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ANTIMICROBIAL PHYTO-PEPTIDES: DISTRIBUTION, STRUCTURAL DIVERSITY AND MECHANISMS OF ACTION

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ABSTRACT

In response to infection by a variety of pathogens, plants display up-regulation of a set of genes associated with systemic acquired resistance. General resistance is accomplished by the generation of pathogenesis-related (PR) proteins. There are at least 17 families that have been detected and isolated which possess a wide range of defense-related properties, including antibacterial, antifungal, antiviral, anti-oxidative activity, as well as chitinase and proteinase inhibitory activities. Plants AMPs have been isolated from roots, seeds, flowers, stems, and leaves of a wide variety of plant species. The majority of plants antimicrobial peptides (AMPs) are Cys-rich; a feature that enables the formation of multiple disulfide bonds that contribute to a compact structure and resistance to chemical and proteolytic degradation. 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. The main families of AMPs comprised; defensins, thionins, lipid transfer proteins, cyclotides, snakins, and hevein-like proteins, according to amino acid sequence homology. Most of the known plants AMPs act by formation of membrane pores, resulting in ion and metabolite leakage, depolarization, interruption of the respiratory processes, and cell death. Conventional antibiotics usually have a narrow spectrum, acting only on bacteria or fungi, whereas plant AMPs have a broad spectrum of activity displaying antibacterial, antiviral, anti-parasitic, and anticancer activities. Plants AMPs have the ability to bypass the common resistance mechanisms that are reducing the effectiveness and safety of the conventional antibiotics. They can be used as a single antimicrobial due to their direct killing actions or as adjuvants with conventional antibiotics

to obtain synergistic interactions. Hence, plants AMPs are potential therapeutic agents for many infectious diseases.

KEYWORDS: Antimicrobial, Plants, Peptides, Proteins, AMPs, Antibiotics.

INTRODUCTION

Plants have a wide range of defense mechanisms to counter physical, chemical and biological stress. In response to infection by a variety of pathogens, plants display up-regulation of a set of genes associated with systemic acquired resistance (Wijaya *et al*., 2000). General resistance is accomplished by the release of secondary metabolites like phytoalexins, tannins and polyphenolic compounds and the generation of pathogenesis-related (PR) proteins. PR proteins were first discovered in the early 1970s in tobacco leaves in response to tobacco mosaic virus infections and were later defined as the induced proteins that are released during pathogenic attacks (Stec, 2006). According to a Hegedus and Marx (2013), there are at least 17 families that have been detected and isolated which possess a wide range of defense-related properties, including antibacterial, antifungal, antiviral, anti-oxidative activity, as well as chitinase and proteinase inhibitory activities (Wong and Ng, 2005).

In plants, the majority of AMPs are Cys-rich, a feature that enables the formation of multiple disulfide bonds (usually two to six) that contribute to a compact structure and resistance to chemical and proteolytic degradation (Hegedus and Marx, 2013).

Antimicrobial peptides 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). AMPs are peptides having 10 to 50 amino acids in length, with molecular weight from 2 to 9 kDa. They are positively charged, and contain high amount of hydrophobic amino acids, and often display a helical structure (Schauber *et al*., 2006). These peptides are categorized into distinct families mainly on the basis of their amino acid sequence, identity, number of cysteine residues, and their spacing (Lay and Anderson, 2005).

Antimicrobial Plant peptides share several common characteristics with those from microbes, insects and animals, which include features such as positive charge and amphipathic nature, all of which are primarily related to their defensive role(s) as membrane-active antifungals,

antibacterials, and antivirals. These features, in addition to being Cys-rich, are well represented by two plant AMP families, thionins and plant defensins. Other families of plant AMPs act on pathogens differently from animal AMPs. For example, hevein-like peptides bind chitins, knottin-type peptides inhibit enzymes such as proteases, and lipid transfer proteins bind lipids to disrupt microbial penetration into cell membranes (Pelegrini *et al*., 2011).

Plant antimicrobial peptides have been isolated from roots, seeds, flowers, stems, and leaves of a wide variety of plant species (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 (Lay and Anderson, 2005).

