# Chapter 5
# Plant Antimicrobial Peptides

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**Abstract** Disease afflicts crop productivity as well as nutritional attributes. Pathogens have the ability to mutate rapidly and thereby develop resistance to pesticides. Despite plant's multilayer of innate defence against pathogens, often the latter are able to penetrate and establish themselves on plant host. The discovery of antimicrobial peptides (AMPs) has the promise of durable defence by quickly eliminating pathogens through membrane lysis. AMPs characteristically are made up of from fewer than 20 amino acids to about 100 amino acids, and yet are structurally diverse. AMPs in plants are classified into cyclotides, defensins, lipid transfer proteins (LTPs), thionins, snakins, hevein-like peptides, knottin-type peptides, and others. It is important to characterize and study mechanism of their action in order to develop a wide range of structures with the potential to provide durable plant immunity against pathogens. We bring together recent information on the mechanisms by which AMPs are able to help the plant to thwart pathogen attack. Although permeabilizing cellular membrane is a major mechanism known for AMP action, new and diverse modes of action have recently been unearthed, including targeting of intracellular function of the pathogen.

## 5.1 Introduction

A serious impediment to sustainable production and yield of crops is the major loss due to environmental factors be it of abiotic or biotic nature. The latter factors mainly involve pathogens and pests which regularly pose significant threat to food security globally. Pathogens find unique ways to establish themselves on their plant

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hosts, particularly because of their variability, diversity, and ability to mutate. The host plant, in return, also employs a medley of processes in response to pathogen attack, but such multilayer nature of host defence also eventually capitulates. R-gene regulated pathogen resistance is well known in plants (Chisholm et al. 2006). Breeders employed selection pressure for identifying resilient cultivars with robust 'R' resistance factors and incorporated such resistance in high yielding cultivars. However, such strategy works as long as the 'R' resistance does not break down. The other caveat is that it is often a slow process and restricted to closely related species. Thus far, elite breeding lines together with the use of chemical pesticides have contained plant diseases to a large extent. Unfortunately, regular and excessive pesticide use has led to environmental and human health issues.

Considerable attention has also been given to understanding plant-pathogen interactions in order to highlight plant genes that durably respond to a pathogen ingression in order to develop durable disease resistance through 'innate' immunity. In addition to hypersensitive defence response and 'R' resistance proteins, plants also employ barriers through the cell wall and synthesize antimicrobial peptides (AMPs). The defence employed through the AMPs has generated much attention as also the recombinant technology as potential alternative strategies to contain pathogens from devouring their host plants.

## 5.2 Structure and Classification

Small peptides are generally made in a cell either as precursor proteins or non-precursor proteins. The precursor protein can possess functional significance or be nonfunctional. The term nonfunctional is used for those having no known biological activity, as for nonfunctional precursors. Interestingly, small peptides form, in certain instances, a part of plant proteins, somewhat buried within the long stretch. Such buried peptides have a distinct biological activity. The non-precursor-derived peptides encoded by sORFs are located in or near five regions of a gene (Tavormina et al. 2015). Some antimicrobial peptides (AMPs) are made as precursor proteins that need to be processed to produce a functional peptide.

AMPs are grouped according to their origin, primary and secondary structure, and the presence of disulfide linkages or net charge. Some have either α helical, β sheets or both αβ secondary structures. Majority of AMPs are rich in basic amino acids providing them a net positive charge at physiological pH and are called cationic AMPs. AMPs are made of fewer than 20 amino acids to about 100 amino acids. Details on each AMP category or family of peptides have been reviewed (Stotz et al. 2013; van der Weerden et al. 2013; Nawrot et al. 2014). As mentioned above, peptides have also been classified based on their synthesis as precursor proteins and/or posttranslational processing into mature peptides (Tavormina et al. 2015). A brief description of the prominent families of AMP members is given below. The 3D ribbon structures of representative AMPs categorized according to the prevalent system are illustrated in Fig. 5.1.

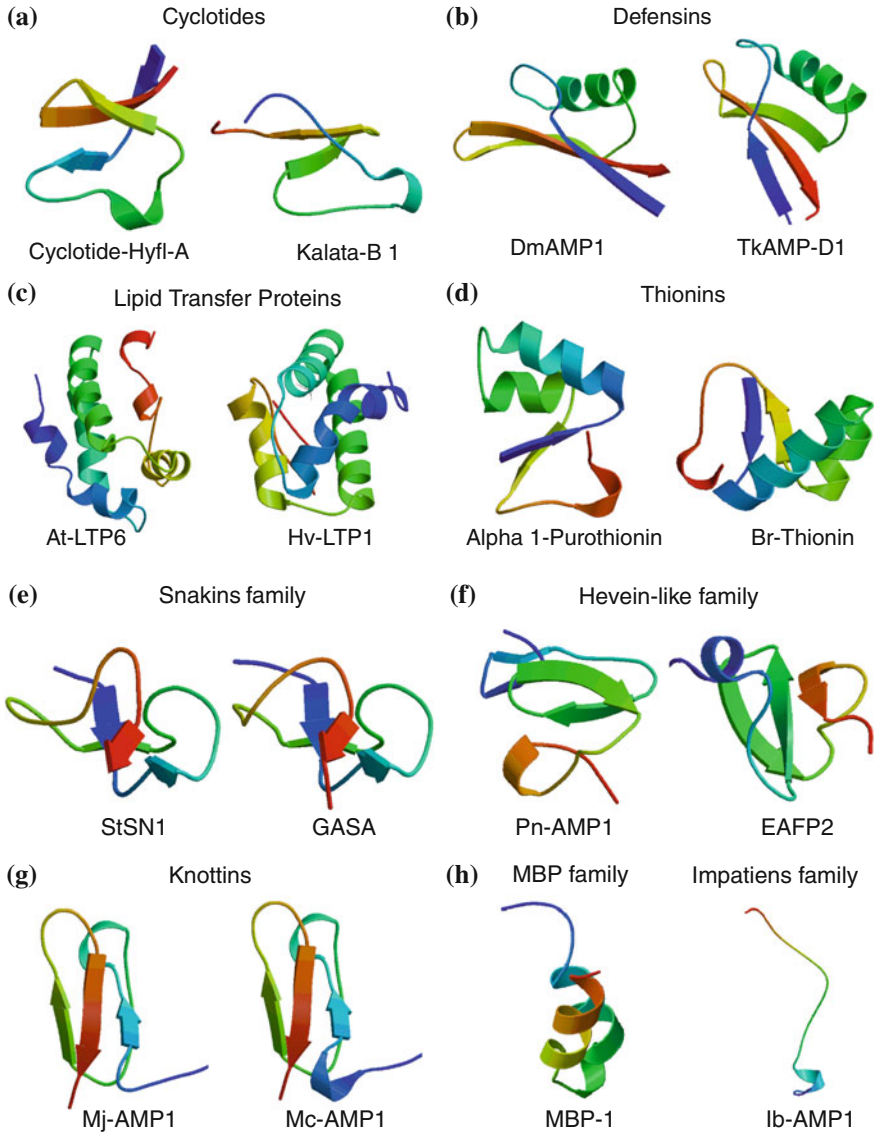


**Fig. 5.1 a–h** 3D ribbon structures of different family members of plant AMPs. The structures were computed using SWISS-MODEL. Cyclotide Hyfl-A: *Hybanthus floribundus* (P84647), Kalata-B1: *Oldenlandia affinis* (P56254), DmAMP1: *Dahlia merckii* (P0C8Y4), Tk-AMP-D1: *Triticum kiharae* (P84963), At-LTP6: *Arabidopsis thaliana* (Q9LDB4), Hv-LTP1: *Hordeum vulgare* (A8YPK3), Alpha-1-Purothionin: *Triticum aestivum* (P01543), Br-Thionin: *Brassica rapa subsp. pekinensis* (Q9SBK8), StSN1: *Solanum tuberosum* (Q948Z4), GASA: *Fagus sylvatica* (Q0VYL5), Pn-AMP1: *Ipomoea nil* (P81591), EAFP2: *Eucommia ulmoides* (P83596), Mj-AMP1: *Mirabilis jalapa* (P25403), Mc-AMP1: *Mesembryanthemum crystallinum* (O81338), MBP-1: *Zea mays L.* (P28794), Ib-AMP1: *Impatiens balsamina* (O24006). AMP name: plant name (GenBank or UniProt ID)

