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ORIGINAL ARTICLE

CHARACTERIZATION OF PLANT ANTIMICROBIAL PEPTIDES

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ABSTRACT: Plant antimicrobial peptides (AMPs) have been isolated from roots, seeds, flowers, stems, and leaves of a wide variety of species and have activities towards phytopathogens,as well as against bacteria pathogenic to humans. They are a component of barrier defense system of plants.Thus,plant AMPs are considered as promising antibiotic compounds with important biotechnological applications.Plant AMPs are grouped into several families and share general features such as positive charge, the presence of disulfide bonds (which stabilize the structure), and the mechanism of action targeting outer membrane structures.
Keywords: Antimicrobial; peptides; thionin; defensin cyclotides.

INTRODUCTION

As a part of defense response, plants produce a high number of toxic molecules, including antimicrobial peptides (AMPs), that kill pathogens by interaction with phospholipids and membrane permeabilization. The other group comprises cell-penetrating peptides (CPPs), capable of introducing into cells a variety of cargoes in the absence of specific receptors by interaction at some point with membrane phospholipids. AMPs and CPPs are a part of the nonspecific host defense system and are active against different types of microorganisms (Eudes and Chugh 2008; Rivas et al. 2010; Pelegrini et al. 2011; Hegedus and Marx 2013). Antimicrobial peptides have been described in a wide variety of species including, insects, amphibians, andmammals. They exhibit a wide range of functions ranging from direct antimicrobial properties to immunomodulatory effects (Choi et al. 2012). AMPs have been demonstrated to inactivate prokaryotic cells by targeting a number of essential or metabolic processes at extracellular, plasma membrane,and/or intracellular sites (Yount and Yeaman 2013).Most of the natural antimicrobial peptides are 10 to 50 amino acids (aa) in length, range in size from2 to 9 kDa, are positively charged, contain a high position of hydrophobic amino acid, and often display a helical structure.AMPs are gene-encoded and they are either constitutively expressed or rapidly transcribed upon induction in eukaryotes by invading microbes and their products, or host cellular compounds, such as cytokines, butyrate, or vitamins (Schauber et al.2006; Lai and Gallo 2009).

These peptides are categorized into distinct familiesmainly on the basis of their amino acid sequence,identity, number of cysteine residues, and their spacing (Lay and Anderson 2005). On the basis of their electrical charge, plant AMPs can be divided into anionic (AAMPs) and cationic peptides(CAMPs) (Pelegrini et al. 2011).

Plant antimicrobial peptides has been isolated from roots, seeds, flowers, stems, and leaves from a wide variety of species and have demonstrated activities towards phytopathogens, as well as against organisms pathogenic to human, viruses, bacteria, fungi, protozoa, parasites, and neoplastic cells (Montesinos 2007). The repertoire of AMPs synthesized by plants is extremely large with hundreds of different AMPs in some plant species.The main families of AMPs comprise defensins, thionins, lipid transfer proteins, cyclotides, snakins, and hevein-like proteins, according to amino acid sequence homology.

STRUCTURAL AND FUNCTIONAL RELATIONSHIPS OF PLANT AMPS

Primary and tertiary structure comparison of plant AMPs

In silico analyses revealed some similarities in tertiar y structures of
lant AMPs, despite significant differences in amino acid
equences between the families (Pelegrini et al. 2011; Fig. 1).
Key features of AMPs are high content of cysteine and/or glycine and the presence of disulphide bridge s, which
are important for enhancing structural stability unde r stress
conditions. Around 17 % of the amino acids in plant AMPs
are charged (mainly ariginines and/or lysines, but als

o aspartic
acid and glutamic acid), what seems to play an essent ial role in
activity towards pathogenic bacteria (Hammami et al . 2009; Pelegrini et al. 2011).