Classification and Characteristics of AMPs

The following defining features are found in plant AMPs:

a. Mostly characterized as moderate-size (MW of 2–6 kDa), basic, CRPs with two to six intra-molecular disulfide bonds.
b. Members within a family are classified based on Cys motif, sequence similarity and are conserved in secondary and tertiary structure.
c. One or two additional disulfide bonds are found in members of thionins, defensins, and hevein-like peptides. These additional bonds bolster structural stability without affecting the general scaffold.
d. In addition to being antimicrobial, AMPs also display “peptide promiscuity”, which refers to the multiple functions displayed by a single peptide.
e. All are ribosomally derived and bioprocessed from precursors, which often contain three domains: N- and C-terminal pro-domains and a mature AMP domain. Mature sequences are often hypervariable and display more variation than the conserved terminal domains in the preproprotein to give sequence diversity for adaptation.
f. Because of cross-bracing by multiple disulfide bonds, most CRP-AMPs with a molecular weight of 2–6 kDa are structurally compact with high thermal, chemical, and enzymatic stability (Yount and Yeaman, 2013).

Structural and Functional Relationships of Plant AMPs

In silico analyses of the primary and tertiary structure of plant AMPs revealed some similarities despite significant differences in amino acid sequences between the families (Pelegrini *et al*., 2011). Key features of AMPs are high content of cysteine and/or glycine and the presence of disulphide bridges, which are important for enhancing structural stability under stress conditions. Around 17% of the amino acids in plant AMPs are charged (mainly arginines and/or lysines, but also aspartic acid and glutamic acid), what seems to play an essential role in activity towards pathogenic bacteria (Hammami *et al*., 2009).

Studies comparing the primary sequences and tertiary structures of antimicrobial peptides from plants show that 33% of them present activity against bacteria, and around 59% are formed by 30 to 50 amino acid residues (Villa-Perello *et al*., 2003). The occurrence of disulphide bridges is also important for enhancing structural stability under diverse stress conditions (Montesinos, 2007). Additionally, it was observed that the percentage of cysteine residues is higher in peptides with known β-sheet structures. This can be compared to an antimicrobial peptide belonging to the glycine-rich family isolated from guava seeds (Hammami *et al*., 2009). The structure of this peptide, inferred by molecular modelling studies, consists only of α-helices and lacks β-sheets. Analysis of the primary sequence revealed no cysteine residues, and thus the peptide is unable to form disulphide bonds (Li, 2011). Therefore, evidence suggests that the presence of cysteine residues and β-sheet structures may go together, but does not imply relevancy for antimicrobial activity.

Mechanisms of Antimicrobial Action of Plant Peptides

Most of the known plants AMPs act by formation of membrane pores, resulting in ion and metabolite leakage, depolarization, interruption of the respiratory processes, and cell death (Pelegrini *et al*., 2011). Amphipathic structure and positive charge at physiological pH may be significant features allowing AMPs to interact with membrane lipids. The cationic residues electrostatically attract negatively charged molecules (e.g., anionic phospholipids, lipopolysaccharides, or teichoic acids) allowing the peptide to accumulate on the membrane surface (Pelegrini and Franco, 2005). When concentration reaches a threshold value, the collapse begins. Three main models explaining this phenomenon were proposed: barrel-stave mechanism, AMPs oligomerize with hydrophobic residues of peptide facing interior of the lipid bilayer and hydrophilic ones oriented towards the lumen of newly formed pore. In the wormhole mechanism, peptide molecules reorient in the membrane during the aggregation

dragging of the lipids with them (through electrostatic interactions between head groups of phospholipids and hydrophilic residues of AMPs) (Pokorny and Almeida, 2004).

In the carpet mechanism, peptides act like detergents, covering the membrane in an electrostatic manner (in monomeric or oligomeric form). This "carpet" of amphipathic molecules causes phospholipid displacement, alters membrane properties, and disrupts the membrane (Pelegrini *et al*., 2011). There are some other models such as the sinking raft model (Pokorny and Almeida, 2004), aggregate model (Wu *et al*., 1999), or the molecular electroporation (Miteva *et al*., 1999); however, they have not received much attention in the field and have not found much experimental confirmation. There are some differences between antifungal and antibacterial activity, mainly connected with different composition of the target membrane. For example, γ-thionins might bind to glucosylceramides and sphingolipids in fungal membrane (instead of phospholipids being their receptors in bacteria) (Hughes *et al*., 2000). However, many AMPs (e.g., γ- thionin SIα1 from Sorghum bicolor) show activity toward both bacteria and fungi (Pelegrini and Franco, 2005). Site-directed mutagenesis studies performed to produce new variants of Rs-AFP1 defensin revealed that a variant in which Gly9 or Val39 was replaced with arginine was more active against certain fungi than wild-type Rs-AFP2 (Lay and Anderson, 2005).