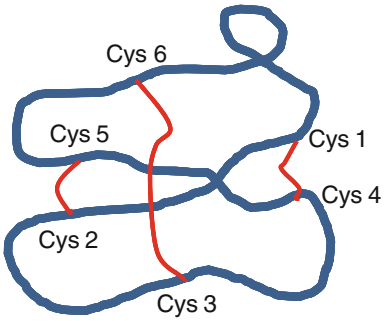


**Fig. 5.2** Cyclotide backbone connected through three disulfide bonds at designated cysteine residues to create a CCK motif

### *5.2.1 Cyclotides*

Cyclotides form the largest family of plant AMPs. Violaceae and Rubiaceae families are the richest source of cyclotides but they have also been detected in Fabaceae, Cucurbitaceae, Poaceae, and Solanaceae families. These AMPs are characterized by their unique structure where N- and C-termini are attached through a peptide bond to form a cyclic backbone (Fig. 5.1a). The cyclic structure is made of approximately 30 amino acids, which contains six cysteine residues engaged in three internal disulfide bonds to give it a cyclic cystine knot (CCK) structural motif (Fig. 5.2). The CCK motif provides extraordinary stability to the peptide as also resistance against proteases. Its surface exposed hydrophobic amino acids influence its antimicrobial activity. In addition to cysteine residues, Glu in loop 1 is highly conserved. Its ability to form hydrogen bond contributes to the cyclotides activity. The ribbon model of two cyclotides, kalata B1 and cyclotide-Hvfl A is presented in Fig. 5.1a. The cyclotides are synthesized as precursor molecules with a conserved signal for endoplasmic reticulum (ER) along with pro-region and a highly conserved N-terminal repeat (NTR). The presence of NTR in multiple numbers can lead to multiple molecules of cyclotides. The structure, isolation, and synthesis of cyclotides have been recently reviewed (Burman et al. 2014).

### *5.2.2 Defensins*

Defensins, representing another large family of AMPs, are widely distributed in plant species. Defensins are the best studied Cys-rich peptides. Initially thought to be localized to seeds, their distribution in almost all plant organs has since become apparent. The defensins are synthesized as two types of precursor molecules. Majority of the defensin precursors contain ER sequence and a mature ‘defensin

domain'. In another category, the precursors are larger in size and contain an additional C-terminal prodomain (Aerts et al. 2008). Defensins are rich in cysteine content, carry a net positive charge, and constituted between 45–54 amino acids. The conserved eight cysteine residues with disulfide bridges favour triple-stranded antiparallel β-sheets and one α-helix structure (Fig. 5.1b). One disulfide bond near N- and C-termini provides extraordinary stability to the peptide. Besides four disulfide bonds present in a majority of defensins, an additional Cys–Cys has been noticed in *Petunia hybrida* peptide (PhD1). The core conserved structure of the defensin is maintained even with an additional disulfide bond. In addition to conserved cysteines, glycine residue (near fifth) and second cysteine residue are conserved with a high priority for an aromatic amino acid before second conserved cysteine residue (Fig. 5.3). The integrity of disulfide bonds and structural conformation is essential for antimicrobial activity but the stability of the structure does not directly correlate with the activity. Structure–activity relationship of defensins has been reviewed (Sagaram et al. 2011; Lacerda et al. 2014). A majority of the

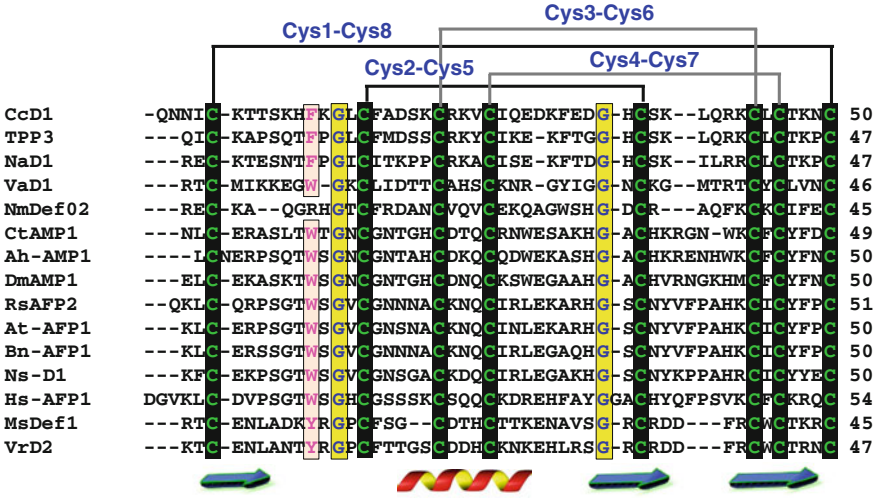


**Fig. 5.3** Alignment of amino acid sequence of plant defensins. CcD1: *Capsicum chinense* (Af128239), TPP3: *Lycopersicon esculentum* (4UJ0), NaD1: *Nicotiana tabacum* (P32026), NmDef02: *Nicotiana megalosiphon* (ACR46857), Ct-AMP1: *Clitoria ternatea* (AAB34971), Ah-AMP1: *Aesculus hippocastanum* (Q7M1F3), DmAMP1: *Dahlia merckil* (P0C8Y4), At-AFP1: *Arabidopsis thaliana* (P30224), Bn-AFP1: *Brassica napus* (Q39313), Ns-D1: *Nigella sativa L.* (P86972), Rs-AFP2: *Raphanus sativus* (P30230), Hs-AFP1: *Heuchera sanguinea* (AAB34974), MsDef1: *Medicago sativa* (Q9FPM3), VrD2: *Vigna radiata* (2GL1). The residues enclosed in eight black bars represent highly conserved cys. The *solid lines* indicate disulfide bonds engaging the two cys residues. The two highly conserved gly residues are shown in *greenish yellow*. The preference for an aromatic amino acid before 1st conserved gly is labelled in *pink*. The *arrows* represent β-sheet and a helix represents α-helical secondary structures corresponding to the amino acids above them. AMP name: plant name (GenBank or UniProt ID)

defensins possesses activity against diverse range of fungi and oomycetes, but some members are toxic to bacteria.

### 5.2.3 Lipid Transfer Proteins

The nonspecific, plant lipid transfer proteins (nsLTPs) were first isolated from potato tubers and later discovered from a wide range of monocotyledonous (monocots) and dicotyledonous (dicots) species. nsLTPs represent small proteins deriving their name from their function of transferring lipids between the different membranes as well as in vitro. They carry lipids nonspecifically, the list includes phospholipids, fatty acids, their acylCoAs or sterols. LTPs with approximately 100 amino acids are relatively larger in size than defensins. Depending on their size, LTPs are subcategorized into LTP1s and LTP2s having a molar mass of 9 and 7 kDa, respectively. These are synthesized with an N-terminal signal sequence directing them to cell walls. Some LTPs possess a C-terminal sequence which enables their posttranslational modification with a glycosylphosphatidylinositol molecule. The latter facilitates the integration of LTP on extracellular side of the plasma membrane. LTPs are structured with eight cysteine residues forming four disulfide bridges like defensins. However, LTPs are distinct in having four α-helices in their tertiary structure (Fig. 5.1c), which carve out a hydrophobic cavity to bind the lipids through hydrophobic interactions. A different arrangement of cysteine residues in disulfide bonds results in two types of folds—Type 1 and Type 2. These folds provide different specificity of lipid binding at the LTP binding site with Type 2 fold relatively more flexible and with lower lipid specificity than Type 1.