DETAILED DESCRIPTION OF MAIN FAMILIES OF PLANT AMPS

THIONINS

Thionins are a family of antimicrobial peptides with low molecular weight (about 5 kDa), rich in arginine, lysine, and cysteine residues. Their structure includes two antiparallel α-helices and an antiparallel double-stranded β-sheet with three or four conserved disulfide linkages. They are positively charged at neutral pH. The groove between the α-helices and β-sheets posses the Tyr 13 residue, the membrane interactions of which may be associated with cell leakage which appears to be a common mechanism of cell lysis of thionins (Majewski and Stec 2001). Thionins are toxic against bacteria, fungi, and yeast (Table 1). Around 100 individual thionin sequences have been identified in more than 15 different plant species (in monocots, dicotyledonous, and rosids; Stec 2006). The first thionin was isolated in 1942 by Balls and collaborators from wheat endosperm Triticum aestivum, later called purothionin (Mak and Jones 1976). The name thionins is used for two distinct groups of plant peptides: α-/β-thionins and γ-thionins. The last group (γ-thionins) have much more in common with a large family of membrane active peptides called defensins, found in plants and animals (Stotz et al. 2009). Thionins have a common gene structure with an ~20 aa-long leader peptide and an ~60 aa-long trailing acid peptide, which neutralizes the basic toxin (Stec 2006). Cleavage of the leader peptide is necessary for toxin activation. All thionins are present in almost every crucial plant tissue from endosperm to leaves. Their toxic effect was postulated to arise from lysis of the membranes of attaching cells. The precise mechanism underlying toxicity remains unknown. Antifungal activity of thionins is a result of direct protein–membrane interactions by electrostatic interaction of the positively charged thionin with the negatively charged phospholipids in fungal membranes, and this result in pore formation or a specific interaction with a certain lipid domain (De Lucca et al. 2005). α-/β-thionins are subdivided into five classes; however, all types appear highly homologous at the amino acid level (Stec 2006).

Table 1 Antimicrobial properties of selected thionins

Protein	Susceptible species
Wheat endosperm crude purothionin	Bacteria:
	Pseudomonas solanacearum
	Xanthomonas phaseoli
	Xanthomonas campestris
	Erwinia amylovora
	Corynebacterium fascians
	C. flaccumfaciens
	C. michiganese
	C. poinsettiae
	C. sepedonicum
Wheat endosperm α-purothionin	Fungi:
	Rhizoctonia solani
Viscotoxin A3 and B from leaves and stems of *Viscum album* L.	Fungi:
	Fusarium solani
	Sclerotinia sclerotiorum
	Phytophtora infestans
Nicotiana attenuate PR-13 thionins	Bacteria:
	Pseudomonas syringae pv. tomato
Pearl millet seed thionin	Fungi:
	Sclerospora graminicola
WBeta (thionin) from *Triticum aestivum*	Fungi:
	Fusarium solani
AX1 thionin from *Beta vulgaris*	Fungi:
	Cercospora beticola

