfully evolved in molecules related to SFTI (Sunflower Trypsin Inhibitor) of the Curcubitaceae family [56].

Additionally, it has been pointed out that the processing of Squash TI family seems to be variable [107]. As shown in (Fig. **3**), members of the same species, often exhibit almost complete sequence identity with the exception of the addition of an N-terminal segment, which exhibit many glutamine residues (Glu). If the existence of two TI peptides is a result of two distinct genes, or the result of post-translational

modifications is yet unknown, although in the peptides of *Cucurbita maxima* (CMTI) and *Momordica charantia* (MCTI) enough replacements can be observed beyond the N-terminus, suggesting individual genes. Interestingly in MCoTI-III, the presence of a precursor sequence in cyclic versions was considered as a consequence of mutations in the N-terminal region of the precursor protein in the short version of TI resulting in cyclization [67]. It has been also proposed that the cyclization process in *M. cochinchinensis* has been acquired to confer resistance to proteolytic activity and/or to increase the peptide stability [57].

However, it should be emphasized that the linear squash inhibitors present a very stable structure, as in the case of EETI-I that bears a melting temperature of 140˚C [108] and, therefore, the conferred advantage would be increased resistance to exopeptidases. Since there is a linear homolog of *M. cochinchinensis* peptide and if other Curcubitaceae species do not contain cyclic trypsin inhibitors, so it is very likely that cyclization is a relatively recent event, possibly caused by mutations in a precursor sequence [67].

2.8. Databanks and Prevalence of CPs in Plants

Cyclic proteins are widely distributed among living organisms, from prokaryotes to eukaryotes (including fungi, animals and plants) [40]. Cybase - the Database of Cyclic Proteins (http://www.cybase.org.au/index.php; Wang *et al.* [67] - is the most comprehensive databank that includes more than 800 entries from 103 different species.

Using the CyPred (http://biomine.ece.ualberta.ca/ CyPred/.method) method, Kedarisetti *et al.* [109] predicted cyclic proteins in 640 complete proteomes of the three domains of life (Archaea, Bacteria and Eukarya). Depending on the group, 89-98 % of proteomes presented at least one predicted cyclic protein (or 45-56 % of proteomes, if considering high confidence; score > 0.9). However, only a small fraction of proteomes had larger counts of CPs, i.e., only between 7 % (for archaea proteomes) and 16 % (for eukaryotic proteomes) comprised over 10 cyclic proteins. There were no proteomes in archaea with more than 10 CPs predicted with high confidence, while 2 % and 11 % of proteomes in bacteria and eukaryote, respectively, exhibited at least 10 CPs that were identified with high confidence. When only the Eukaria domain was analyzed, a variation in proteome size was evident between species. Considering this domain, animals showed high proportions of CPs with high confidence levels when compared to fungi. In turn, plants presented a wide range of situations, including species that have no putative CP up to some with high number of candidate molecules as in Violaceae and Rubiaceae that exhibit a large number of cyclic proteins reaffirming previous observations of Gruber *et al.* [105].

Among prevalent plant CPs, cyclotides stand out, with 532 entries, regarding 55 different taxa (Cybase: http://www.cybase.org.au/index.php), although it is estimated that this number may well achieve 50,000 representatives [105]. In turn, Sunflower Trypsin Inhibitor (SFTI-1) was the less represented, with only three peptides described for two species (Cybase: http://www.cybase.org.au/ index.php), being considered the most potent BBI inhibitor homolog actually known [73]. Regarding MCoTI-I and II that belong to squash family, they have been grouped within the cyclotides, even though some structural evidences consider this classification as contradictory [110].

3. PERSPECTIVES AND POTENTIAL APPLICATIONS

Based on vast biological properties, such as bactericidal, insecticidal, antiviral, antifungal, nematicidal, molluscicidal, antitumoral and hemolytic, evaluated in different plant species within the families Rubiaceae, Violaceae, Curcubitaceae, Asteraceae and Fabaceae (summarized in Table **2**), CPs represent promising potential targets for agricultural development and pharmaceutical applications [103, 111-114].

Natural and linear peptides are considered promising resources for pharmaceuticals purposes, presenting often perceptible effects and high specificity to physiological targets. Despite of that, some of them have been reported to present some disadvantages with respect to low bioavailability and *in vivo* stability. In addition, peptides have been regarded as expensive to produce compared to drugs with small molecules. In turn, CPs present advantages over their acyclic counterparts. For example, they show resistance to exopeptidases. The cyclization also allows the blocking of conformation of another peptide, reducing entropic losses, thus resulting in a more efficient binding interaction, besides being small, what reduces production costs [33, 114].