DESCRIPTION OF SOME PLANT ANTIMICROBIAL PEPTIDES

A-/B-Thionins

Thionins are the prototypic plant AMP, they are cationic peptides of 45–48 amino acids with three or four disulfide bonds (Pelegrini *et al*., 2011). Initially, they were known as plant toxins because of their toxicity towards bacteria, fungi, plant and animal cells, as well as insect larvae (Hughes *et al*., 2000). The prototypic thionin with antimicrobial activity, α-purothionin, was isolated from the endosperm of wheat (Lai and Gallo, 2009). Following the discovery of α-purothionin, subsequent thionins isolated from other plants are labeled with descending letters of the Greek alphabet in the order of their discovery (e.g., α-thionins, β-thionins, and γ1/γ1-thionins). Classification of thionins is largely based on α-purothionin and includes the α-/β-thinoins of crambin, viscotoxins, phoratoxin A, hordothionins, and purothionins. However, γ-thionins are considered part of the plant defensin family based on structural considerations. Thus, α-/β-thionins share a similar structural fold different from that of γ-thionins. For convenience, α/β-thionins with eight Cys residues will be hereafter referred to as 8C-thionins and those with six Cys designated 6C-thionins (Wu *et al*., 1999).

Occurrences and Distribution: Thionins have been identified from monocots and dicots and are expressed in different tissues, such as seeds, leaves, and roots (Lai and Gallo, 2009). The expression of thionins can be induced by infection with various microbes and has been shown to be related to the release of the hormone methyl jasmonate upon plant wounding or microorganism invasion (Hughes *et al*., 2000). Thionins are ribosomally derived and expressed as preproproteins, wherein the prothionin domain is flanked by two conserved sequences, the N-terminal signaling peptide and C-terminal acidic domain (Wu *et al*., 1999). Mature thionin sequences display more variation than the conserved terminal domains in the preproprotein due to evolutionary pressure (Pelegrini *et al*., 2011). The three-domain precursor of plant AMPs in Cyclic AMP (Adenosine Monophosphate) Receptor Protein – AMP (CRP-AMPs) families is typical and found in other CRP families, including defensins, hevein-like peptides, and knottin-type peptides (Pokorny and Almeida, 2004).

Structure: From the limited number of members identified thus far, thionins have relatively conserved amino acid sequences compared to other plant defense peptides. They also share a conserved β1-α1-α2-β2-coil secondary structural motif, which forms a gamma (Γ) fold, a special turn consists of three amino acid residues with the first and third residue connected by a hydrogen bond in the tertiary structure.

Mechanism of Action: Thionins are hydrophobic and likely elicit their toxicity to bacteria, fungi, and animal and plant cells via membrane interactions with their hydrophobic residues or/and positive surface charge (Eudes and Chugh, 2008). The proposed mechanism of toxicity is attributed to lysis of cell membranes (Rivas *et al*., 2010). Thionins are known to directly interact with membrane lipids apart from protein receptors (Villa-Perello *et al*., 2003). *Pyrularia* thionin from the nuts of *Pyrularia pubera* mediates the influx of Ca^{2+} during certain cellular responses, while Tyr iodination reduces its haemolysis, phospholipase A2 activation, and cytotoxicity. Structure-function studies have demonstrated that Lys1 and Tyr13 in thionins are highly conserved and proposed to be crucial to their toxicity, with the exception of non-toxic, non-lytic crambin. Instead, crambin contains Thr1 and Phe13 residues (Hughes *et al*., 2000). Furthermore, Arg10 is suggested to be important to the folding stability of all thionins, as it is an abundant source of hydrogen bonds between β1, α1 and the C-terminal coil (Villa-Perello *et al*., 2003).