### 5.2.4 Thionins

The first plant thionin AMP was isolated in 1942 from wheat flour and labelled as purothionin. Thionins are yet another class of cysteine-rich peptides that are present in a wide range of plants. They are smaller in size, ~5 kDa containing 45–47 amino acids. Thionins comprise of two distinct groups of plant peptides —α/β-thionins and γ-thionins with distinguished structural features. Based on γ-thionins' more resemblance with defensins than the other group of thionins, it has been suggested that they should be placed along with defensins (Stec 2006). Both groups of peptides share about 25 % sequence similarity. Thionins, rich in basic amino acids providing the peptides a net positive charge, have highly conserved Lys1, Arg10 and Tyr13 in addition to six Cys residues. The secondary structure contains two antiparallel α-helices and an antiparallel β-sheet (Fig. 5.1d). α/β-Thionins are further divided into five sub-types based on the number of disulfide bonds, net charge, length, or the origin. Thionins I and II contain eight Cys residues bonded with each other to make four disulfide bridges. Type I are more

basic and contain 45 amino acids compared to Type II, which have 46–47 amino acids. Type III are 45–46 amino acids long, containing three disulfide bridges and being basic as the Type IIs. Like Type III, the Type IV thionins have three disulfide bonds but possess no charge at neutral pH. Type V are the truncated forms of thionins demonstrating no activity. Thionins are synthesized as precursor molecules and demonstrate antimicrobial activity after acidic C-terminal domain is removed (Ponz et al. 1983). Interestingly, an unprocessed thionin has been identified in Arabidopsis, providing an example of peptides derived from a functional protein, without the involvement of a precursor (Tavormina et al. 2015). Further, a thionin proprotein processing enzyme has been isolated and characterized from barley that releases the acidic domain of leaf-specific thionin (Plattner et al. 2015). Thionins have broad-spectrum antimicrobial activity targeting bacterial, fungal and mammalian cells.

### 5.2.5 *Snakins*

Snakins too are cysteine-rich peptides differing from other cysteine-abundant AMPs in having relatively more number of disulfide bonds. As the name suggests, there is a structure motif similarity between snakins and the hemotoxic desintegrin-like snake venoms. The first snakin, Snakin-1 (StSN1), was isolated from potato tubers (Segura et al. 1993). The sequence of 63 amino acids long StSN1 did not relate to previously purified protein sequence. Instead, it depicted homology with some sequences deduced from plant cDNAs that were induced by the plant hormone gibberellic acid. This led to their being categorized as GASA (gibberellic acid stimulated in Arabidopsis) protein family. Later, another snakin, Snakin-2 (StSN2) was identified in potato tubers that was inducible by certain phytopathogens. The precursor form of StSN2 is processed into 66 amino acids long peptide that has low identity (38 %) with StSN1. The members of snakin/GASA family contain N-terminal signal sequence of 15–20 residues followed by a variable region both in terms of length, amino acid composition and a region of approximately 60 residues at C-terminus. The latter contain 12 Cys conserved residues forming six disulfide bonds and nine other conserved amino acids. Later studies found snakins expressed in different plant organs and widely spread in both monocots and dicots. Like other AMPs, the mature snakins are enriched in basic amino acids and thus are positively charged. The 3D Swiss-Models of some snakins depicted only β-sheets and the absence of α-helices was conspicuous (Fig. 5.1e).

### 5.2.6 *Hevein-like Peptides*

Hevein was discovered in the latex of rubber tree (*Hevea brasiliensis*). Due to its chitin-binding property, it inhibits the hyphal growth and confers protection against

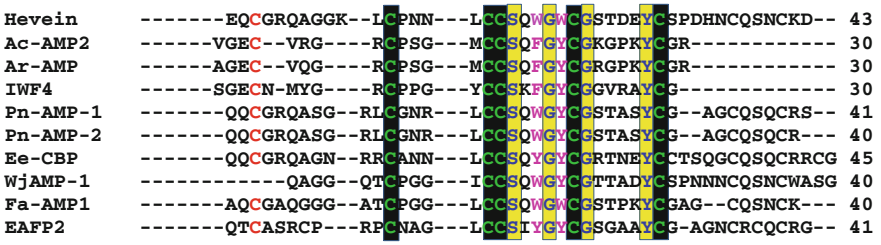


**Fig. 5.4** Alignment of amino acid sequences of Hevein-like peptides. Hevein: *Hevea brasiliensis* (P02877), Ac-AMP2: *Amaranthus caudatus* (Q9S8Z7), Ar-AMP: *Amaranthus caudatus* (Q512B2), IWF4: *Beta vulgaris*, Pn-AMP1: *Ipomoea nil* (P81591), Pn-AMP2: *Ipomoea nil* (P81591), Ee-CBP: *Euonymus europaeus* (Q7X9R9), WjAMP-1: *Eutrema wasabi* (Q8H950), Fa-AMP-1: *Fagopyrum esculentum* (P0DKH7), EAFP2: *Eucomia ulmoides* (P83596). The highly conserved cys residues are enclosed in *black bars*. The cys residues labelled in *red* depict a nearly conserved cys at this position. The other conserved amino acids (ser, gly, and tyr) are shown in *greenish yellow*. The preference for an aromatic amino acid before and after conserved gly is labelled in *pink*. AMP name: plant name (GenBank or UniProt ID)

fungal phytopathogens (Van Parijs et al. 1991). Hevein is a relatively small peptide with 43 residues (4.7 kDa) and contains 3–5 disulfide bridges. The other chitin-binding peptides that display similarity in their antifungal activity but differ in amino acid composition from Hevein are grouped in hevein-like peptides. Several hevein-like peptides have been isolated from plants, including *Beta vulgaris* (IWF4), *Pharbitis nil* (Pn-AMP1) and *Eucommia ulmoides* (EAFP2). These peptides contain 6, 8 and 10 Cys residues, respectively. The 3D structure of hevein-like peptides contains three antiparallel β-sheets (Fig. 5.1f). The presence of α-helical turns varies from peptide-to-peptide. The sequence alignment of hevein-like peptides shows five Cys residues that are highly conserved and another towards N-terminus is nearly conserved (Fig. 5.4). The four conserved Cys residues near C-terminus are part of a conserved domain in the peptides, which seems to follow a pattern of Cys–Cys–Ser–X–(aromatic amino acid)–Gly–(aromatic amino acid)–Cys–Gly–$X_4$–Tyr–Cys. Initially, binding to chitin in fungus cell wall was thought to be an essential property of hevein-like peptides. However, when other hevein-like peptides were isolated (e.g. Pn–AMP1 and EAFP2) these were found to target fungi irrespective of the presence of chitin (see van der Weerden et al. 2013). Pn–AMP1 being highly basic (pI 12.02), with a net positive charge belongs to a broad category of cationic AMPs. The distribution of hevein-like peptides from aerial parts of plants to seeds indicate this group of peptides may contribute to plant immune system.

### *5.2.7 Knottin-Type Peptides*

Knottin peptides contain six Cys residues forming three disulfide bonds with one disulfide bond crossing through the other two like in cyclotides. Like with

cyclotides, the Cys-stabilized structure supports a knotted fold. However, the free N- and C-termini make them distinct from cyclotides (Fig. 5.1g). The first plant knottin-type peptides, Mj-AMP1 and Mj-AMP2, were identified from *Mirabilis jalapa* L. (Cammue et al. 1992), and subsequently from *Phytolacca americana* (PAFP-S) and *Mesembryanthemum crystallinum* (Mc-AMP1). These peptides are synthesized as precursor proteins and after maturation display antimicrobial activity against both fungi and bacteria. The structures of Mj-AMP1 and Mc-AMP1 consist of triple-stranded, antiparallel β-sheets connected through a loop (Nawrot et al. 2014).

### *5.2.8 Other AMPs*

There are several other AMPs that do not relate with the abovementioned categories of peptides but possess unique amino acid composition or secondary structures. Some of them are named after the plant source from where they were isolated. For instance, 1b-AMP1 (Impatiens family) and Shepherin-1 (Shepherin family) were isolated from *Impatiens balsamina* and *Capsella bursa-pastoris* (Shepherds purse), respectively. The others include vicilin-like, 2S albumin peptides, MBPs, puroindolines, hairpinins, β-barrelins, glycine-rich cysteine-free, and glutamic acid-rich peptides. A 3D structure of two such AMPs is shown in Fig. 5.1h. A recently isolated peptide from *Benincasa hispida* seeds called Hispidulin containing 49 residues does not show homology with any known sequence in the database. As we make more discoveries, the number of unique peptides that do not share similarity with known peptides is likely to grow. A wide spectrum of peptides should in the future provide a better classification rationale.

