

Innate Immunity in Plants: The Role of Antimicrobial Peptides

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Abstract Antimicrobial peptides (AMPs) are part of innate immunity, establishing a first line of defense against pathogens. All plant organs express AMPs constitutively or in response to microbial challenges. Plant AMPs are structurally and functionally diverse. Five classes of AMPs are considered in this review, the thionins, defensins, lipid transfer proteins (LTPs), snakins, and a group of related knottins, cyclotides and hevein-like AMPs. Besides targeting fungal, bacterial, and oomycete pathogens, certain AMPs can be directed against other organisms, like herbivorous insects. The biological activity of plant AMPs primarily depends on interactions with membrane lipids, but other modes of action exist as in the case of defensins with α -amylase activity or a defensin-like peptide that interacts with a receptor kinase. Limited information exists on the regulated expression of plant AMPs, their processing, and posttranslational modification. Conclusive data on the role of certain AMPs in plant defense have only recently become available. This review can therefore only be considered as a snapshot of the progress in this field of research.

1 Introductory Remark

Protection of plants and animals against infectious microorganisms depends on both constitutive and induced defense mechanisms. Antimicrobial peptides (AMPs) are an important component of constitutive and induced epithelial defenses, contributing to the first line of defense in animals (Schröder 1999). Plants produce

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AMPs in all organs either constitutively or in response to microbial infection, but information about the expression of AMPs in epidermal cells is limited. As plants also biosynthesize protective secondary metabolites, AMPs may not be as crucial for the first line of defense as in animals. In light of the induction of specific AMPs, a brief review of the plant immune system will be given first.

2 Innate Immunity in Plants

The innate immune system of plants consists of two branches (Jones and Dangl 2006). Initial recognition of microbes by host plants is analogous to the animal system in that pattern recognition receptors (PRRs) on the surface of the host cell detect the presence of pathogen-associated molecular patterns (PAMPs), representing small motifs of larger molecules that are essential for microbial survival (Janeway 1989). PAMP-triggered immunity (PTI) activates a myriad of processes, including mitogen-activated protein kinase (MAPK) cascades, production of reactive oxygen species (ROS), hormone signaling, and gene expression (Schwessinger and Zipfel 2008). Successful pathogens suppress PTI by delivering virulence effector proteins to the host. In turn the second branch of plant immunity, which is defined by another set of largely intracellular plant receptors, the resistance (*R*) gene products, is activated. These receptors recognize specific effector proteins directly or indirectly to activate effector-triggered immunity (ETI), a stronger plant defense response that can culminate in programmed cell death (Jones and Dangl 2006).

PTI and ETI stimulate distinct hormone biosynthesis and signaling pathways. ETI triggers salicylic acid (SA) biosynthesis and signaling, leading to local and systemic acquired resistance (SAR) against biotrophic pathogens (Mettraux et al. 1990; Delaney et al. 1994). SAR is the induction of broad-spectrum disease resistance in uninfected distal tissues activated by local pathogen infection that results in tissue necrosis, also known as the hypersensitive response (Ward et al. 1991). Conversely, PTI stimulates ethylene (ET) biosynthesis (Felix et al. 1999). The cross talk between PTI and ET biosynthesis and signaling has recently been reviewed (Trujillo and Shirasu 2010). ET and jasmonic acid (JA) synergistically activate plant defenses against necrotrophic pathogens (Thomma et al. 1998). Moreover, antagonistic interaction exists between JA/ET and SA signaling (Niki et al. 1998). Both pathways induce the expression of AMPs in different ways, as will be discussed later.

3 General Characteristics of Plant AMPs

Like AMPs of animal origin, the molecular diversity of AMPs from plants is striking (Padovan et al. 2010). Plant AMPs were assigned to different classes according to their tertiary structures (Fig. 1). The most common classes are

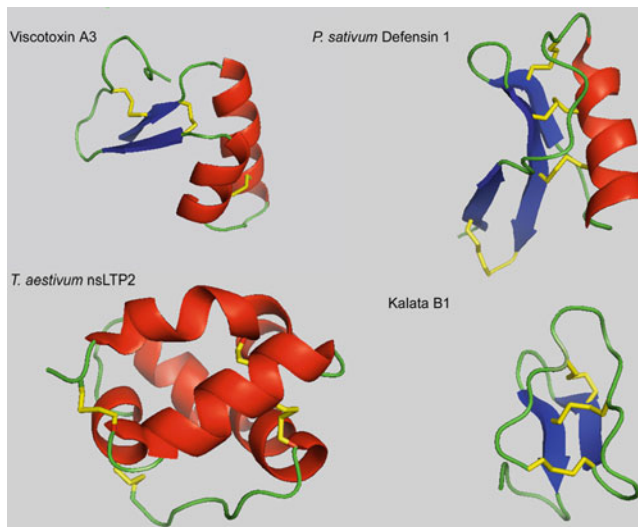


Fig. 1 3D structures of representative antimicrobial peptides (AMPs) belonging to different classes of AMPs from plants. Viscotoxin A3 (PDB entry 1ED0), a thionin from mistletoe (*Viscum album*) is a representative of thionins. Defensins are represented by defensin 1 (PDB entry 1JKZ) from pea (*Pisum sativum*). An example of lipid transfer proteins (LTPs) is the non-specific nsLTP2 (PDB entry 1TUK) from wheat (*Triticum aestivum*). Kalata B1 (PDB entry 1NB1) is a cyclotide from the perennial herb *Oldenlandia affinis*. Disulfide bridges are highlighted in yellow; α -helices and β -sheets are color-coded in red and blue, respectively

thionins, defensins, and lipid transfer proteins. Plant AMPs share the following important features: They are small cationic peptides with molecular masses of 2–10 kDa. The structures of these small peptides are stabilized through formation of 2–6 disulfide bridges. The activities of plant AMPs are primarily directed against fungal, oomycete, and bacterial microorganisms, but certain members of a class can be directed against other targets, including herbivorous insects.

