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Identification and Rational Design of Novel Antimicrobial Peptides for Plant Protection

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bioactive peptides, antibacterial, antifungal, peptide libraries, plant resistance, cell penetrating peptides, peptide production

Abstract

Peptides and small proteins exhibiting antimicrobial activity have been isolated from many organisms ranging from insects to humans, including plants. Their role in defense is established, and their use in agriculture was already being proposed shortly after their discovery. However, some natural peptides have undesirable properties that complicate their application. Advances in peptide synthesis and high-throughput activity screening have made possible the de novo and rational design of novel peptides with improved properties. This review summarizes findings in the identification and design of short antimicrobial peptides with activity against plant pathogens, and will discuss alternatives for their heterologous production suited to plant disease control. Recent studies suggest that peptide antimicrobial action is not due solely to microbe permeation as previously described, but that more subtle factors might account for the specificity and absence of toxicity of some peptides. The elucidation of the mode of action and interaction with microbes will assist the improvement of peptide design with a view to targeting specific problems in agriculture and providing new tools for plant protection.

INTRODUCTION

AMP: antimicrobial peptides

Pathogenic microorganisms, the leading cause of plant diseases and crop losses, require the continued use of chemicals for control as well as to meet world food needs. However, the emergence of resistant isolates and pathogens, the limited spectrum of action, and negative long-term repercussions on human health and the environment have propelled the search for new alternatives as substitutes for the chemicals currently in use.

Peptides and small proteins exhibiting direct antimicrobial activity have been characterized from a vast number of organisms ranging from insects to humans (21, 191). The general biological role of antimicrobial peptides (AMP) in defense against challenging microbes is recognized. Antimicrobial peptides look like promising alternatives as novel therapeutics in combating the increasing incidence of antibiotic resistances in pathogenic microbes; and several examples are undergoing clinical trials (55, 57, 191).

Plants have specific defense mechanisms that include small antimicrobial proteins or peptides with activity against phytopathogens (19, 50, 176). In addition to their in vitro antimicrobial activity, some of these endogenous proteins are involved in active defense against disease. A recent study has suggested that these proteins might account for up to 2%–3% of the genes predicted in plant genomes (162).

In parallel to what has occurred in medicine, antimicrobial peptides and proteins have been proposed for potential use in agriculture (24, 67, 114, 152, 175). However, initial studies soon revealed that some natural peptides have undesirable properties such as nonspecific toxicity, low stability, and poor bioavailability that would compromise their application on crops.

Advances over the past decade have made possible the rational design of novel nonnatural AMP with the goal of improving their properties. The short sequence length of AMP favors structure/activity studies in a holistic approach to enhance their stability, potency, and specificity toward certain microbes. Their sequence length also makes feasible the chemical synthesis to high purity and facilitates the design of synthetic genes for their heterologous production through biotechnology. All these features boost scientific interest in this class of molecules.

In this review, we summarize and describe the processes of identification and characterization of AMP active toward phytopathogens, improvement of peptide properties by the rational modification of the amino acid sequence, and the different strategies for use of the resulting peptide in plant protection.

OVERVIEW ON NATURAL ANTIMICROBIAL PEPTIDES AND PROTEINS

AMP are a broad class of peptides and small proteins of short size (below 30–40 amino acid residues), which have antimicrobial activity against microorganisms, at least under in vitro assay conditions. Most of the conclusions from previous reviews on the identification, characterization, and activity of AMP of interest in medicine (21, 57, 68, 140, 191) can be extrapolated to plant pathology. We deal only with the application of AMP to plant protection, and discuss only examples/topics outside of our field that are relevant.

Although posttranslational modification, the inclusion of nonnatural amino acids, and peptide-like (mimetic) compounds can modulate (and increase) substantially the activity and stability of AMP (57), we focus our discussion mostly on ribosomally synthesized peptides. The properties and applications of nonribosomal or posttranslationally modified peptides in agriculture and food science have been reviewed elsewhere (32, 114).