## 5.3 Mechanism(s) of Action

Most of the AMPs are able to target several different fungal and bacterial pathogens. This broad-spectrum ability suggests that AMPs interfere with the structural and/or functional cellular components essential for the survival and proliferation of a pathogen. The protective cell wall of a microorganism is likely the first contact point with AMPs. The fungal cell wall is complex, assembled in many layers comprising 80 % heteropolysaccharides (Fig. 5.5a). The inner most layer or plasma membrane is composed of lipid bilayer with interspersed proteins surrounded by chitin and β-glucan layers. The outer envelope is made mainly of mannosylated glycoproteins that aid in host cell wall receptor recognition and interaction. A Gram-negative bacterial cell wall contains inner lipid bilayer membrane surrounded by a thin layer of peptidoglycan (Fig. 5.5b). Additionally, there is an outer membrane composed of phospholipids and lipopolysaccharides. The latter are highly charged molecules providing a net negative charge to the membrane surface.

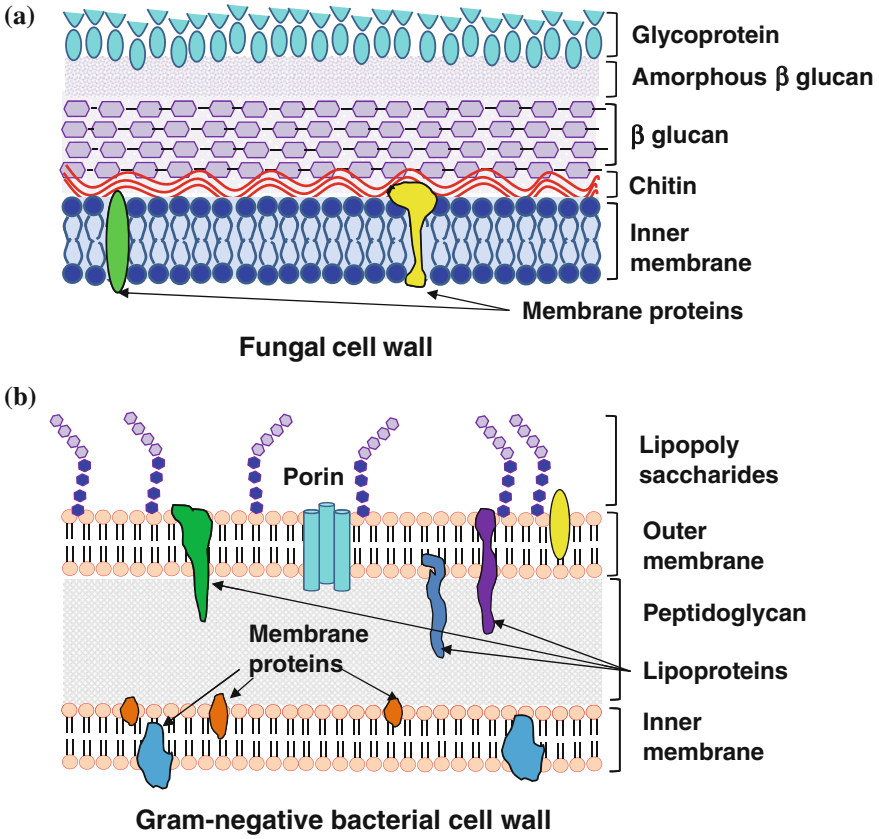


**Fig. 5.5** Composition of typical fungal and Gram-negative bacterial cell walls

The Gram-positive bacteria, however, contain only a very thick layer of peptidoglycan adjacent to inner membrane. The enrichment of peptidoglycan layer with acidic polysaccharides such as teichoic and teichuronic acids confers a negative charge to the membrane. The plant cell wall has distinguishable features of cellulose, hemicellulose, lignins and the absence of peptidoglycan, chitin and β-glucan. In mammalian membrane phospholipids, phosphatidylcholine and phosphatidylethanolamine are neutral in charge.

AMPs from plants or mammalian sources can distinguish the host from its microbial targets. The studies indicate that structural disparity of prokaryotic and eukaryotic membranes contribute towards the AMP selectivity (Zasloff 2002; Yeaman and Yount, 2003; Yount and Yeaman 2013). In spite of significant structural differences in prokaryotic organisms, the AMPs are known to establish interaction at the surface of these microbes. The structure of AMPs with hydrophobic regions and net positive charge supports the interaction with negatively-charged polar heads and hydrophobic core of the microbial membranes.

A high positive charge in cationic AMPs forges an electrostatic interaction, thus facilitating their initial binding to the membranes.

The structure of an AMP is critical to its antimicrobial activity. There are several structural parameters such as conformation, charge, hydrophobicity, hydrophobic moment, amphipathicity and polar angle that contribute to the toxicity and target specificity. The topic has been comprehensively reviewed (Yeaman and Yount 2003). Experimental evidence showed the presence of specific binding sites for AMPs on targeted pathogen envelope. For example, mannosylinositol phosphoryl-ceramide, an acidic complex sphingolipid in fungal cell wall, was identified as a high-affinity binding target for a defensin (DmAMP1) from *Dahlia merckii* (Thevissen et al. 2003). Thus, it would mean that the binding of an AMP to a pathogen is not necessarily dictated only by electrostatic interactions but also recognition of specific cell wall component(s). Once the contact has been made via an initial interaction and subsequent binding of an AMP with the target, the toxic effect on the pathogen can then be exerted in two broad ways, membrane permeabilization and impairment of intracellular functions as discussed below.

### *5.3.1 Membrane Permeabilization*

Membrane permeabilization occurs after an AMP interacts with the target site of the pathogen. It results in dissipation of electrochemical gradient across the membrane, membrane fragmentation, leakage of ions and other cellular contents and ultimately cell death (Shai 2002). A threshold concentration of an AMP is required for inducing permeabilization and the phenomenon is time-dependent (Wimley 2010). More structural deformity of the membrane occurs over time. Membrane permeabilization can occur in different ways depending on the interaction dictated by the structure of an AMP. Models have been proposed to explain the disruptive effect of AMPs on the membranes (Fig. 5.6).

#### 5.3.1.1 Barrel-Stave Model

In this model, AMP molecules bind to the target membrane as monomers. After self-aggregation, the molecules get inserted across the membrane to form a transmembrane pore (Fig. 5.6b). The hydrophobic regions of α-helix or β-sheets of an AMP align with the hydrophobic core of target membrane and hydrophilic surfaces form the lining or lumen of the pore. The peptides engaged in a pore are oriented parallel to the lipid bilayer. The pore size may vary depending on the peptide and the degree of aggregation. It can be further expanded in a cooperative manner by assembling more molecules into the pore. A case study with alamethicin, a 20-residue peptide produced by fungus *Trichoderma viride*, lent evidence in favour of the Barrel-Stave pore mechanism.

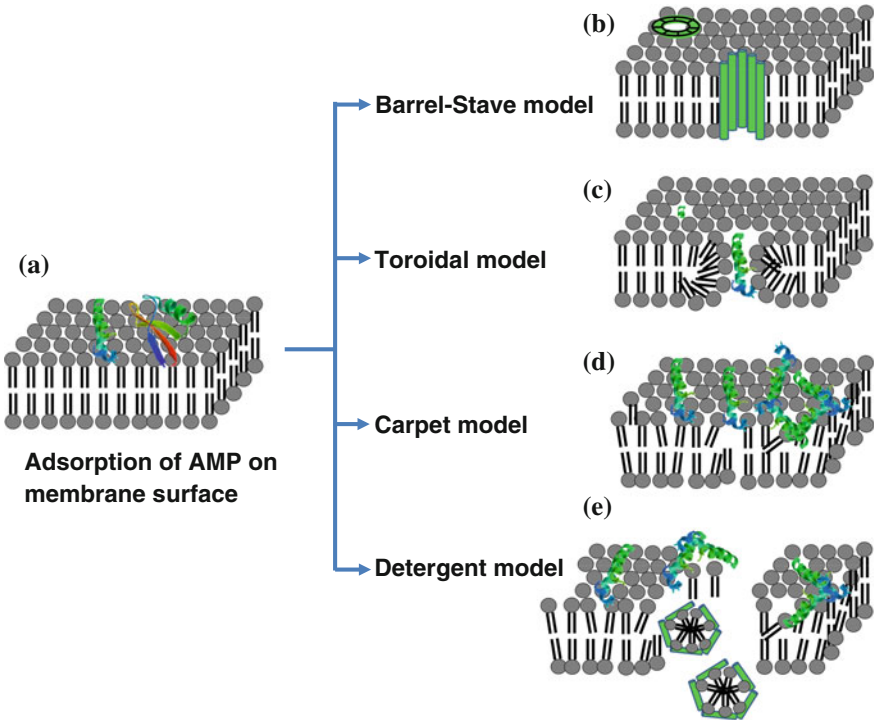


**Fig. 5.6** Models of membrane permeabilization by antimicrobial peptides. After initial attachment of an AMP on the membrane surface (**a**), it can disrupt the membrane structure/function either through pore formation (**b, c**) or by other mechanisms (**d, e**)