Different classes of plant AMPs will be discussed next. Special attention will be given to their structures and functions, their regulated expression, and their modes of action.

4 Thionins

The first AMP isolated from plants was a thionin from the endosperm of wheat (Balls et al. 1942). The protein moiety of a proteolipid was later shown to be a mixture of two forms, purothionins α and β (Nimmo et al. 1968). Additional thionins were isolated, including α - and β -hordothionins from barley endosperm, viscotoxins and phoratoxins from mistletoe species, and crambin from the cruciferous plant *Crambe abyssinica* (Bohlmann and Apel 1991). Thionins from cereals

and *Pyricularia pubera* have four disulfide bonds. Other dicotyledonous thionins have three disulfide bonds. The structural feature common to all thionins is the Γ (gamma) fold consisting of two antiparallel α -helices that form a stem and antiparallel β -sheets that form an arm (Padovan et al. 2010). A groove exists between the helical and β -sheet segments. Thionins are cationic peptides with amphipathic properties with the exception of crambin, which is hydrophobic and carries no net charge.

Both purothionins differentially inhibit the growth of and kill several bacterial plant pathogens but not mycelial fungi (Fernandez de Caley et al. 1972). Thionins from the endosperm and leaf of barley inhibit the fungi *Drechslera teres*, a pathogen of barley, and *Thielaviopsis paradoxa*, a pathogen of sugar cane (Bohlmann et al. 1988). The thionin from *P. pubera* has antifungal as well as antibacterial activities (Vila-Perello et al. 2005). Thionins also affect organisms other than plant pathogens. Purothionins are toxic to small mammals when injected intravenously or intraperitoneally but not when administered orally (Coulson et al. 1942). The reason behind these broad biological activities lies in the thionin structure.

Thionin exerts its primary effect on the membrane through binding of phospholipids (Stec et al. 2004). Amino acid residues 1 and 2 as well as residues 9–14 are highly conserved. Specifically, Lys1 and Arg10 contribute to phosphate binding. Ser2 and Tyr13 form the glycerol-binding site. Modeling of thionin interactions with phospholipids implied that the acyl chain of the phospholipid fits into the groove of the toxin (Stec et al. 2004). In the absence of phospholipids, thionins form dimers that bind inorganic phosphate and are stabilized by Asn11 and Asn14. Upon association with membranes, monomers are formed that insert into the membrane and segregate phospholipids. This action destabilizes the membrane, leading to ion leakage and eventually to lysis.

Mature thionins are derived from preproteins that contain an N-terminal signal peptide for targeting to the endoplasmic reticulum (ER) and a C-terminal acidic peptide that is thought to neutralize the activity of the cationic thionin. Thionins are targeted to the vacuole (Romero et al. 1997). The function of vacuolar-targeted thionins is probably analogous to the action of basic chitinase and β -1,3-glucanase (Mauch and Staehelin 1989). These enzymes accumulate in the vacuole and are released during host cell lysis caused by pathogen attack. The sudden release of high concentrations of antimicrobial peptides is thought to overwhelm invading pathogens without time to adapt to the challenge.

The genome of the model plant *Arabidopsis thaliana* also encodes thionins. The *Thi2.1* and *Thi2.2* genes are regulated differently (Epple et al. 1995). *Thi2.1* is constitutively expressed at high levels in flowers and siliques and inducible in seedlings in response to methyl jasmonate and inoculation with the fungal pathogen *Fusarium oxysporum*. *Thi2.2* is constitutively expressed in seedlings but not inducible. The octadecanoid pathway, which is analogous to the inflammatory response pathway in animals that leads to the production of prostaglandins from arachidonic acid, culminates in JA biosynthesis and induction of *Thi2.1* expression (Bergey et al. 1996; Bohlmann et al. 1998). This was proven with the help of JA-insensitive

and JA-deficient mutants, which were no longer able to induce *Thi2.1* expression in response to stimuli like wounding, leading to JA accumulation and signaling (Bohlmann et al. 1998). Overexpression of *Thi2.1* in transgenic *A. thaliana* plants decreased susceptibility to *F. oxysporum* (Epple et al. 1997). Hyphae had more growth anomalies, including hyperbranching, when the fungal pathogen was grown on cotyledons of *Thi2.1*-overexpressing lines than on cotyledons of untransformed plants (Epple et al. 1997). A phenotypically similar disruption of fungal growth was observed when *F. oxysporum* was exposed to thionins in vitro (Vila-Perello et al. 2005). To address the endogenous function of thionins, a loss-of-function approach is needed. For this purpose, lines with T-DNA insertions in exon regions of both *Thi2.1* and *Thi2.2* genes are available from The Arabidopsis Information Resource (TAIR). However, their analysis still needs to be performed. As will become clear from the next chapter, knockout or knockdown mutants are useful for determining the in vivo function of AMPs.