Plant Defensins

Plant defensins are the best known, and likely most abundant, of all plant AMPs with membranolytic functions, according to data mining of selected plant genomes. They are cationic peptides of 45–54 amino acids with four to five disulfide bonds (Lai and Gallo, 2009). Plant defensins have diverse biological functions which include antifungal, antibacterial, and α-amylase and trypsin inhibitory activity (Pelegrini *et al*., 2011). In addition to being antimicrobial, plant defensins are also involved in the biotic stress response, as well as plant growth and development. Plant defensins were first identified as γ-thionins, γ1-hordothionin, and γ1-/γ2-purothionins from wheat and barley grains (Hughes *et al*., 2000). Thus, they were initially classified as γ-thionins due to their limited sequence identity (25%) with α-/β-thionins. Later, they were found to be unrelated to thionins based on structural features (Villa-Perello *et al*., 2003). In, 1995, they were grouped as plant defensins based on their sequence, structure, and function similarities with mammalian and insect defensins (Eudes and Chugh, 2008).

Occurrences and Distribution: Plant defensins include over 100 members from a wide range of plants, including wheat, barley, tobacco, radish, mustard, turnip, arabidopsis, potato, sorghum, soybean, cowpea, and spinach, among others (Stotz *et al*., 2009). They were identified in many tissues, tubers, leaves, pods, and flowers (Yount and Yeaman, 2013), with the majority identified from seeds and roots (Wu *et al*., 1999). Two types of precursors have been identified in plant defensins, wherein the dominant group is composed of the N-terminal signal peptide and a mature plant defensin domain, while the minor group is composed of an extra C-terminal acidic pro-domain of thirty three amino acids reportedly associated with the vacuolar sorting mechanism since defensins with this domain were found in vacuoles and those without were found in the outer cell layers (Hegedus and Marx, 2013).

Structures: Plant defensins are generally characterized by four conserved disulfide bonds with the outer disulfide pair as an end-to-end inner disulfide bridge and the inner three pairs of disulfide bonds forming a cystine knot (Wong and Ng, 2005). A secondary structure characteristic of plant defensins is a Cys-stabilized αβ (CSαβ) motif of the cysteine knot (Schauber *et al*., 2006).

Mechanism of Action: The structure-function relationship of plant defensins has been suggested to correlate with their positive charge and amphipathic nature. Thus, plant defensins could initially bind to microbial membranes through interactions with specific

binding sites (receptors), as reported for Rs-AFP2, Hs-AFP1, and Dm-AMP1 (Lai and Gallo, 2009). Binding of plant defensins, such as Rs-AFP2 and Dm-AMP1, to the cell membrane results in the influx and efflux of positive ions like Ca^{2+} and K^{+}. Lastly, Ms-Def1 is able to block the Ca^{2+} channel in a manner similar to the Ca^{2+} channel blocker KP4 (Pokorny and Almeida, 2004). Van der Weerden *et al.*, (2015) demonstrated that NaD1 does not cause membranes permeabilization via a canonical mechanism which involves nonspecific insertion into membranes but rather a cell wall dependent process, likely requiring a specific receptor. The mechanism of the fungicidal action of *Nicotiana alata* Defensin 1 (NaD1) is likely through permeabilization of the hyphae of *Fusarium oxysporum*, entering into the cytoplasm of the cell and inducing reactive oxygen species (ROS) oxidative stress (Hughes *et al*., 2000). Choi *et al*., (2012) reported that the high-osmolarity glycerol (HOG) pathway is involved in the protection of the cell against NaD1, indicating that the inhibition of the HOG pathway increases the activity of antimicrobial peptides against *Candida albicans* (Pokorny and Almeida, 2004).

Hevein-Like Peptides

Hevein-like peptides are basic peptides of 29–45 amino acids with three to five disulfide bonds. They are rich in Gly and contain conserved aromatic residues found in the hevein domain of lectins. Hevein domains bind to chitin, which is their primary target. Hevein was first identified as the most abundant protein component from the latex of the rubber tree *Hevea brasiliensis* and displays strong antifungal activity *in vitro*. It was also reported to be a major allergen from latex involved in human latex-fruit syndrome (Hughes *et al*., 2000). Similar to hevein, hevein-like peptides inhibit the growth of chitin-containing fungi and defend plants against attack from a wide range of fungal pathogens (Choi *et al*. 2012).