#### 5.3.1.2 Toroidal Model

The Toroidal model or Wormhole mechanism is also a pore forming way of membrane disruption. Its major difference from the Barrel-Stave model is that the AMP intercalation in lipid bilayer induces positive curvature of phospholipid polar heads perpendicular to the membrane plane. The peptide provides a stronger alternative of both hydrophobic and hydrophilic interactions than intramolecular interactions of lipid molecules. The presence of a peptide, thus, breaks hydrophobic–hydrophobic interactions of lipid molecules and favours their realignment to create toroidal pores (Fig. 5.6c). In contrast to Barrel-Stave model, the lipid headgroups in toroidal pores are exposed to the lumen of a pore. In vitro studies with peptide and membrane vesicles have suggested that the threshold of peptide-to-lipid (P/L) ratio for magainin is 1:30, which is consistent with the micromolar quantities of peptides required for their toxic effect on pathogen membranes.

#### 5.3.1.3 Carpet Model

Carpet model is a non-pore forming mechanism of AMP action. In contrast to forming pores, the peptide does not insert into the hydrophobic core of the membrane as observed in pore inducing models but instead orients itself parallel to the membrane surface and covers it like a carpet. A strong electrostatic interaction between negatively charged phospholipid polar headgroups and the cationic peptide distorts the structure of the membrane and its fluidity (Fig. 5.6d). As the peptide reaches its threshold membrane disintegration or cell lysis is induced. The model was first proposed to explain the toxic effect of a moth hemolymph cecropin P1, which aligns parallel with the membrane surface. Its activity was noticeable only at relatively high concentrations or at high P/L ratio. Dermaseptins from *Phyllomedusa spp* are thought to follow carpet model to induce membrane damage.

#### 5.3.1.4 Detergent Model

This is an extended version of the carpet model of AMP action. The peptide interacts through a mechanism similar to the carpet model, leading to catastrophic collapse of the membrane. The peptide molecules form micelles with the fragmented membrane like a detergent (Fig. 5.6e). The comprehensive breakdown of the membrane cannot hold its contents and results in cell death.

### *5.3.2 Impairment of Intracellular Functions*

Membrane permeabilization is considered an important attribute of antimicrobial activity. However, increasing evidence suggests other modes of AMP action in addition to disruption of membrane functions (Brogden 2005; Muñoz et al. 2013). The degree of permeabilization for some peptides did not correlate with their activity, in some the microorganism survived for an extended time period after membrane disruption. In another study, the active fragments of a bovine peptide Bac7 did not permeabilize the *Escherichia coli* membrane but a 2–5 log reduction in viable cell count was apparent (Gennaro and Zanetti 2000). In vitro studies showed the ability of an AMP to associate with intracellular targets such as nucleic acids, proteins or enzymes, which suggested a mechanism of action other than merely involving the membranes. It is now accepted that AMP action is a combined outcome of membrane permeabilization and inhibition of intracellular functions. To facilitate internalization of the peptide into cytoplasm there could be either transient or permanent disruption of the membrane structure, which may enhance the lethal effect of an AMP. It is not known how much contribution membrane

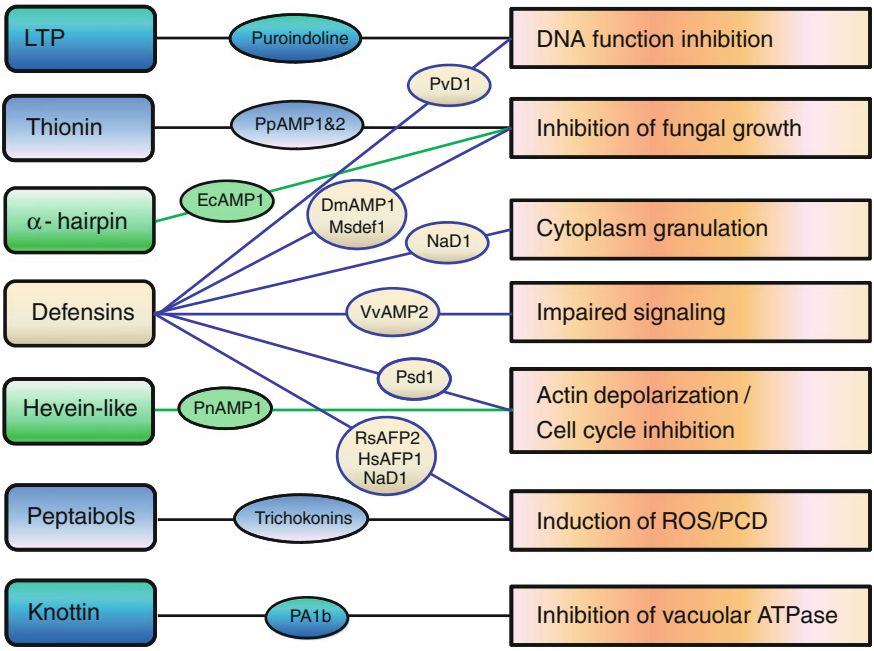


**Fig. 5.7** Plant AMPs targeted cellular functions. The name in *oval* shape represents AMPs, which are connected by *solid lines* to the class they belong to (on the *left*) and their intracellular targets (on the *right*)

permeabilization makes to the potency of such peptides. Other possibilities such as passive peptide transport (Henriques et al. 2006) and/or an active energy-dependent process (Kim et al. 2001) are meaningful. Many plant antifungal defensins interact with fungal membrane sphingolipids or phospholipids. Subsequently, some of them are internalized and induce cell death. For example, cellular uptake for MtDef4, NaD1 and Psd1 has been observed.

Mechanistic details of action for some defensins have been reviewed (Vriens et al. 2014). Once inside the cell, an AMP can target cellular organelles and affect their associated functions. Several plant AMPs have been identified that display cellular toxicity through diverse modes of action. Detailed information on the targets and pathogens have been reviewed (Goyal and Mattoo 2014). A brief summary is illustrated in Fig. 5.7. Different members of AMPs interact with diverse intracellular targets and interfere with their functions. Also, different intracellular functions can be targeted by the same class of AMPs. There is a large repertoire of plant AMPs that target a variety of cellular functions (Goyal and Mattoo 2014).

## 5.4 AMP-Mediated Defence Is Highly Conserved

Protection against life-threatening challenges is a critical determinant of self-survival. Thus defensive mechanisms evolve as organisms combat living in changing environments. Based on organisms and their growth environment some of the defensive mechanisms are widespread although some are unique to a certain class of organisms. In addition to other modes of protection, plants have developed a hypersensitive defence response (HR) against biotic and abiotic stresses. In HR, plants recognize pathogen's presence through structural signatures called pathogen-associated molecular patterns (PAMP) via transmembrane receptors such as receptor-like kinases/proteins. Once the process of recognition is established, a cascade of signalling is initiated to mount a comprehensive defence against the pathogen. The immunity conferred through this mechanism is referred to as PAMP-triggered immunity. In a *tug-of-war* with the pathogen there is an evolutionary trail to keep the effectiveness of defence in place. The plants have evolved strategies to counter the evasion mechanisms developed by the pathogen (Chisholm et al. 2006). It is expected that any type of defence would require allocation of resources in proportion to its magnitude. The comprehensive changes associated with HR defence to pathogens divert the resources that would otherwise be used for growth and development of the plant. Consequently, biotic stresses lead to reduced plant productivity by down-regulation of photosynthetic genes and reduced photosynthetic activity. Thus, in HR defence of plant immunity, there is a fitness cost associated with heightened defence response (Brown 2002; Bolton 2009). Perhaps, this explains why HR, which is an effective response to contain the pathogen, is not all-time-deployment (constitutive) defence feature but activated/induced only in response to a pathogenic or non-pathogenic threat to the plant.