5 Defensins

Defensins were originally grouped with the thionins and defined as γ -thionins. However, structural comparisons to insect defensins required a reclassification of this group of AMPs as defensins (Bruix et al. 1993). Characteristically, defensins consist of a well-defined triple-stranded antiparallel β -sheet and a single α -helix that lies parallel to the β -sheet. The α -helix is connected to the β -sheet with the help of two disulfide bridges, forming a characteristic Cys-stabilized α -helix β -sheet (CS $\alpha\beta$) motif (Cornet et al. 1995). Typical plant defensins form two additional disulfide bonds. Plant and arthropod defensins consist of a $\beta\alpha\beta\beta$ pattern, whereas mammalian β -defensins contain an N-terminal α -helix and an overall $\alpha\beta\beta\beta$ -fold. Plant defensins are small 45–54 amino acids long cationic peptides. Defensins are widely distributed among dicots and monocots. The genome of *A. thaliana* alone was shown to encode more than 300 defensin-like (DEFL) peptides, 78 % of which have a CS $\alpha\beta$ motif (Silverstein et al. 2005). An even larger diversity of DEFL genes is present in legume species (Graham et al. 2008).

Unlike animal defensins, few plant defensins are active against bacteria (Franco et al. 2006; Yokoyama et al. 2008). Instead, the most common activity of these peptides is directed against diverse fungi (Osborn et al. 1995). Besides these antimicrobial activities, specific defensins have been reported to inhibit protein synthesis (Mendez et al. 1990), protease trypsin (Wijaya et al. 2000), or α -amylase activity (Bloch and Richardson 1991; Lin et al. 2007; Pelegrini et al. 2008). The α -amylase inhibitors are insecticidal. The defensins VrD1 from *Vigna radiata* and VuD1 from *Vigna unguiculata* use the L3 loop and the N-terminus, respectively, to inhibit α -amylase activity (Lin et al. 2007; Pelegrini et al. 2008).

Plant defensins also influence plant growth and development. *Medicago* spp. do not express MsDef1 and MtDef2 in roots, but when roots of *A. thaliana* are exogenously treated with MsDef1, MtDef2, or RsAFP2, their growth is retarded

and root hair elongation is inhibited (Allan et al. 2008). According to the authors, constitutive expression of MsDef1 in *A. thaliana* did not alter root or root hair growth. Altered expression of the tomato defensin DEF2 in transgenic tomato plants reduced pollen viability and seed production (Stotz et al. 2009). Constitutive expression of this defensin had pleiotropic effects on plant development. In contrast to the above examples, the following developmental process has been studied at the mechanistic level. The S-locus protein 11 (SP11), also known as S-locus Cys-rich (SCR) protein, is a DEFL peptide that interacts with the S-locus receptor kinase (SRK) to trigger self-incompatibility, a response that prevents self-fertilization via inhibition of pollen tube growth and that favors outcrossing (Nasrallah 2002). The L3 loop connecting $\beta 2$ and $\beta 3$ and the α -helical region are important for recognition of SP11 by SRK (Sato et al. 2004).

A group of DEFL peptides with four to six Cys residues was recently shown to control symbiotic interactions. Nodule-specific Cys-rich (NCR) peptides govern terminal differentiation of nitrogen-fixing endosymbiotic *Rhizobium* bacteria in root nodules of leguminous host plants (Van de Velde et al. 2010). NCR peptides are delivered to the bacterial symbiont via a nodule-specific secretory pathway (Wang et al. 2010). These peptides cross the bacterial membrane and accumulate inside the bacteria to inhibit cytokinesis and to stimulate DNA synthesis and cell enlargement (Van de Velde et al. 2010). These examples demonstrate that defensins and DEFL peptides are functionally diverse, probably reflecting their evolutionary diversification.

The defensins DmAMP1 from *Dahlia merckii* and RsAFP2 from *Raphanus sativus* induce rapid potassium efflux and calcium influx in the hyphae of the fungus *Neurospora crassa* in combination with medium alkalization (Thevissen et al. 1996). Inhibition of fungal growth in response to both defensins was associated with cation-resistant membrane permeabilization (Thevissen et al. 1999). Using radiolabeled HsAFP1, a defensin from *Heuchera sanguinea*, specific high-affinity binding sites were identified on the plasma membrane of *N. crassa* (Thevissen et al. 1997). A genetic approach was used to identify these binding sites as complex lipids (Thevissen et al. 2000). Mutation in the *IPT1* gene, encoding an enzyme that catalyzes the last step in biosynthesis of the sphingolipid mannose-(inositol-phosphate)₂-ceramide, conferred DmAMP1 resistance to *Saccharomyces cerevisiae*. Sensitivity of the yeasts *Candida albicans* and *Pichia pastoris* to RsAFP2 was a function of the gene *GCS*, encoding UDP-glucose:ceramide glucosyltransferase (Thevissen et al. 2004). RsAFP2 was shown to interact with membrane components of lipid rafts to elicit the production of reactive oxygen species, leading to fungal cell death (Aerts et al. 2007). NaD1, a defensin from *Nicotiana glauca*, also causes permeabilization of the fungal membrane, but it enters fungal cells via a cell wall-dependent mechanism possibly reaching intracellular targets (van der Weerden et al. 2008, 2010). Thus, even with respect to antifungal activity, different modes of defensin action exist.