Among naturally occurring CPs, there are potent trypsin inhibitors such as the Bowman-Birk inhibitor SFTI-1 and squash family of cystine-knotted peptides [115]. A member of squash family isolated from the bitter gourd *M. cochinchinensis* (MCoTI) and the squirting cucumber *Ecballium elaterium* (EETI) act as molecular scaffolds that are important to design drugs with better stability for uses as, for example, agents for non-invasive molecular imaging of tumors in living subjects [116, 117]. MCoTI are interesting from a pharmaceutical perspective because of their ability to penetrate cells and interact with intracellular targets [118]. Studies carried out by Greenwood *et al.* [119] showed that MCoTI-II is capable to enter human macrophages and are non-hemolytic and non-toxic to human cells. In addition, SFTI-1 presented potential applications in the treatment of prostate cancer because it selectively inhibits human KLK4 (kallikrein-related peptidase 4) [120].

Considering CP toxicity with the RTD-1 peptide indicated low cytotoxic and hemolytic activity in human red cells with concentrations up to 100 μg/mL [121]. Many approaches have tested CP activity against tumor line cells, as in the case of the study by Park *et al.* [122], indicating that most cyclotides present high cytotoxic activity against GTB-line U937 lymphoma cells, while Huang *et al.* [123] using a modified MCoTI-II cyclic peptide identified characteristics required as a model for graft desired bioactivities and identified no cytotoxicity against HeLa cells at concentrations up to 64 μM.

It has been proposed that the natural function of cyclotides is associated to protection of the host plants from pathogens or pests, especially insects [103]. Some studies have been carried out *in vivo* or *in vitro*, to determine the effects of cyclotides on insect survival and development. The

Table 2. Plant cyclic peptides, including their sources and known activities.

Peptide	Source species	Family	Activity	References
Cycloviolacin O12	*Viola abyssinica* *Viola arvensis,* *Viola baoshanensis,* *Viola tricolor,* *Viola yedoensis,*	Violaceae	Antitumor, hemolytic	[152]
Circulin A/B	*Chassalia parvifolia*	Rubiaceae	Antibacterial, anti-HIV, hemolytic	[47]
Circulin C/D/E/F			Anti-HIV	[153]
CT1/CT4	*Clitoria ternatea*	Fabaceae	Antibacterial	[76]
CT2/CT4/CT7/CT12			Cytotoxic, antitumor	[133]
Cyclopsychotride A	*Oldenlandia affinis*	Rubiaceae	Antimicrobial	[127]
Cycloviolacin (Y1/Y4/Y5)	*Viola yedoensis*	Violaceae	Anti-HIV, hemolytic	[134]
Cycloviolacin H3	*Viola hederaceae*		Nematocidal	[140]
Cycloviolacin H4	*Viola hederaceae*		Hemolytic	[17]
Cycloviolacin O1	*Viola odorata*		Anthelminthic, molluscicidal	[137, 140]
Cycloviolacin O13-O14	*Viola odorata*		Anti-HIV, hemolytic, nematicidal	[140, 154]
Cycloviolacin O15-O16	*Viola odorata*		Nematocidal	[140]
Cycloviolacin O2	*Viola biflora* *Viola odorata*		Antibacterial, molluscicidal, cytotoxic, antitumor, hemolytic, Anthelminthic	[17, 138-140, 165, 167]
Cycloviolacin O24	*Viola odorata*		Anti-HIV, hemolytic	[154, 132]
Cycloviolacin O3-O8	*Viola odorata*		Nematocidal	[141]
Cycloviolin A-D	*Leonia cymosa*		Anti-HIV	[135]
EETI	*Ecballium elaterium*	Curcubitaceae	Trypsin inhibitor	[155]
Hypa A	*Hybanthus parviflorus*	Violaceae	Insecticidal	[156]
Kalata B1	*Oldenlandia affinis* *Viola odorata*	Rubiaceae Violaceae	Antibacterial, anti-HIV, hemolytic, insecticidal, Anthelminthic, molluscicidal	[74, 101, 127, 133-137]
Kalata B2	*Oldenlandia affinis*	Rubiaceae	Antibacterial, insecticidal, Anthelminthic, molluscicidal	[103, 124, 157-158]
Kalata B5			Hemolytic	[159]
Kalata B6/B7			Anthelminthic	[139]
Kalata B8			Anti-HIV, antitumor	[160]
MCoTI I/II	*Momordica cochinchinensis*	Curcubitaceae	Trypsin inhibitor	[56, 63]
Palicourein	*Palicourea condensate*	Rubiaceae	Anti-HIV	[161]
Parigidin-br-1	*Palicourea rigida*		Insecticidal	[93]
SFTI-1	*Helianthus annuus*	Asteraceae	Trypsin inhibitor	[70]

(Table 2) cond….