Keeping in view the cost and benefit, the natural selection is likely to favour the retention of a defensive apparatus that has minimal maintenance cost but has high deterrence value. A defence through the deployment of AMPs likely incurs low cost taking into consideration their size and complexity—thus AMPs fit the criterion of a defensive apparatus that has minimal maintenance cost with a high deterrence value. It is therefore not surprising that, in addition to animal and plant sources, AMPs have been identified also in microorganisms including bacteria and fungi (Paiva and Breukink 2013). This reflects the ubiquitous presence of AMPs, ranging from microorganisms to higher eukaryotes.

Like eukaryotic AMPs, bacterial species and members of Archaea domain synthesize peptides with antimicrobial activity involving ribosomal machinery. These peptides are named bacteriocins, which are active against human and animals pathogens. The most commonly known bacteriocin is nisin, which is a 34-amino acid long, cationic and hydrophobic peptide produced by a Gram-positive bacterium. Interestingly, nisin uses membrane disruption of the target through pore formation as observed for eukaryotic AMPs. The negative charge on bacterial membrane lipids facilitates the binding of cationic nisin and subsequently peptide molecules aggregate along with lipids to create a pore. Besides being similar in their

mechanism of action, an evolutionary conservation is apparent in structural relatedness of fungal defensin-like peptides. The first defensin-like peptide, plectasin, identified from a saprophytic fungus, *Pseudoplectania nigrella* is structurally similar to defensins from primitive arthropods and molluscs. Like plant defensins, many fungal defensin-like peptides are cysteine rich and have α-helix and β-sheet structures. Among the six families of defensin-like peptides predicted by computational studies in fungal genome three families display high similarity with insect, invertebrate and plant defensins. A genetic closeness study between certain eukaryotic AMPs revealed a structural conservation in evolutionary divergent group of organisms suggesting minimal speciation events during evolution (Goyal and Mattoo 2014). These observations point to AMPs as integral components of innate immune defence in organisms early during evolution, while the retention of the close-to-basic form suggests their importance in the defence architecture of living organisms. A broad-spectrum activity and protective function across kingdoms highlights the importance of AMP-mediated defence.

## 5.5 AMP Potency Across Kingdoms

AMPs display effectiveness at low concentrations and relatively within short exposure times against pathogens. Their potency has been assessed through in vitro studies involving a purified candidate peptide and a targeted pathogen grown in culture media, and generally expressed as $IC_{50}$ (a concentration of peptide required to inhibit 50 % growth) or as MIC (minimum inhibitory concentration: a minimum concentration of peptide required to completely inhibit the growth). The $IC_{50}$ values for plant AMPs ranges from <1.0 to >100 μg/ml. For each AMP the $IC_{50}$ value varies from pathogen to pathogen. The composition of growth media also affects the $IC_{50}$ or MIC values. The effective in vivo concentrations of AMP that provide immunity against pathogens, however, are largely unknown. The $IC_{50}$ or MIC values for some AMPs are given in Table 5.1.

AMPs are known to possess broad-spectrum antimicrobial activity. Both in vitro studies and in vivo expression of AMPs in transgenics suggested that the antimicrobial activity of peptides isolated from an organism is not restricted against its own pathogenic population but also well beyond the phylum or kingdom. This characteristic of AMPs is evident from their mode of action where AMPs target microbial cell walls in addition to binding to specific domains. The activity across the kingdoms has been observed in plant isolated AMPs, which showed antimicrobial activity against mammalian pathogens, including human ones. Conversely, AMPs isolated from insects, arthropods, amphibians, humans, etc., display toxicity against a variety of phytopathogens. In cross-kingdom scenarios, the AMPs do not exhibit cytotoxicity to the host cells. This property of AMPs has enhanced the scope of their application in disease management of humans or other mammals of

**Table 5.1** Plant AMP IC50 or MIC values against specified microbial pathogens

| Peptide | Class/family | Source | Pathogen | $IC_{50}$ or MIC |
|---|---|---|---|---|
| Circulin-A | Cyclotide | *Chassalia parviflora* | *Staphylococcus aureus* | MIC: 0.19 μM |
| – | – | – | *Candida kefyr* | MIC: 18.6 μM |
| Circulin-C | Cyclotide | *Chassalia parviflora* | HIV-1 | $IC_{50}$: 50–275 ηM |
| Kalata-B1 | Cyclotide | *Oldenlandia affinis* | *Staphylococcus aureus* | MIC: 0.26 μM |
| – | – | – | *Pseudomonas aeruginosa* | MIC: >500 μM |
| – | – | – | *Candida albicans* | MIC: >500 μM |
| Rs-AFP2 | Defensin | *Raphanus sativus* | *Pyricularia oryzae* | $IC_{50}$: 0.4 μg/ml |
| – | – | – | *Verticiliium dahliae* | $IC_{50}$: 1.5 μg/ml |
| – | – | – | *Alternaria brassicola* | $IC_{50}$: 2 μg/ml |
| Ah-AMP1 | Defensin | *Aesculus hippocastanum* | *Bacillus subtilis* | $IC_{50}$: 100 μg/ml |
| – | – | – | *Leptosphaeria maculans* | $IC_{50}$: 0.5 μg/ml |
| Psd2 | Defensin | *Pisum sativum* | *Neurospora crassa* | $IC_{50}$: <0.5 μg/ml |
| – | – | – | *Fusarium solani* | $IC_{50}$: 8.5 μg/ml |
| Ace-AMP1 | LTP | *Allium cepa* | *Alternaria brassicola* | $IC_{50}$: 2.5 μg/ml |
| – | – | – | *Verticillium dahliae* | $IC_{50}$: 0.25 μg/ml |
| – | – | – | *Botrytis cinerea* | $IC_{50}$: 3 μg/ml |
| La-LTP | LTP | *Leonurus artemisia* | *Ralstonia solanacearum* | $IC_{50}$: 15 μM |
| – | – | – | *Botrytis cinerea* | $IC_{50}$: 7.5–15 μM |
| Cw18 | ns-LTP | *Hordeum vulgare* | *Fusarium solani* | MIC: 174 μg/ml |
| Pp-AMP1 | Thionin | *Phyllostachys pubescens* | *Erwinia carotovora* | $IC_{50}$: 22 μg/ml |
| – | – | – | *Clavibacter michiganensis* | $IC_{50}$: 14 μg/ml |
| – | – | – | *Fusarium oxysporum* | $IC_{50}$: 2 μg/ml |
| Tu-AMP1 | Thionin | *Tulipa gesneriana* | *Erwinia carotovora* | $IC_{50}$: 11 μg/ml |
| – | – | – | *Fusarium oxysporum* | $IC_{50}$: 2 μg/ml |
| AX1 | Thionin | *Beta vulgaris* | *Cercospora beticola* | MIC: 4 μg/ml |
| AC-AMP1 | Hevein-like | *Amaranthus caudatus* | *Fusarium culmorum* | $IC_{50}$: 2 μg/ml |
| – | – | – | *Alternaria brassicola* | $IC_{50}$: 7 μg/ml |
| – | – | – | *Bacillus megaterium* | $IC_{50}$: 40 μg/ml |

(continued)