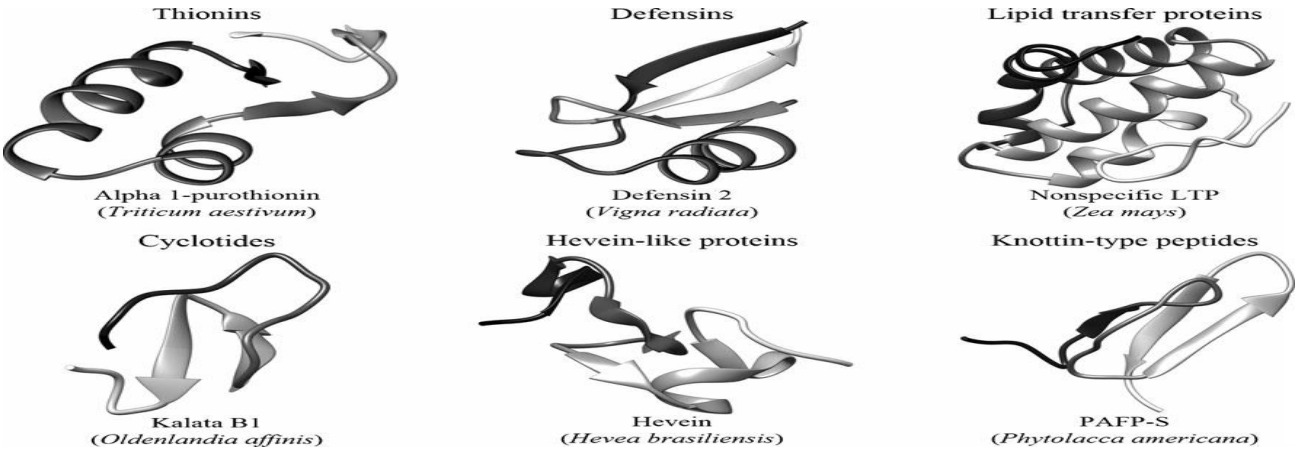

Fig. 1 Three-dimensional structures of selected antimicrobial peptides from different families. The structures were retrieved from RCSB Protein Databank and visualized with UCSF Chimera package.

DEFENSINS

The first plant defensins were isolated from wheat T. aestivum and barley Hordeum vulgare and initially classified as γ- thionins. Plant defensins are small (ca. 5 kDa), basic,cysteine-rich peptides ranging from 45 to 54 amino acids,and are positively charged. Biological activities reported for plant defensins include antifungal, antibacterial, proteinase, and insect amylase inhibitor activities (Table 2; Wijaya et al.2000; Stotz et al. 2009). The plant defensins have quite diverse amino acid composition and conserved threedimensional structure, which comprises a triple-stranded β- sheet with an α-helix in parallel stabilized by four disulfide bridges.Plant defensins are very similar to defense peptides of mammals and insects what suggest their ancient and conserved origin. Generally, plant defensins are composed by one subunit, being found in monomeric forms. On the other hand, the defensins from Pachyrrhizus erosus and other from Vigna unguiculata showed the ability to dimerism (Pelegrini and Franco 2005). The mode of action of plant defensins is still unclear and not all plant defensins have the same mode of action. Probable defensins used glucosylceramides as receptors for fungi cell membrane insertion. Then, repulsion of defensins into cellmembrane by their positive charges leads tomembrane disruption, membrane destabilization, and ion efflux (Pelegrini and Franco 2005). Plant defensins can be divided in two groups: (1) plant defensins that inhibit fungal growth through morphological distortions of the fungal hyphae and (2) plant defensins that inhibit fungal growth without morphological distortion (Hegedus and Marx 2013). Most plant defensins were isolated from seeds. In radish, defensin RS-AFPs represents 0.5 % of total protein in seeds. Defensins were also isolated from leaves, pods, tubers, fruits, roots, bark, and floral organs of such plants as Heuchera sanguinea (Hs-AFp1), Raphanus sativus (Rs-AFP1), Aesculus hippocastanum (Ah-AMP1), Dahlia merckii (Dm-AMP1), and Clitoria ternatea (Ct-AMP1; De Lucca et al. 2005). Defensins are expressed during normal plant growth and development and induced by environmental factors and biotic and abiotic stress (Pestana- Calsa and Calsa 2011). The defensins gene induced upon pathogen infection has been identified in pea, tobacco, Arabidopsis, and spruce (Lay and Anderson 2005).

Two classes of defensins are produced. The first class, the precursor protein, contains an amino signal peptide that targets the peptide to the extracellular space. The second class of defensins have C-terminal prodomains.

Plant defensins are best known for their antimicrobial activity against a broad spectrum of plant pathogens as bacteria,yeast, oomycetes, and necrotrophic pathogens (Segura et al. 1998; Portieles et al. 2010; van der Weerden et al. 2010). They also show activities important for medical applications as anticancer activity and antiviral activity (Ngai and Ng 2005; Wong and Ng 2005). Plant defensins interact with glucosylceramides in membranes of susceptible yeast and fungi and induce membrane permeabilization and fungal cell death (Thevissen et al. 1996, 2004).