Different defensins are expressed throughout the plant. Defensins were first isolated from seeds of monocot and dicot species. Importantly, the defensins from radish seeds RsAFP1 and RsAFP2 are released during seed germination after

disruption of the seed coat (Terras et al. 1995). The amount of released peptides was shown to be sufficient for inhibition of fungal growth around the germinating seedlings. RsAFP peptides are expressed in surface cell layers and in spaces between different organs of the seed. Moreover, RsAFP peptides are secreted into the middle lamella region of plant cell walls, which are important for cellular adhesion. Expression of RsAFPs is induced after pathogen challenge, and constitutive expression of RsAFP2 in transgenic tobacco resulted in increased resistance against the foliar pathogen *Alternaria longipes* (Terras et al. 1995). Different defensins are constitutively expressed in every organ of *A. thaliana* (Thomma and Broekaert 1998). *PDF1.2* has been established as an important marker gene to study the activation of the JA/ET signaling pathway (Manners et al. 1998; Mitter et al. 1998; Brown et al. 2003; Nandi et al. 2003; Ndamukong et al. 2007; Zander et al. 2009). *PDF1.2* is regulated by an amplification loop that involves recognition of the endogenous peptide elicitors AtPEP1-6 by the receptors AtPEPR1 and AtPEPR2 (Huffaker et al. 2006; Huffaker and Ryan 2007; Pearce et al. 2008; Yamaguchi et al. 2010). *AtPEPR1* and *AtPEPR1-3* are induced after inoculation of *A. thaliana* with the necrotrophic ascomycete *Sclerotinia sclerotiorum*, which may be responsible for the dramatic induction of *PDF1.2* in response to pathogen infection (Stotz et al. unpublished). To determine the endogenous function of PDF1.1 and PDF1.2, knockout and knockdown lines were generated (De Coninck et al. 2010). However, no difference in pathogen susceptibility was observed between the knockout lines and wild-type plants, probably because of the functional redundancy of multiple *AtPDF* genes. Still, overexpression of *PDF1.1* resulted in reduced susceptibility to the necrotrophic fungus *Cercospora beticola* (De Coninck et al. 2010). More conclusive results were obtained in tobacco. Silencing of the tobacco defensin PR-13 resulted in increased susceptibility of *Nicotiana attenuata* to *Pseudomonas syringae* pv. *tomato* DC3000 under glasshouse conditions and increased susceptibility to opportunistic *Pseudomonas* spp. and mortality in the native habitat (Rayapuram et al. 2008). PR-13 is closely related to the defensin peptide NaD1 and more distantly related to thionins (Fig. 2). Based on these data, defensins are clearly important for plant defense, and their evolutionary diversification is the basis for various ecological functions.

Most defensins consist of a signal sequence that targets the peptides to the ER, followed by the mature peptide. Solanaceous floral defensins are an exception because they also contain an acidic C-terminal extension, which perhaps prevents inappropriate activation of mature cationic peptides (Stotz et al. 2009). This difference in proteolytic processing adds to the complexity of plant defensins. It is to be expected that additional information on the impact of defensins on membrane lipids and proteinaceous receptors will soon become available. To appreciate the function of plant defensins, this is the most urgent problem that needs to be addressed.

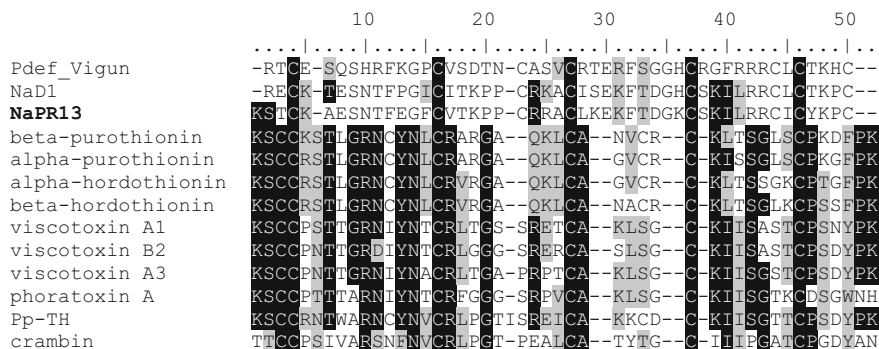


Fig. 2 Alignment of defensin and thionin amino acid sequences. The predicted mature peptide sequence of PR-13 from *Nicotiana attenuata* NaPR13 (GenBank AY456268) is highlighted in bold and compared to the related defensins NaD1 from *Nicotiana glauca* (GenBank Q8GTM0) and Pdef_Vigun from *Vigna unguiculata* (GenBank ACN93800). Note that all eight Cys, two Gly, and one Glu residue are highly conserved and shared among these three peptides. Less closely related to NaPR13 are the thionins: β -purothionin from *Triticum urartu* (GenBank 218767043), α -purothionin from *Triticum aestivum* (GenBank 4007850), α - and β -hordothionins from *Hordeum vulgare* (GenBank 19110 and 225008, respectively), viscotoxins A1 (GenBank 190613619), B2 (GenBank 190613410) and A3 (GenBank 7245963) from *Viscum album*, phoratoxin A from California mistletoe (GenBank 135797), thionin from *Pyricularia pubera* (GenBank 135798), and crambin from *Crambe abyssinica* (GenBank 6226577). Boxes indicate identical (black) and similar amino acids (gray)

6 Lipid Transfer Proteins

Lipid transfer proteins (LTPs) were named based on their ability to facilitate transfer of phospholipids between a donor and an acceptor membrane *in vitro* (Bloj and Zilversmit 1977; Kader et al. 1984). As these LTPs have broad substrate specificity, they are also referred to as nonspecific LTPs (nsLTPs) (Kader 1996). Genome-wide analysis of *nsLTP* gene families resulted in detection of 52 and 49 members in rice and *A. thaliana*, respectively (Bureau et al. 1996).