**Table 5.1** (continued)

| Peptide | Class/family | Source | Pathogen | $IC_{50}$ or MIC |
|---|---|---|---|---|
| Ee-CBP | Hevein-like | *Euonymus europaeus* | *Botrytis cinerea* | $IC_{50}$: 0.2 μM |
| – | – | – | *Alternaria brassicola* | $IC_{50}$: 0.6 μM |
| – | – | – | *Pythium ultimum* | $IC_{50}$: 6.6 μM |
| Fa-AMP1 | Hevein-like | *Fagopyrum esculentum* | *Clavibacter michiganensis* | $IC_{50}$: 14 μg/ml |
| – | – | – | *Fusarium oxysporum* | $IC_{50}$: 19 μg/ml |
| – | – | – | *Geotrichum candidum* | $IC_{50}$: 36 μg/ml |
| StSN1 | Snakins | *Solanum tuberosum* | *Listeria monocytogenes* | MIC: 10 μg/ml |
| – | – | – | *Botrytis cinerea* | $IC_{50}$: 2 μM |
| – | – | – | *Colletotrichum graminicola* | $IC_{50}$: 10 μM |
| MJ-AMP1 | Knottins | *Mirabilis jalapa* | *Bacillus megaterium* | $IC_{50}$: 6 μg/ml |
| – | – | – | *Cercospora beticola* | $IC_{50}$: 10 μg/ml |
| – | – | – | *Ascochyta pisi* | $IC_{50}$: 200 μg/ml |
| Pa-AMP1 | Knottins | *Phytolacca americana* | *Staphyanococcus sp.* | $IC_{50}$: 11 μg/ml |
| – | – | – | *Fusarium oxysporum* | MIC: 40 μg/ml |
| Ib-AMP4 | Impatiens | *Impatiens balsamina* | *Micrococcus luteus* | $IC_{50}$: 5 μg/ml |
| | | | *Penicillium digitatum* | $IC_{50}$: 3 μg/ml |
| Shepherin I | Shepherin | *Capsella bursa-pastoris* | *E. coli* | $IC_{50}$: <2.5 μg/ml |
| – | – | – | *Fusarium culmorum* | $IC_{50}$: 72 μg/ml |
| MBP-1 | MBP | *Zea maize* | *Fusarium graminearum* | MIC: 60 μg/ml |
| MiAMP2c-3 | Vicilin-like | *Macadamia integrifolia* | *Phytophthora cryptogea* | $IC_{50}$: 5–10 μg/ml |

*Source* http://phytamp.pfba-lab-tun.org/main.php (Hammani et al. 2009)

commercial interest as well as of plants. Interestingly, plant AMPs have shown promising results in specifically targeting the human cancerous cells. Some of the examples of cross protection by AMPs are illustrated in Fig. 5.8.

## 5.6 Production of AMPs in Plants

The therapeutic application of AMPs in medicine and their ability to protect plants from host of diseases generated interest in devising strategies to produce AMPs on a mass scale. A good number of peptide-based drugs have been approved by US

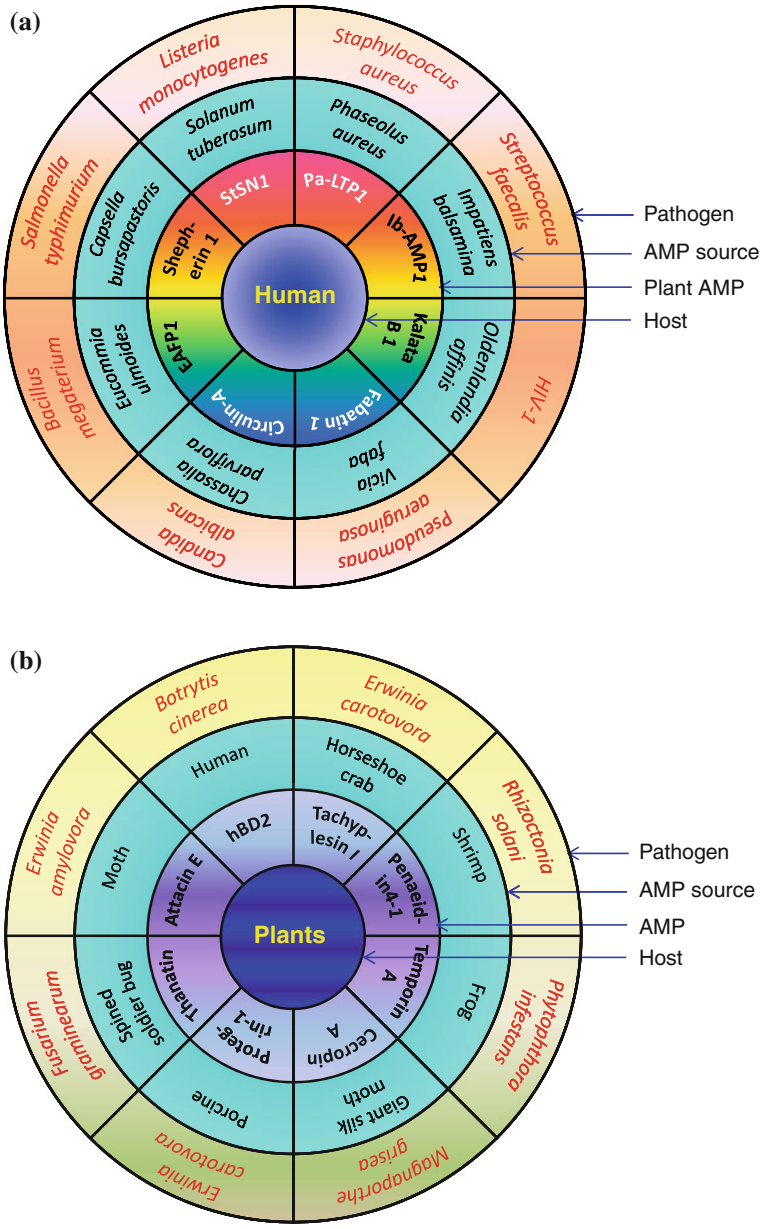


**Fig. 5.8** AMPs showing antimicrobial activity in distantly related hosts to their pathogenic microbes. **a** The AMPs from different plant sources (2nd ring from the outside) active against a variety of human pathogens. **b** The AMPs from different animal sources possess activity against plant pathogens

Food and Drug Administration. Currently, majority of these peptides are produced through chemical synthesis using either *solid-phase or solution-phase* syntheses. Despite high cost of production and environmental effects, chemical synthesis continues to dominate the peptide synthesis industry. Chemical synthesis has advantage of incorporating non-natural components or performing other modifications. Alternate eco-friendly strategies such as biological sources have been explored to produce AMPs in large quantities. High yield, stability, solubility, ease of purification of proteins and scalability of the process are generally some of the criteria for any commercial process. Bacteria and yeast meet most of these criteria with the ease of being genetically transformed and therefore provide the most suitable choice to develop platforms for biomolecule production. However, AMPs are cytotoxic to microorganisms, especially bacteria, and this presents challenges for their deployment as biofactories. Nevertheless, when a snakin peptide SN1 was fused with thioredoxin, it was expressed in *E. coli.* The thioredoxin fusion increased the solubility of the expressed AMP while rendering it ineffective as a toxic compound. Yeast offers another avenue for improving AMP yields once the conditions are optimized. For example, an enhancement in yield of a specific peptide was obtained using a constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase, which is essential to carbohydrate metabolism. Also, the protozoic options, though less common, have been explored.

The successful heterologous, ectopic and overexpression of AMPs in plants is another alternative choice to microbial production. A large leaf biomass and high seed or tuber yields provide a strong platform for the large scale production commonly termed as *molecular farming* of biomolecules. Plant-based systems could be cost effective and proteins can be synthesized with post-translational modifications such as disulfide bond formation and glycosylation. Various strategies have been formulated to increase the proportion of AMPs in soluble fraction of host cells. For enhanced expression, AMP-genes are driven by strong constitutive and inducible promoters. The *cauliflower mosaic virus 35S RNA* and *ubiquitin* are among the commonly used constitutive promoters. Some of the inducible promoters tested include wound, *win3.12T*, and pathogenic, *mannopine synthase,* specific and heat shock responsive *Os.hsp82* promoters. Plants offer many opportunities to manipulate the expression of AMPs for desired results. Some of the strategies employed are briefly described here. For a more descriptive review on plant-based expression systems, the readers are suggested to go to these recent ones (Holaskova et al. 2015; Liew and Hair-Bejo 2015).

### *5.6.1 Sub-cellular Localization of Recombinant Proteins*

The recombinant proteins without a signal peptide usually end up in the cytosol. They accumulate at relatively low concentrations in the soluble forms. A major portion of these tend to be present as insoluble form perhaps due to the lack of chaperons or other cellular factors. Also, such ‘free’ proteins tend to become targets

of endogenous proteases. Therefore to enhance their yield and stability, the candidate protein genes are constructed such that they get targeted to sub-cellular locations such as endoplasmic reticulum, chloroplasts, amyloplasts or extracellular spaces. Chloroplasts in particular have received researchers' attention due to their high number in green tissues like leaves. More importantly, these organelles have their own genome that can be suitably transformed with the desired genes. Compared to nuclear genome transformation chloroplasts can potentially generate more than 20,000 copies per cell. Also, chloroplasts being maternal tissue offer a better control on unintended genetic spread of the recombinant gene.