Occurrence and Distribution: As a chitin-binding domain, the hevein domain is found in several plant lectins, natural variants of heveins (pseudo-hevein, wheat germ agglutinin, *Urtica dioica* agglutinin), and AMPs (Hughes *et al*., 2000). Similar to other families of CRP-AMPs, the hevein-like peptide is processed from a three-domain precursor. For example, the cDNA of the Ar-AMP precursor comprises a twenty five amino acids N-terminal signal sequence, thirty amino acids mature peptide, and thirty four amino acids C-terminal region which is cleaved during post-translational processing (Lai and Gallo, 2009). In 10C-hevein (hevein-like peptide containing ten cysteine residues), there are two different precursor peptide structures in the C-terminal prodomain. The WAMP 10C-hevein from *Triticum*

kiharae have precursor similar to other families CRP-AMPs, with a forty five amino acid C-terminal region (Pelegrini *et al*., 2011), while Ee-CBP 10C-hevein from *Euonymus europaeus* is produced as a chimeric precursor consisting of the mature peptide domain linked to a long C-terminal chitinase-like domain (Hughes *et al*., 2000). Wu *et al*. (1999) also showed that the WAMP-1 and WAMP-2 gene may have originated from ancestral chitinase genes and that a frame-shift deletion of the coding region for the catalytic domain led to the WAMP gene formation (Choi *et al*., 2012).

Structure: Hevein-like peptides share conserved Cys, Gly, and several aromatic amino acid residues. They vary substantially in their primary sequences and number of disulfide bonds (from three to five).

Mechanism of Action: As a chitin-binding domain, hevein is an excellent model for studying the carbohydrate-peptide interaction, which is reportedly mediated by hydrogen bonding and van der Waals forces. The carbohydrate-induced conformational change to the hevein domain is small based on NMR investigations of pseudo-hevein, a wheat germ agglutinin and truncated hevein mutant (Choi and Lee, 2012). The interaction between the hydrophobic C-H groups of carbohydrates and the π-electron systems of aromatic amino acids (Trp21, Trp23, and Tyr30 in hevein) of hevein-like peptides appear to play an important role in chitin binding, as observed in Ac-AMP synthetic mutants, hevein, and a truncated form of hevein (hevein32) at key interacting positions (Pokorny and Almeida, 2004). Studies on Pn-AMPs showed that they rapidly penetrate fungal hyphae, leading to hyphal tip bursting, which disrupts the fungal membrane causing leakage of cytoplasmic materials. In addition to the chitin binding function of hevein, Slavokhotova *et al.*, (2013), showed an alternative function in which hevein plays a role in the plant defense against fungal infection (Koren and Torchilin 2012). WAMPs which contains an additional Ser at position 36 is able to inhibit the proteolytic activity of the secreted fungal protease fungalysin (Fv-cmp), a Zn-metalloproteinase, isolated from *Fusarium verticillioides*. This protease is able to truncate corn and Arabidopsis class IV chitinases by cleaving within the Gly-Cys site located in the chitin-binding domain of the plant chitinase. The presence of Ser36 prevents WAMP from being digested by Fv-cmp, allowing it to bind to fungalysin, and displace the plant chitinase, thus enabling the chitinase to remain intact and active (Nasrollahi *et al*., 2012).

Knottin-Type Peptides

Plant knottins belong to a superfamily, with members containing approximately thirty amino acids. They include inhibitors of α-amylase, trypsin and carboxypeptidase families as well as cyclotides. In general, they are among the smallest in size, but most diverse in functions. Knottins typically comprise six Cys residues with conserved disulfide bonds between CysI-CysIV, CysII-CysV, and CysIII-CysVI, forming a cystine knot, but their Cys motifs differ among different subfamilies. Both plant defensins and hevein-like peptides also contain a cysteine-knot motif but they differ in their cysteine spacing. One characteristic of this family is that they display a very broad range of bioactive functions which include hormone-like functions as well as enzyme-inhibitory, cytotoxic, antimicrobial, insecticidal, and anti-HIV activities (Greewood *et al*., 2007). Certain cysteine-knot (CK) peptides with identical scaffold structures involved in multiple biological functions have been viewed as "peptide promiscuity" **(**Wang and Wang, 2017). Historically, the knottin-type peptides were discovered as protease inhibitors sharing in common only in a cysteine knot motif, and they are named collectively as cysteine-knot inhibitor peptides, knottins. The prototypic knottin scaffold was first discovered in the subfamily of potato carboxypeptidase inhibitor (PCI) in 1982. The use of knottins also distinguishes the CK-CRPs from those initially described in the structures of the protein growth factors found in animals (Milletti, 2012). As a superfamily, they are believed to be the largest group of plant peptides associated with AMPs, surpassing defensins in the number of molecular forms and sequence diversity. Knottins in the cyclotide and trypsin inhibitor families are found in two molecular forms, cyclic and linear, based on the presence or absence of backbone (head-to-tail) cyclization. In literature, cyclic knottins of the squash trypsin subfamily are often included in the cyclotide subfamily. Apart from their Cys residues, cyclic knottins and cyclotides share little sequence identity. Currently, both linear (acyclotides) and cyclic (cyclotides) forms of the cyclotide subfamily are found in plants (Hong and Su, 2011).