Peptide	Source species	Family	Activity	References
Vaby A/D	*Viola abyssinica*	Violaceae	Cytotoxic	[162]
Varv A/F	*Viola arvensis* *Viola odorata*		Antitumor	[131]
Varvpeptide D/E/H	*Viola tricolor*		Cytotoxic	[163]
Vhl-1	*Viola hederaceae*		Anti-HIV	[164]
Vibi D/E/G/H	*Viola biflora*		Cytotoxic	[165]
Vila A/B	*Viola labridorica*		Cytotoxic	[166]
Vitri B/C/D/E/F	*Viola tricolor*		Cytotoxic	[163]

cyclotides kalata B1 and B2, isolated from *Oldenlandia affinis* (Rubiaceae) presented effects on the development of Lepidoptera (*Helicoverpa armigera*), causing larval growth delay and consequently reducing insect population [74, 124]. The insecticidal activity of cyclotides appears to occur by depletion of cell membranes, as judged from recent microscopic examination of the guts of *H. armigera* larvae after ingestion of kalata B1 in artificial diets [125]. Similarly, the bracelet cyclotide, cycloviolacin O2 caused potent membrane disruption and showed correlation between membrane interaction and biological activity [94]. In a subsequent study, a cyclotide from *Palicourea rigida* (Rubiaceae), named "parigidin-br1", showed potent insecticidal activity against neonate larvae of *Diatraea saccharalis*, causing 60% mortality. The effect of parigidin-br1 was observed on insect cell lines from *Spodoptera frugiperda* (SF-9) and was supported by *in vivo* trials of insecticidal activity [93]. Since cyclotides exhibit potential insecticidal activity, testing against a range of insect species, including agricultural pests, are needed as well as the generation of transgenic plants expressing cyclotides [126].

The antimicrobial activity of cyclotides has been reported by different groups. For kalata B1 conflicting results have been reported. For example, Tam *et al.* [127] reported that kalata B1 was active against *Staphylococcus aureus* and inactive against *Escherichia coli*, whereas Gran *et al.* [128] showed that this peptide had the reverse effect over strains of both mentioned bacteria. Similarly, the kalata peptides KB1, KB2, KB5-9, cycloviolacin O2 and tricyclon A (tcA) presented no activity against *E. coli* and *S. aureus*, except for cycloviolacin O2 that was active against *E. coli*. Interestingly, cyclotides CT1 and CT4 from *Clitoria ternatea* (Fabaceae) showed antimicrobial activity against strains of *E. coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* [76].

Some authors suggested that interaction between cyclotides and microbial cell membrane is salt dependent, and that the lack of antimicrobial activity in some trials may be related to the physiological relevant salt conditions, suggesting electrostatic interactions [101, 127]. No activity was observed against any microorganisms with 100 μM of KB1 in physiologically relevant saline conditions (150 mM NaCl). The lack of antimicrobial activity under physiological salt conditions was consistent with previous studies. In further studies with Kalata B1 when the level of salt (100 mM NaCl) was lower, an action against Gram-positive bacteria was observed [101]. Further studies are necessary to investigate the mode of action of cyclotides given the growing occurrence of antibiotic-resistant microorganisms in human medicine. Moreover, there is a critical need to understand the effect of cyclotides in bacterial plant pathogens [129]. Cyclotides with cytotoxic and antitumoral activities have also been reported, especially from *Viola* species and in *C. ternatea*. Three cyclotides named vitri A, varv A and varv E were isolated from *Viola tricolor* and they all showed cytotoxicity and applicability in cancer therapy [130]. Another CP, the Cycloviolacin O2 isolated from *V. odorata* has been a particular focus of these studies due to its cytotoxic activity against a variety of human tumor cell lines, tested with different primary cultures of human tumor cells *in vitro* relative to normal cells, including myeloma, leukemia, lymphoma and renal adenocarcinoma [131, 132]. Additionally, five cyclotides isolated from *C. ternatea* (named CT2, CT4, CT7, CT10 and CT12) showed significant cytotoxic activity against human lung cancer cells [133].

A raw extract of *V. tricolor* was fractionated, guided by the fluorometric microculture cytotoxicity assay (FMCA) using cancer cells [GTB U-937 (lymphoma) RPMI-8226/s (myeloma)]. After fractioning, three cyclotides (vitri A, Varv A and E) presented cytotoxicity after 72 h treatment depending on the dose. Another test on the effect of seven *Clitoria ternatea* cyclotides in human lung cancer cells of the lineage A549 (and its sub-line A549/paclitaxel) pointed out that five among them showed significant cytotoxicity [130, 133].