γ-Hordothionin belongs to plant defensins (molecular weight, 5,250 Da; contains four disulfide bridges), which inhibits translation in cell-free systems. The others are defensin PhD1 from Petunia hybrida with antifungal activity and defensins 1 and

2 (VrD1 and VrD2) isolated from the seeds of the mung bean, Vigna radiata (Padovan et al. 2010). However, only VrD1 exhibits insecticidal activity and α- amylase inhibitory activity. PhD1 has 47 residues and five disulfide bonds.Other features of plant defensins are related to the regulation of growth, development, and fertilization (Oomen et al. 2011).

Table 2 Antimicrobial properties of selected plant defensins

Defensin	Susceptible species
MsDef1 from *Medicago sativa*	Fungi:
	Magnaporthe grisea
	Erwinia carotovora
	Botrytis cinerea
WT 1 from *Wasabia japonica* L.	Fungi:
	Magnaporthe oryzae
	Rizoctonia solani
Dm-AMP1 from dahlia	Fungi:
	Fusarium culmorum
Ah-AMP1 from *Aesculus hippocastanum*	Fungi:
	Fusarium moniliforme
Rs-AFP1 from *Raphanus sativus*	Fungi:
	Fusarium culmorum
	Botritis cinerea
RsAFP2 from *Raphanus sativus*	Fungi:
	Baker's yeast
	Candida albicans
Hc-AFP1 Hc-AFP2 HcAFP3 Hc-AFP4 from *Heliophila coronopifolia*	Fungi:
	Botrytis cinerea
	Fusarium solani
HsAFP1 from *Heuchera sanguinea*	Fungi:
	Aspergillus flavus
	Candida albicans
	Candida krusei
Ns-D1 Ns-D2 from *Nigella sativa* seeds	Fungi:
	Aspergillus niger
	Fusarium oxysporum
	Fusarium graminearum
	Fusarium culmorum
	Bipolaris sorokiniana
	Botritis cinerea

CYCLOTIDES

The cyclotides are group of naturally occurring circular proteins that have been discovered in bacteria, plants, and animals (Pelegrini et al. 2007; Craik 2010). Cyclotides appear to have high sequence similarities and a structural identity. Plant cyclotides comprise 28–37 amino acids, contain a head-to-tail cyclised backbone, and three intramolecular disulfide bonds arranged in a cysteine backbone knot topology (cyclic cysteine knot, CCK). The cysteine knot is formed by the disulfide bonds Cys-1-Cys-4 and Cys-2-Cys-5 and their interconnecting backbone form a ring that is penetrated by Cys-3-Cys-6 disulfide bonds (Colgrave and Craik 2004).CCK is largely responsible for the exceptional stability of cyclotides. It forces the hydrophobic parts of the protein to be exposed at the molecular surface. The hydrophobic residues form a patch on the surface, making the overall structure amphipathic (Pränting et al. 2010). They are esilient to various proteolytic and degradative processes (Ireland et al. 2010). The cyclotide structures contain six backbone loops between the conserved Cys residues and different degrees of sequence diversity in the different loops (Ireland et al. 2010). For example, loops 1 and 4 are highly conserved in both size and residue type, whereas the other loops are more variable.

Cyclotides were isolated from the plants belonging to family Violaceae, Rubiaceae, Cucurbitaceae, and Poaceae belong to Asterids, Rosids, and Monocots (Gruber 2010). Based on structural similarities, cyclotides are divided into two subfamilies: Mobius and the bracelets based on the presence or absence of a cis-proline, respectively (Craik et al. 1999). Another type of cyclotide structure has katata B8 isolated from Oldenlandia affinis. It appears to be a hybrid between Mobius and bracelet subfamilies (Pelegrini et al. 2007). The plant cyclotides are geneencoded peptides generated via ribosomal biosynthetic pathways.The cyclotide precursor contains an endoplasmic reticulum ER signal, a pro-region, an N-terminal repeat (NTR), and a cyclotide sequence domain, followed by a short tail (Craik 2010). Individual cyclotide genes encode between one and three repeats of the NTR and cyclotide domain to form multiple cyclotide from a single precursor.