Occurrences and Distribution: Linear knottins are found not only in plants, but also in other biological sources, including fungi, insects, and spiders. Thus, CK peptides with identical or related scaffold structures found in diverse life forms provide an example of parallel evolution of protein structures. Cyclotides and their acyclic variants are found only in plants, from the dicot plants of the *Rubiaceae*, *Violaceae*, *Cucurbitaceae*, *Fabaceae*, and *Solanaceae* families to a monocot plant of the *Poaceae* family, with predicted wide and abundant distribution (Veldhoen *et al*., 2008). Cyclotides and certain members of cyclic

knottins of the squash family are produced from precursor proteins encoding one or more cyclotide domains. The precursor is composed of an endoplasmic reticulum signal region, pro-domain, one (or more) mature cyclotide domain(s), and a short C-terminal tail (Milletti, 2012). However, there are variations in their biosynthesis. A recent report on cyclotides such as cliotides (cT1-cT12) identified from *Clitoria ternatea* showed that they originate from chimeric precursors consisting of Albumin-1 chain A and cyclotide domains (Koo *et al*., 1998). Studies have shown that an asparaginyl endoproteinase could be involved in the backbone cyclization of cyclotides (Candido *et al*., 2011). Linear variants of cyclotides share high sequence identity and contain a similar knottin scaffold but are biosynthetically unable to cyclize from their precursors (Milletti, 2012). Violacin A, a naturally occurring linear cyclotide from *Viola odorata*, lacks the essential bioprocessing signal, the C-terminal Asn residue required for cyclization due to the presence of a stop codon earlier in the C-terminal sequence (Veldhoen *et al*. 2008). Cystine knot α-amylase inhibitors (CKAIs) are plant-derived α-amylase inhibitors originally isolated from *Amaranthus hypocondriacus* (Candido *et al*., 2011). They are the smallest family of proteinacous α-amylase inhibitors among the seven known families. Unlike other knottins, these peptides are rich in proline residues, with at least one of them existing in a *cis*- configuration. In recent studies, Nguyen *et al,* (2018) have isolated an additional three members of CKAIs from the leaves and flowers of *Wrightia religiosa* and another five members from the leaves of *Allamanda cathartica* (Hong and Su, 2011). These CKAIs contain 30 residues, two residues shorter than AAI, and share high sequence homology to each other.

Mechanism of Action: Generally, knottin-type peptides with membranolytic functions are amphipathic in nature like other AMPs, a characteristic necessary for membrane interactions which implement their antimicrobial effects. For example, the surface plots of photoactivatable fluorescent proteins (PAFP-S) and kalata B1 show hydrophobic patches surrounded by several hydrophilic residues. However, in contrast the strongly cationic-charged thionins and plant defensins, most cyclotides are unlikely to have a strong electrostatic interaction with membranes since they are normally weakly positive or neutral at physiological pH (Koren and Torchilin, 2012). The interaction of cyclotides with membranes has been previously investigated *in vitro* using the detergent dodecylphosphocholine (Nasrollahi *et al*., 2012). However, studies on kalata B2 and cycloviolacin O_2 suggest that different cyclotides have different membrane binding modalities because of the varied location of hydrophobic patches in cyclotides (Slavokhotova *et al.*, 2013).