Plant lipid transfer proteins are quite abundant and comprise of two families, LTP1 and LTP2. Members of the plant LTP1 family are about 10 kDa in size, consist of 90–95 amino acids, and are basic, with isoelectric points between 9 and 10. These LTPs have eight Cys residues conserved at similar positions in their primary structure, which form four disulfide bridges stabilizing the tertiary structure (Kader 1996). The LTP2 family members share the properties of the LTP1 family but are only about 7 kDa in size, possessing about 70 amino acids on average. LTPs contain a signal peptide at the amino terminal end, which is cleaved and targets the mature peptide to the cellular secretory pathway resulting in export to the apoplast.

The extracellular localization was confirmed for LTPs from a variety of plants (Serk et al. 1991; Terras et al. 1992; Molina and Garcia-Olmedo 1993; Segura et al. 1993). Expression studies showed that LTP transcripts are abundant in epidermal

and peripheral cell layers (Sossountzov et al. 1991; Sterk et al. 1991; Fleming et al. 1992; Molina and Garcia-Olmedo 1993; Thoma et al. 1994). In broccoli, an LTP was found to be the main protein of the wax layer (Pyee et al. 1994).

Structural data were obtained for LTP1 proteins from wheat (Gincel et al. 1994; Charvolin et al. 1999), maize (Shin et al. 1995; Gomar et al. 1996), barley (Heinemann et al. 1996; Lerche and Poulsen 1998), and rice (Lee et al. 1998). LTP2 proteins from wheat and rice were also analyzed by solution NMR and X-ray diffraction (Samuel et al. 2002; Hoh et al. 2005). Most LTP proteins have a globular structure consisting of a bundle of four α -helices linked by flexible loops and contain a large central hydrophobic cavity. The size of this cavity differs between LTP1 and LTP2, the latter being more spacious, enabling binding of a planar sterol (Samuel et al. 2002). The cavity of LTP1 is variable and can adapt its volume to bind one or two mono- or diacylated lipids or other hydrophobic molecules. LTP1 proteins cannot load sterols or molecules with a rigid backbone (Douliez et al. 2000, 2001; Pato et al. 2002), suggesting that plasticity of the cavity and flexibility of the hydrophobic molecules limit the “nonspecificity” of the nsLTPs.

Antifungal and antibacterial activities were among the first functions shown for LTPs from barley, maize, spinach, and *A. thaliana* (Terras et al. 1992; Molina et al. 1993; Segura et al. 1993). Relative activities of different plant LTPs vary among pathogens, indicating some degree of specificity (Molina et al. 1993; Sun et al. 2008). Also, synergistic activity of an LTP with a thionin against the bacterial pathogen *Clavibacter michiganensis* ssp. *sepedonicus* was observed in vitro, while only additive effects were observed for a fungal pathogen (Molina et al. 1993; Garcia-Olmedo et al. 1995). Overexpression of a barley LTP2 in tobacco as well as in *A. thaliana* plants reduced disease symptoms after infection of leaves with the bacterial pathogen *P. syringae* (Molina and Garcia-Olmedo 1997), and overexpression of an onion LTP in wheat plants decreased growth of the fungal pathogen *Blumeria graminis* f.sp. *tritici* (Roy-Barman et al. 2006).

The mechanism of the toxicity observed for plant LTPs toward fungi and bacteria still remains to be elucidated. It is likely that lipid-binding properties and antimicrobial activity are independent of each other. Unlike related cereal LTPs, Ace-AMP1, an LTP from onion with antifungal activity, was not able to bind diacylphospholipids (Cammue et al. 1995; Tassin et al. 1998). The toxicity and lipid-binding activity of several wheat LTPs were not correlated (Sun et al. 2008). Mutational analysis of the rice nsLTP1 gene indicated that lipid binding and antimicrobial functions are unrelated (Ge et al. 2003). These data support the view that, with respect to LTP structure, lipid binding and antimicrobial activities are spatially separated.

It was recently suggested that a new subfamily of plant LTPs, *signaling LTPs*, should be formed, consisting of LTPs related to two characterized LTPs with signaling function (Pii et al. 2010). This group would consist of the *Medicago truncatula* LTP MtN5, which is expressed in response to a root pathogenic fungus and in root nodules colonized by the symbiotic bacterium *Sinorhizobium meliloti* (Pii et al. 2009, 2010), together with a closely related protein from *A. thaliana*, defective in induced resistance 1 (DIR1). DIR1 is required for inducing SAR in

distal tissues after initial infection with a necrotizing pathogen (Maldonado et al. 2002). There is also evidence that DIR1 itself might be the mobile signal, as antibodies directed against the tomato DIR1 homolog, Le-DIR1, recognized this protein in the phloem sap of tomato plants (Mitton et al. 2009). Another LTP, azelaic acid induced 1 (AZI1), was recently shown to be required for production of the signal, which induces priming of defense responses for SAR (Jung et al. 2009a). Several genetic studies strongly indicated that a lipid or lipid-derived compound is required for the establishment of SAR in *A. thaliana* (Nandi et al. 2004; Chaturvedi et al. 2008; Shah 2009). However, the identity of the SAR signal, the molecular properties of LTPs, which render them active, and the identity of the bound lipid substrate (if any) *in vivo* remain to be determined. Interestingly, wheat LTP1 was shown to bind to a plasma membrane-located receptor for elicitors (Buhot et al. 2001). Elicitins are small proteins secreted by oomycete pathogens capable of binding phospholipids and fatty acids in competition with sterols (Ponchet et al. 1999; Osman et al. 2001). Elicitins are able to trigger plant defense responses reminiscent of SAR (Keller et al. 1996). It is tempting to speculate that some LTPs could mediate pathogen recognition and thereby fulfill yet an additional role in plant defense processes.