### 5.6.2 Tissue/Organ Specific Accumulation

Besides leaves that constitute a large biomass, cereal grains, oilseeds, tubers or roots, which are the primary storage organs of plant photosynthates, can also serve as platforms for *molecular farming*. The monoclonal antibody against hepatitis B surface antigen (HBsAg), expressed in tobacco, was the first commercialized plant-derived antibody (Liew and Hair-Bejo 2015). Transformed rice grains are able to accumulate human lysozyme up to 14 % of total soluble protein. A stronger promoter such as the '*rice glutelin 1*' can empower the accumulation of a recombinant protein up to 40 % of total cellular protein. A human lactoferrin protein essential for iron binding was expressed at 25 % of the total proteins. Maize is another cereal crop that has been used for *molecular farming*. Although economically unviable compared to cereals, a higher percentage of recombinant protein can also be obtained in Arabidopsis seeds.

The large genomes of cereal grains have some merits as well as demerits in being employed for protein-making factories. First, it is relatively cumbersome to transform monocots as compared to dicots. The redundancy in the genomes and associated differentially-active regions can limit the expression of the gene especially when a single copy gets inserted into those regions. The large genomes on the other hand are more tolerant to recombinant gene insertions compared to a small genome like in Arabidopsis. The grains offer a better storage medium of the product vis-à-vis other tissues with high moisture content. Like in bacteria and yeast, the stable incorporation of gene in plant genome can be inherited and the seeds then act as mode of continuum propagation. It is estimated that the production cost of a recombinant protein in plants could be 10–50 times less than *E. coli*. Various veterinary vaccines have been expressed in edible portion of plants.

### 5.6.3 Plant Cell Cultures and Protein Production

Cell suspension cultures (CSC) are rapidly dividing cells in liquid medium with appropriate nutrients. They are maintained in closed environments with control of

light and temperature. The tissue culture resembles with CSC except that the dividing cells grow on solid support producing a mass of cells. The CSC has added advantage of excreting the product into liquid medium with appropriate secretary signals. It makes the product recovery process easier thus cutting down the cost of production. CSC has better control over the growth process leading to high batch-to-batch reproducibility, which helps compliance with good manufacturing practise (cGMP). Both these platforms are more contained in nature than the whole plant system. They are considered safe therapeutically, environmentally and in controlling the proliferation of transgene. These factors make the regulatory approval process easier compared to whole transgenic plants. Taliglucerase alfa (TGA) developed by Protalix and Pfizer was the first plant-made pharmaceutical drug approved by FDA. It is a glucocerebrosidase used to treat Gaucher's Disease. TGA employs carrot cells and its production on commercial scale involves a series of bioreactors that can process thousands of litres of growth media. Like TGA and many more pharmaceutical drugs that are at different stages of commercial production and regulatory approval, AMPs can be synthesized on large scale. The selection of new plant sources and optimization of growth conditions of cell culture media are being explored. A recombinant human serum albumin has been tested on a laboratory scale using rice suspension cells in a simplified bioreactor process leading to sixfold increase in yield. With increasing demand of AMPs as pharmaceuticals, the CSC advances are expected to be extended to commercial production of AMPs.

## 5.7 AMPs Are More than Just Antimicrobial Compounds

It has become apparent in recent years that the role of AMPs is larger than strictly being toxic to pathogens. These peptides now appear to be involved in different phases of plants' life cycle (Marshall et al. 2011; Stotz et al. 2013; Pelegrini et al. 2011; Goyal and Mattoo 2014). A few examples are: (a). Defensins share structural similarity with nodule specific cysteine-rich peptides (CRPs) and are abundantly expressed in seeds (Graham et al. 2004). That plant defensins are multi-taskers stems from the fact that CRPs are expressed early during bacterial symbiotic relationship, permeable across bacterial membrane, inhibitory to cell division and suppress reproduction, and released by nodule-specific secretary pathway (Marshall et al. 2011; Penterman et al. 2014). Defensin-like polypeptides—LUREs, DEFL, ZmES-1, DEF2—seem involved in one or the other biological process associated with pollen tube (pollination) in plants. LUREs mediate guidance of the pollen tube (Okuda et al. 2009; Takeuchi and Higashiyama 2012); ZmES-4 leads to pollen tube burst, discharging sperms by targeting potassium channel KZM1 (Amien et al. 2010); PCP–A1 and SP11 peptides contribute to self-incompatibility in Brassica pollen (Doughty et al. 1998; Takayama et al. 2001). Forward and reverse genetic manipulation of DEF2 in tomato resulted in traits showing roles of this gene product in pollen viability, seeding, and morphology (Stotz et al. 2009); while

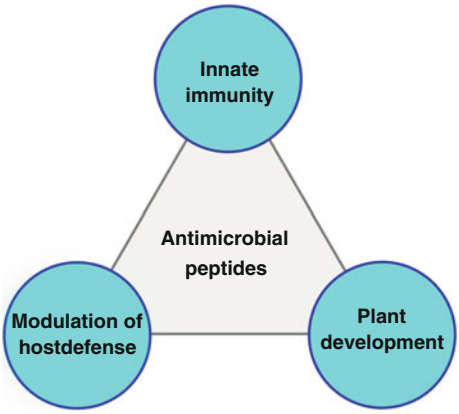


**Fig. 5.9** Plant AMPs are multifunctional

silencing of snakin-1 resulted in negative effects on plant development in potato (Nahirñak et al. 2012). (b) LTPs are involved in cuticular wax synthesis, pollen adhesion, guiding the pollen tube towards fertilization, oxylipin-mediated SAR, and cell wall loosening (Molina and García-Olmedo 1997; Park et al. 2000; Nieuwland et al. 2005; Chae et al. 2009; DeBono et al. 2009). (c) A synthetic heterologous AMP, *msrA3*, when expressed in potato was found to alter floral development and mitigate normal plant response to abiotic and biotic stresses (Goyal et al. 2013). The transgenic potato plants were resistant to *Fusarium solani* and the tuber yield was significantly higher than the control plants. Detailed investigation showed suppression of HR, wound-induced JA and ROS, in concert with changes in transcript profiles of related gene markers under both biotic and abiotic stresses (Goyal et al., 2013). Among other functions, AMPs interact with cellular signalling processes include oxidative stress and its components ROS and NO, MAPK signalling, HR, and systemic acquired resistance (SAR) (reviewed in Goyal and Mattoo 2014). Thus, plant AMPs are potent defence molecules while they also have moonlighting functions related to plant development processes, similar to what is known about mammalian AMPs which, in addition to immunomodulating host defence, also modify physiological responses of the cell (Choi et al. 2012; Hilchie et al. 2013). Multifunctional role of plant AMPs is summarized in Fig. 5.9.

## 5.8 Conclusions

Disease afflicts crop productivity as well as nutritional attributes. Pathogens have the ability to mutate rapidly and thereby develop resistance to pesticides. Despite plant's multilayer of innate defence against pathogens, often the latter are able to penetrate and establish themselves on plant host. The discovery of antimicrobial

peptides (AMPs) has the promise of durable defence by quickly eliminating pathogens through membrane lysis, and positively impacting the host's cellular machinery for development. AMPs characteristically are made up of from fewer than 20 amino acids to about 100 amino acids, structurally diverse, and amenable for higher potency by either alteration of their chemical structure and/or engineering them to produce higher amounts in heterologous systems in order to provide durable plant immunity against pathogens. For achieving this, it will be important to first characterize them, understand their mechanism(s) of action, and develop a wide range of structures. Although permeabilizing cellular membrane is a major mechanism known for AMP action, new and diverse modes of action have recently been unearthed, including targeting of intracellular function of the pathogen.

Crop protection against pathogens is inimical to global food security. Immense focus on the 'R' gene defence for crop survival against pathogens has demonstrated the short half-life of such a strategy and breakdown of such defence. The discovery of antimicrobial peptides (AMPs) as generators of durable plant resistance against target pathogens together with their broad-spectrum activity across kingdoms has shown their promise in enabling crop resistance to disease.

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