Lipid Transfer Proteins

Plant lipid transfer proteins (LTPs) are two families of CRP-AMPs with MW >7 kDa, and are considered proteins. LTPs are cationic proteins of approximately 70 and 90 amino acids with eight Cys residues. They are distinguished from other CRP-AMPs by their lipid transfer activity, in which they bind a wide range of lipids including fatty acids (C10–C14), phospholipids, prostaglandin B2, lyso-derivatives, and acyl-coenzyme A. Consequently, they are also called non-specific LTPs. LTPs can inhibit growth of fungus and some bacterial pathogens and are involved in the plant defense system. LTPs are subdivided into LTP1s (MW = 9 kDa) and LTP2s (MW = 7 kDa) based on their molecular mass (Hong and Su, 2011).

Occurrences and Distribution: Plant LTPs have been identified in various species, such as seeds of the radish, barley, maize, Arabidopsis, spinach, grapevine, wheat, and onion (Veldhoen *et al*., 2008). They are synthesized as precursors containing a signal peptide of 20–25 amino acids and a mature protein with eight Cys (Milletti, 2012).

Structure: Although LTPs vary in their primary sequence, they share a defining structural feature, a conserved inner hydrophobic cavity surrounded by α-helices (Candido *et al*., 2011).

Mechanism of Action: Initially, LTPs were reported to facilitate lipid transfer between membranes of vesicles or organelles *in vitro* (Hegedus and Marx, 2013). However, later discoveries have shown that LTP1s are extracellular cell wall proteins, making *in vivo* intracellular lipid transfer activity unlikely. Thus, LTPs promote membrane permeabilization in pathogens rather than host cells. Although structural studies have shown that LTPs can ‘cage’ lipid molecules in their hydrophobic cavity, a detailed mechanism of antimicrobial activity mediated by lipid transport remains unclear. However, further studies may provide new evidences for their function on bacterial cell wall (Pelegrini *et al*., 2011).

APPLYING PLANT AMPs TO REDUCE ANTIBIOTIC RESISTANCE

Plant Antimicrobial peptides have the ability to bypass the common resistance mechanisms that are reducing the usefulness and safety of conventional antibiotics. The novel antimicrobial peptide dendrimer G3KL (synthetic) was demonstrated to be a promising antimicrobial agent with antibacterial activity against multidrug-resistant and extensively drug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Silva *et al*., 2016). The antimicrobial peptide defensin from *Tribolium castaneum* displays *in vitro* and *in vivo*

antimicrobial activity against drug resistant *Staphylococcus aureus* probably via disruption of the bacterial cell membrane (Lewies *et al*., 2017). In addition, the human antimicrobial peptide LL-37 exhibits significant antimicrobial activity against multidrug-resistant *Acinetobacter baumannii* (Nwokoro *et al*., 2016).

However, it is inevitable that pathogens have evolved mechanisms that resist deleterious damage by AMPs. These include degradation of AMPs by production of proteolytic enzymes, repulsion of the peptides by alternation of net surface charges, expulsion of the peptides using membrane efflux pumps, and reducing the bacterial membrane fluidity through alternations in Lipid A. Even with such defensive mechanisms in pathogens, plant AMPs still provide protection. Compared with the conventional antibiotics, one of the strengths of AMPs is their low propensity for resistance development (Li, 2011). Three mechanisms are important in their low level of induced resistance. First, the positively charged peptides interact directly with the negatively charged cellular membranes of the target cells due to the electrostatic binding. The peptide-membrane interactions result in membrane permeabilization, which leads to a rapid cell death. This physicochemical mechanism lessens development of bacterial resistance because the target of AMPs is the bacterial membrane and membrane redesign by bacteria would be a "costly" solution for most microbial species **(**Wang and Wang, 2017); Secondly, Plant AMPs have multiple mechanisms for attacking bacteria, increasing the probability of success and decreasing the probability of bacterial survival, which might be the best strategy to prevent bacteria from developing resistance (Elmogy *et al*., 2015). It has been demonstrated that nisin (artificial) is a multi-function antimicrobial peptide with at least four different antimicrobial activities combined in one molecule, including inhibition of cell-wall synthesis, increasing pore formation in bacterial membranes, activation of cell wall autolytic enzymes and inhibition of bacterial spore germination; Thirdly, increasing evidence suggests that AMPs are modulators of innate immunity. Because AMPs act through a diverse innate immune system rather than direct action on bacteria, increased resistance is less likely (Shin *et al*., 2016).