It was originally suggested that LTPs facilitate intracellular transfer of lipids. However, most LTPs have been extracellularly localized. The following diverse functional data also indicate that LTPs play important roles in the apoplast. This includes direct antimicrobial activity, and for some LTPs, their role in plant defense signaling was shown. DIR1, for instance, appears to be a signaling molecule in itself. Other LTPs may act via binding to plant elicitor receptors. Plant LTPs were assigned additional divergent functions, including a role in beta-oxidation (Tsuboi et al. 1992), cutin synthesis (Pyee et al. 1994), pollen adherence (Park et al. 2000), and somatic embryogenesis (Serk et al. 1991). In the future, we will have to address the exact mechanism by which LTPs mediate signaling, identify the *in vivo* substrates of LTPs, and determine the mode of antimicrobial action.

7 Hevein-Like AMPs, Knottins, and Cyclotides

These three types of AMPs are treated together because of their structural similarities. All of them form a triple-stranded β -sheet that is stabilized by at least three disulfide bridges.

7.1 Hevein-Like AMPs

Hevein is the most abundant protein in latex of rubber trees. The mature peptide consists of 43 amino acids with four disulfide bridges and contains a chitin-binding domain (Lee et al. 1991). Homologous chitin-binding domains are found in multidomain proteins, like chitinase (Iseli et al. 1993), and in hevein-like AMPs (Broekaert et al. 1992).

Two classes of hevein-like AMPs exist. The first class of peptides is similar to hevein and contains eight Cys residues. Examples are PnAMP1 and PnAMP2, two peptides that are produced in seeds of *Pharbitis nil* (Koo et al. 1998). PnAMP1 and PnAMP2 are 41 and 40 amino acids long, respectively. Their antimicrobial activity is not dependent on microbial chitin production as oomycete pathogens were also inhibited by these peptides. Fluorescently labeled PnAMP1 rapidly penetrates hyphae, leading to membrane disintegration and disruption of hyphal tips.

The second class of hevein-like AMPs is shorter and contains only six Cys residues. Examples are AcAMP1 and AcAMP2, two peptides from seeds of *Amaranthus caudatus*, consisting of 29 and 30 amino acids, respectively (Broekaert et al. 1992). Both peptides were found to be potent inhibitors of fungal growth when six fungal pathogens and one saprophyte were tested. The antibacterial activity of these peptides is much lower. As in the case of thionins, divalent cations inhibited the antimicrobial activity of these peptides. Intercellular wash fluids from leaves of sugar beet contain another peptide, designated as IWF4 (Nielsen et al. 1997). The chitin-binding activity of this peptide was stronger than that of class I and class IV chitinases (Hamel et al. 1997). IWF4 is 30 amino acids long and inhibits growth of the foliar pathogen *Cercospora beticola* (Nielsen et al. 1997). Its mRNA is constitutively expressed in leaves and flowers but not induced after inoculation of sugar beet leaves with *C. beticola*.

Hevein-like AMPs are produced as preproteins. They contain a signal peptide that targets them for secretion and a C-terminal extension (De Bolle et al. 1996; Nielsen et al. 1997). Overexpression of *pnAMP-h2* in transgenic tobacco elevated resistance to *Phytophthora parasitica* (Koo et al. 2002). PnAMPs therefore possibly protect seeds of *P. nil* against pathogens.

7.2 Knottins

Knottins are structurally different from hevein-like AMPs in that all three disulfide bridges take part in the reinforcement of the sheet structure and a helix found in hevein-like AMPs is absent (Chagolla-Lopez et al. 1994). The solution structure of a knottin from seeds of *Phytolacca americana* was solved (Gao et al. 2001b).

The 38 amino acids long knottin from *P. americana* has broad-spectrum antifungal activity (Gao et al. 2001). Earlier, two knottins were isolated from seeds of *Mirabilis jalapa* (Cammue et al. 1992). The 37- and 38-amino-acid long *MjAMP1* and *MjAMP2*, respectively, associate to form dimers and effectively inhibit a wide range of fungal pathogens and, to a lesser degree, Gram-positive bacteria. Seeds of *Amaranthus hypochondriacus* contain a knottin that inhibits α -amylase activity (Chagolla-Lopez et al. 1994), demonstrating that these AMPs have multiple biological activities.

An unusual AMP with two knottin motifs was isolated from the cycad *Cycas revoluta* (Yokoyama et al. 2009). The recombinant peptide is capable of binding to chitin and has antifungal and antibacterial activity. Mutant forms of recombinant

CyAMP1 were generated by site-direct mutagenesis of amino acids that are conserved with knottins and hevein-like AMPs. These mutant peptides were no longer able to bind to chitin and lost their antifungal activity. However, the antibacterial activity was maintained, suggesting a different mode of action against prokaryotes.