The NTR region has the amphipathic helical nature and might assist in directing the connect folding of the cyclotide domain (Ireland et al. 2010). The role of C-terminal region (CTR) is unclear. The conserved Asn (or Asp) residue in this region suggests that this part of protein is a target of an asparaginyl endoproteinase. The first described cyclotide kalata B1 was isolated from the plant O. affinis (Mylne et al. 2010).Kalata B1 was used by women in Africa to accelerate labor and childbirth. These peptides have a diverse range of biological activities, including uterotonic, anti-HIV, antimicrobial, insecticidal, antihelmintic, and molluscidal properties (Table 3; Craik 2010). Their natural function appears to be as plant defense molecules based on their insecticidal properties (Gruber 2010).Thus, cyclotides have potential applications in both the pharmaceutical and agricultural industries. The cyclotides Vitri isolated from Viola tricolor demonstrated cytotoxicity to human lymphoma and myeloma cells. Similarly, cycloviolacin H4 isolated from Viola hederaceae is able to cause hemolysis in human erythrocytes (Pelegrini et al. 2007). It has been suggested that membrane interactions might be involved in the various biological activities of cyclotides; however, the mechanism of their action remains unknown. These proteins have specific membrane-disrupting activity (Svangård et al. 2007; Burman et al. 2011). Kalata B1 interacts directly with the membrane by targeting phosphatidylethanolamine phospholipids, probably leading to membrane bending and vesicle formation. This protein together with cyclotide Momordica cochinensis trypsin inhibitor II (McoTI-II) extracted from seeds and sunflower trypsin inhibitor I (SFT1) from seeds belong also to cyclic cell-penetrating peptides CCPs (Greewood et al. 2007). McoTI-II has been reported to be internalized into cells by macropinocytosis, probably by interacting with phosphatidylinositides and phosphatidic acid, but the specific mechanism by which this occurs is not known (Cascales et al. 2011). The mechanism of penetration of SFTI-1 across the plasma membrane of living cell remains unresolved but is independent of phospholipid and differs from McoTI-II and kalata B1 (Greewood et al. 2007).

Table 3 Biological activity of selected cyclotids

Cyclotide	Activity
Kalata B1 from *Oldenlandia affinis*	Insecticidal, molluscidal, hemolytic, nematocidal, antibacterial, anti-HIV
Kalata B2 from *Oldenlandia affinis*	Insecticidal, molluscicidal, nematocidal, antibacterial,
Cyrulin A&B from *Chassalia parviflora*	Hemolytic, antibacterial, anti-HIV
cycloviolacin O1 from *Viola odorata*	Nematocidal, molluscidal
Cycloviolacin O2 from *Viola odorata*	Gram-negative bacteria
MCoTI-II from *Momordica cochinensis*	Trypsin inhibitor

RESULTS AND DISCUSSION

Plant AMPs are diverse peptides differing in their amino acid composition and structure that generally display rapid killing and broad spectrum antimicrobial activities. Therefore, AMPs have a high potential for therapeutic use in healthcare and agriculture, and can be used as natural antibiotics as

alternativefor their chemical counterparts, for protection of plants,and/or animals against diseases. AMPs offer a good alternative for treating infections in relation to conventional antibiotics based on their broad spectrum activity and efficiency (Pinheiro da Silva and Machado 2012). Despite their many promising features, not one AMP has yet reached the status of a clinically approved drug. However, the cationic AMPs have been applied in the formation of aerosol sprays for patients with cystic fibrosis.

Numerous transgenic plants expressing AMPs that confer different degrees of protection against diseases have been developed; therefore, AMPs could play strong roles in agriculture as plant protection products. Unfortunately, the commercial cultivars have not been marketed because of regulatory limitations and social concerns. The other problems comprise the intrinsic toxicity and low stability of some of the compounds and the need for inexpensive products in plant protection. Therefore, future areas of commercial plant AMPs use consist of developing less toxic and more stable compounds as well as decreasing production costs mainly by improving biotechnological procedures or preparative peptide synthesis (Montesinos 2007).

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