Antibiotic adjuvant therapy can be employed to combat antibiotic resistance. Adjuvants can be divided into two classes; class I adjuvants, which affect bacteria by inhibiting active and intrinsic (passive) antibiotic resistance in bacteria, and class II adjuvants, which enhance the ability of the host to neutralize bacteria (Tran *et al*., 2015). Many AMPs can be considered class I adjuvants that interact synergistically (the total effect is greater than the sum of the

individual effects) with antibiotics, or as class II adjuvants (enhances the effectiveness) due to the ability of AMPs to reinforce the host defense system through immunomodulation (Naghmouchi *et al*., 2012).

ADVANTAGES OF ANTIMICROBIAL PLANT PEPTIDES OVER CONVENTIONAL ANTIBIOTICS

Compared to conventional antibiotics, one of the major incentives for the use of plant AMPs is their diverse applications. Antibiotics usually have a narrow spectrum, acting only on bacteria, whereas plant AMPs have a broad spectrum of activity displaying antibacterial, antiviral, anti-parasitic, and anticancer activities. The rate of acquired resistance is lower for plant AMPs compared to antibiotics (Choi and Lee, 2012). Also, compared to antibiotics, cationic AMPs do not elicit bacterial stress pathways such as SOS and rpoS, and therefore does not increase bacterial mutagenesis (Lewies *et al*., 2017). Furthermore, plant AMPs can be used as a single antimicrobial due to their direct killing actions or as adjuvants with conventional antibiotics to obtain synergistic interactions, but said AMPs can be employed to address issues relating to bacterial infections for which antibiotics have not been proven to be successful. These include septicemia (which has an estimated 30% death rate) and infections in individuals who are immune-comprised (due to immunosuppressive diseases or chemotherapy) who cannot provide immune support for antibiotic therapy (Silva *et al*., 2016).

AMPs not only possess the potential of being applied as immune-modulatory/stimulatory compounds but are also able to neutralize endotoxins and prevent sepsis. Septic shock caused by the release of LPS from the cell wall of Gram-negative bacteria, associated with several antibiotic treatments, poses a major problem. Due to the ability of AMPs to neutralize LPS, they make attractive proxies for use in combination with antibiotic treatment to prevent septic shock (Nwokoro *et al*., 2016).

Finally, ribosomally-synthesized AMPs hold better potential than secondary metabolites, including conventional antibiotics and non-ribosomally synthesized AMPs, due to the fact that they are gene-encoded and therefore more susceptible to bioengineering strategies in an attempt to enhance their activities and possibly circumvent bacterial resistance. For example, nisin has activity against most Gram-positive bacteria but lacks activity against Gram-negative bacteria. A bioengineering approach was used to produce nisin A serine 29 derivatives using a site-saturation mutagenesis approach. These derivatives displayed

enhanced activity against MRSA and other Gram-positive bacterial species as well as Gram-negative food associated pathogens, which included *E. coli, Salmonella enterica serovar Typhimurium*, and *Cronobacter sakazakii* (Silva *et al*., 2016). Foregoing the direct development of AMPs as antimicrobials, structural and mechanistic studies of AMPs could also lead to the development of new classes of antibiotics (Pettersen *et al*., 2004).

CONCLUSION

Plant antimicrobial peptides (plant AMPs) share several common characteristics with those from microbes, insects and animals. They are divided into families based on their sequence similarity, Cys motifs, and distinctive disulfide bond patterns which, in turn, determine their tertiary structure folding. Generally, the mechanism of AMP interaction with microbes is believed to be associated with cell lysis due to membrane disruption and/or peptide penetration of lipid membranes followed by attack of intracellular targets. Most of the known plant AMPs act by formation of membrane pores, resulting in ions and metabolites leakages, depolarization, interruption of the respiratory processes, and cell death. Plant AMPs have the ability to bypass the common resistance mechanisms that are reducing the effect and safety of conventional antibiotics. Since membrane integrity is essential for bacterial survival regardless of the metabolic stage of the cell and because AMPs target the membrane, they show good potential to kill even resistant microbes.

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