Knottins are encoded as preproteins and not proteolytically processed like hevein-like AMPs (De Bolle et al. 1995). Expression of *MjAMP2* in transgenic tobacco showed that the peptide is secreted and functional because it inhibits in vitro growth of *Botrytis cinerea* (De Bolle et al. 1996). However, *MjAMP2* did not protect transgenic tobacco against infection from *B. cinerea* or *A. longipes*. Further research is therefore needed to determine the role of knottins in protection of plants against pathogens.

7.3 Cyclotides

Kalata B1 was the first identified member of a new family of cyclic AMPs (Saether et al. 1995). These peptides are covalently joined by a peptide bond between the N- and C-terminal amino acids. Cyclotides consist of 27 to 37 amino acids with an embedded cystine knot (Padovan et al. 2010). Unlike other AMPs, cyclotides are not cationic peptides, but they contain a solvent-exposed hydrophobic patch. Two major subfamilies of cyclotides exist. The Möbius subfamily contains a twist in the peptide backbone, owing to the presence of a Pro residue in loop 5 that is preceded by a *cis*-peptide bond located between Cys residues five and six (Craik et al. 2006). The bracelet subfamily does not contain a Pro residue in loop 5, but it contains a short helical segment in loop 3 that lies between the third and fourth Cys residues. Aside from the Cys residues, the Glu residue in loop 1 between the first and second Cys residues is most highly conserved throughout the cyclotide family (Goransson et al. 2009). This Glu residue forms a hydrogen bond network that stabilizes the cyclotide framework for efficient aggregation in membranes (Goransson et al. 2009).

Cyclotides are present in Cucurbitaceae and Apocynaceae, in every analyzed species of the Violaceae, and in a few species of the coffee family Rubiaceae (Gruber et al. 2008). Linear cyclotide-like sequences are present in monocots (Poaceae), suggesting that these peptides evolved prior to the divergence of monocots and dicots. Presence of a single intron in Rubiaceae genes but absence thereof in Violaceae genes suggests that cyclization evolved independently after the divergence of Asterids and Rosids. Within a single species, *Viola hederacea*, 66 different cyclotides were identified (Trabi and Craik 2004). Cyclotide diversity within a single plant family is estimated to be in the order of 10,000 (Craik et al. 2006; Gruber et al. 2008).

Cyclotides are generated from linear precursor proteins that contain one, two, or three cyclotide domains (Dutton et al. 2004; Gillon et al. 2008). Precursor proteins consist of an ER signal sequence, an N-terminal pro-domain, an N-terminal repeat,

the cyclotide domain, and a C-terminal tail. Oak1 is the precursor protein of kalata B1 from the African plant *Oldenlandia affinis*. Foliar extracts from *O. affinis* contain an 11-kDa protein without the ER signal sequence, a 6-kDa processing intermediate without the N-terminal pro-domain, and mature 4-kDa kalata B1. The cyclotide processing sites are highly conserved (Gillon et al. 2008). A protein-disulfide isomerase was shown to be essential for correct oxidative folding of kalata B1 and production of biologically active cyclotides (Gruber et al. 2007). An asparaginyl endopeptidase was shown to catalyze peptide bond formation between the N-terminal Gly and C-terminal Asn residues in kalata B1 (Saska et al. 2007). Violacin A from *Viola odorata* is a naturally occurring linear cyclotide that contains a mutation introducing a stop codon and preventing translation of the key Asn residue required for cyclization (Ireland et al. 2006).

Cyclotides have multiple biological activities. Kalata B1 accelerates contractions during childbirth. Cyclotides, including kalata B1, have antimicrobial activity that is salt-sensitive (Tam et al. 1999). Kalata B1 also has insecticidal activity, disrupting epithelial cells in the midgut of lepidopteran larvae (Jennings et al. 2001; Gruber et al. 2007; Barbetta et al. 2008). Various cyclotides possess cytotoxic, hemolytic, and anti-HIV activities (Chen et al. 2006; Ireland et al. 2006, 2008). This diversity of biological activities together with the marked resistance against chemical, thermal, and enzymatic degradation conferred by the closed cysteine knot structure has sparked interest in using cyclotides as scaffolds for protein engineering and drug design (Craik et al. 2006).

The biological activity of cyclotides depends on membrane interactions. The size of the surface-exposed hydrophobic patch determines cytotoxicity, hemolytic, and anti-HIV activities of cyclotides (Chen et al. 2006; Ireland et al. 2008). Bracelet cyclotides are generally more hydrophobic than Möbius cyclotides with hydrophobic residues on both faces of the molecule (Ireland et al. 2008). Insertion into lipid bilayers differs between members of both subfamilies (Wang et al. 2009). Whereas Möbius cyclotides interact with the membrane via loops 2 and 6, bracelet cyclotides interact via loops 2 and 3. Strategically located charged residues modulate hydrophobic interactions between cyclotides and target membranes and influence the therapeutic index of these peptides (Ireland et al. 2008). Evidently, only a portion of the cyclotide molecule binds to the membrane. These peptides are not buried deeply into the membrane. In the case of bracelet cyclotides, hydrophobicity has been linked to their membrane-disrupting ability (Svangard et al. 2007).

Production of cyclic kalata B1 has been reduced in transgenic tobacco via silencing of asparaginyl endopeptidase (Saska et al. 2007). This would make it possible to test effects of altered cyclotide expression on biotic interactions, but such studies have not yet been performed. It should be noted that another family of circular plant proteins exists that is distantly related to the cyclotides and has trypsin inhibitor activity (Felizmenio-Quimio et al. 2001).