Another area of research that is of particular interest regarding the therapeutic use of CPs is the inhibitory activity of some cyclotides against human immunodeficiency virus (HIV). Wang *et al.* [134] identified five new and three known cyclotides from *Viola yedoensi* using RP-HPLC (Reversed Phase-High Performance Liquid Chromatography), and observed the existence of correlation between hydrophobicity and an anti-HIV action of new described cyclotides. They proposed that this trend tracks with their ability to disrupt membranes, which was inferred from hemolytic assays on human erythrocytes. Recently, the bracelet cyclotide cycloviolacin Y5 from the same plant species was demonstrated to be the most potent anti-HIV cyclotide tested so far

[134]. Moreover, cyclotides named cycloviolin and palicourein [isolated from *Leonia cymosa* (Violaceae) and *Palicourea consensata* (Rubiaceae), respectively] also displayed activity against HIV [17, 135].

Circular retrocyclin peptides, synthesized from RTD-1, have antimicrobial and anti-HIV activity, being smaller than cyclotides (only 18 amino acid residues, with six cysteine and three disulfide bonds). Due to their significant stability, cyclotides can be synthesized with a number of substituted amino acids in the sequence, allowing them to be modified or optimized for their inherent activity or, still, to be used as a scaffold for biologically active epitopes. Similar synthetic approaches have been used to create retrocyclins congeners, increasing their antiviral or antimicrobial activity or to select other desirable attributes such as the development of therapeutic or preventive agents [136], thereby allowing cytotoxic effects are controlled. As previously reported, some cyclotides have toxic effects [74, 124, 125, 137-141], although none of these activities are directed to mammals, being therefore not a concern in the development of human therapeutic cyclotides, although the existence of any degree of toxicity should be taken into account. For example, in accordance to Henriques and Craik [142] the toxic effects of Kalata B1 are not an impediment to the future pharmaceutical development synthetically modified cyclotides using bioengineering.

CONCLUDING REMARKS

The importance of CPs for plant survival is evident and their use has been considered for the production of transgenic plants, more resistant to microorganism attack or as natural insecticides, potentially benefiting farmers and considerably reducing losses and costs. In addition, the high biodiversity, small size, cyclic structure, stability and vast bioactivity revealed by cyclotides have triggered a great interest in the pharmaceutical area, as precious source in the production of new drugs.

Although many answers still need to be answered, as those addressed to the evolution of these peptides in plants, surveys are increasingly committed to unravel the evolutionary paths taken by these peptides, especially considering the large scale generation of genomic, transcriptomic and proteomic data, and their use in biotechnological approaches .

LIST OF ABBREVIATIONS

A1b	=	Albumin 1 chain b
aa	=	amino acids
AEP	=	Asparaginyl Endopeptidase
AMPs	=	Antimicrobial Peptides
BBI	=	Bowman-Birk Inhibitor
CCK	=	Cyclic Cystine Knot
CCL	=	Cyclic cystine ladder
CMTI	=	*Cucurbita maxima* Trypsin Inhibitor
CPs	=	Cyclic Peptides
cT	=	Cyclotide
CTPP	=	C-terminal propeptide
CTR	=	C-terminal repeat
EETI	=	*Ecballium elaterium* Trypsin Inhibitor
ER	=	Endoplasmic Reticulum
FMCA	=	Fluorometric Microculture Cytotoxicity Assay
HR	=	Hypersensitive Response
ICK	=	Inhibitor Cystine Knot
kDa	=	kilodalton
KLK4	=	kallikrein-related peptidase 4
LTPs	=	Lipid Transfer Proteins
MCoTI-I	=	*Momordica cochinchinensis* Trypsin Inhibitor I
MCoTI-II	=	*Momordica cochinchinensis* Trypsin Inhibitor II
MCTI	=	*Momordica charantia* Trypsin Inhibitor
NTPD	=	N-terminal precursor domain
NTPP	=	N-terminal propeptide
NTR	=	N-terminal repeat
pa1	=	Albumin 1 from Pisum sativum
PDI	=	Protein Disulfide Isomerase
PR	=	Pathogenesis Related
PR-proteins	=	Pathogenesis-Related proteins
RTD-1	=	*Rhesus Theta* Defensin-1
SAR	=	Systemic Acquired Resistance
SF-9	=	Spodoptera frugiperda-9
SFTI	=	Sunflower Trypsin Inhibitor
TI	=	Trypsin Inhibitor

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support and fellowships.

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