8 Snakins

Yet another class of AMPs was found in solanaceous plants. These AMPs isolated from potato (*Solanum tuberosum*) and closely related *Solanum* species were termed snakins based on their sequence similarity to hemotoxic desintegrin-like snake venoms (Segura et al. 1999). The amino acid sequences of snakins are also related to gibberellin-stimulated transcripts GAST and GASA from a variety of plant species, including *A. thaliana*. The mature snakin-1 (StSN1) and snakin-2 (StSN2) peptides are cationic and contain 63 and 66 amino acids, respectively, with 12 Cys residues (Segura et al. 1999; Berrocal-Lobo et al. 2002). Whereas StSN1 is preceded by a signal sequence, StSN2 is derived from a preproprotein that contains an additional N-terminal acidic peptide and requires proteolytic processing.

Snakins have antifungal and antibacterial activities (Segura et al. 1999; Berrocal-Lobo et al. 2002). StSN1 and StSN2 cause rapid aggregation of Gram-positive and Gram-negative bacteria. Interestingly, StSN1 caused aggregation of *Ralstonia solanacearum* at concentrations that were not toxic to these Gram-negative bacteria (Segura et al. 1999). Although snakins do not lyse artificial lipid membranes, they can promote aggregation of liposomes (Caaveiro et al. 1997). This mode of action is clearly different from other AMPs and responsible for the synergistic activities of StSN1 and potato defensin PTH1 against bacterial and fungal pathogens.

Another AMP that aggregates bacteria prior to killing is hydramacin-1 from the freshwater polyp Hydra (Jung et al. 2009). The structure of hydramacin-1 has been solved and shown to consist of two hydrophobic hemispheres sandwiched by a belt of positive charges. The cationic StSN1 peptide consists of a central hydrophobic stretch flanked by highly polar N-terminal and C-terminal domains (Segura et al. 1999). Further comparisons will have to await the structure of snakins to be solved.

Developmental expression of *StSN1* and *StSN2* mRNAs differs but it overlaps (Segura et al. 1999; Berrocal-Lobo et al. 2002). *StSN1* expression is particularly high in axillary and floral buds, in the stem and in petals, but tubers and carpels also express this gene. *StSN2* is strongly expressed in tubers, petals, carpels, stamen, and leaves, but stems and floral buds also express this gene. Wounding and treatment with the phytohormone abscisic acid induce *StSN2* expression in leaves (Berrocal-Lobo et al. 2002). *StSN2* expression is also induced after infection of tubers with *B. cinerea* but suppressed after inoculation with the bacteria *R. solanacearum* and *Erwinia chrysanthemi*. The expression patterns of *StSN1* and *StSN2* are therefore compatible with roles in constitutive and induced resistance, respectively.

Overexpression of *ScSN1* from *Solanum chacoense* in transgenic potato increased resistance to the fungal pathogen *Rhizoctonia solani* and the Gram-negative bacterium *Erwinia carotovora* (Almasia et al. 2008; Kovalskaya and Hammond 2009). Conversely, silencing of *snakin-2* in *Nicotiana benthamiana* reportedly resulted in an increase in susceptibility to *C. michiganensis* subsp. *michiganensis* (Balaji et al. 2010). These results point toward an important role of snakins in defense of solanaceous plants against pathogens.

9 Conclusions

Plant AMPs are functionally and structurally diverse. Structural features common to plant AMPs are disulfide bridges and secondary structures like α -helices and β -sheets. These structural features generate compact molecules that are resistant to chemical and physical insults and can survive hostile environments like the plant cell wall and the vacuole. Another general feature is that plant AMPs interact with lipids, phospholipids in the case of thionins, sphingolipids in the case of defensins, and various lipids in the case of LTPs. Interactions between other plant AMPs and lipids appear to be less specific. Moreover, other molecular functions, like chitin binding, in the case of hevein-like AMPs and knottins, and interactions with other proteins as observed for defensins and LTPs are important.

Certain plant AMPs, like thionins and cyclotides, are inherently toxic, while others, including defensin and LTPs, are not. The latter category of AMPs has been shown to fulfill important functions in plant signaling. The exact mechanism by which LTPs and defensins modulate plant signaling will be of interest not only to plant scientists.

The vast diversity of Cys-rich AMPs in the plant kingdom suggests that these peptides fulfill important ecological functions. The molecular evolution of the different classes of plant AMPs is incompletely understood. Research on this topic is desperately needed to better understand interactions of plants with symbiotic and pathogenic microbes and with herbivorous and beneficial insects.

In this review, we provide clear evidence that AMPs are an intricate part of the plant immune system, not merely executors of a defense program designed to kill enemies. AMPs therefore fill similar niches in the immune systems of plants and animals, although the molecules involved and the processes are different. As animal defensins are known to link innate and adaptive immune systems (Yang et al. 1999; Biragyn et al. 2002; Funderburg et al. 2007), plant LTPs play essential roles in SAR.

As their animal counterparts, plant AMPs can be exploited for pharmaceutical purposes. In the presence of multiple-drug-resistant bacteria, peptide antibiotics are clearly needed, and plant AMPs may add their share to the antimicrobial cocktail that may be used to fend off infectious diseases.

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Antimicrobial Peptides and Innate Immunity

Hiemstra, P.S.; Zaat, S.A.J. (Eds.)

2013, XI, 384 p. 43 illus., 20 illus. in color., Hardcover

ISBN: 978-3-0348-0540-7