Properties of Antimicrobial Peptides

AMP are diverse (see examples in **Table 1**) and can be subdivided into several groups based on their origin, composition, and structure (21, 41). However, they also share certain common structural characteristics such as (*i*) amino

Table 1 Representative natural short antimicrobial peptides

Peptide	Amino acid sequence[a]	Source
Apidaecin	GNNRPVYIPQPRPPHPRI	Insect
Cecropin A	KWKFKKIEKMGRNIRDGIVKAGPAIEVIGSAKAI	Insect
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	Insect
Ib-AMP1	QWGRRCCGWGPGRRYCVRWC	Plant
Indolicidin	ILPWKWPWWPWRR	Bovine
Magainin 2	GIGKFLHSAKKFGKAFVALKAL	Frog
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	Insect
PR-39	RRRPRPPYLPRPRPP	Porcine
Tritrpticin	VRRFPWWWPFLRR	Porcine

[a]One letter amino acid code is shown to indicate peptide sequences. Positive charged residues (R, K) are indicated in red; aliphatic hydrophobic (I, L, V) in blue; and aromatic hydrophobic (W, F) in green.

acid composition: the residues most abundant in AMP are cationic (arginine and lysine) and hydrophobic (tryptophan, phenylalanine, leucine, and isoleucine) (see colored residues in **Table 1**); (*ii*) net charge: most AMP are positively charged at physiological pH, although a minor subgroup includes anionic peptides; (*iii*) amphipathicity: conferred by their amino acid composition and arrangement; and (*iv*) a remarkable diversity of structures and conformations, including α-helices, β-sheets, nonconventional structures, or even extended conformations (the latter two are specially abundant in short AMP). Most of these structures are amphipathic and are induced under specific experimental conditions that also facilitate their interaction with lipid bilayers. In fact, biological membrane-induced amphipathicity of AMP is a hallmark property related to antimicrobial activity (see below).

Some cationic AMP are enriched in certain amino acids. A highly abundant class is that of peptides rich in cysteines, which can form disulfide bonds that make peptide structure compact and remarkably stable against adverse biochemical conditions and protease degradation. Most of the antimicrobial peptides found in plants belong to this class (Ib-AMP1, **Table 1**). There is much sequence and structural diversity within this group, which includes animal, insect, and fungal defensins; plant proteins such as thionins, defensins, and lipid transfer proteins (LTP) (19, 50, 176); and antifungal proteins (AFP) isolated from certain fungal genera including *Aspergillus* or *Penicillium* (102, 123).

Other relevant groups rich in specific residues are short cationic peptides with a high proportion in tryptophan (29), among which representative examples are Indolicidin, with activity against phytopathogens, or Tritrpticin (**Table 1**). Likewise, there are peptides enriched in histidines such as histatins, or in prolines such as PR-39.

DESIGN OF ANTIMICROBIAL PEPTIDES AGAINST PLANT PATHOGENS

The development of efficient methods for the synthesis of peptides and their analogs, peptide collections and libraries, has resulted in substantial advances in the identification and characterization of bioactive peptides. Rationally designed peptide modifications, additions, or deletions can be assayed for their effects on peptide activity with a view to improving their properties in terms of specificity against pathogens, reduced toxicity against plant or animal cells, greater stability, or modulation of the spectrum of action. In the first steps of the procedure, assays are performed by using suitable in vitro methods.

In most cases, the primary screen for peptide activity is a cell-based assay of in vitro growth inhibition that enables miniaturization in the high-throughput formats required for the

Amphipathic: an amphipathic peptide contains both polar and nonpolar domains

AFP: antifungal protein

Analog: a sequence derivative of a given peptide

analysis of complex collections of compounds (i.e., libraries) (20, 153). Most of the AMP assayed in vitro have 50% inhibitory and minimum completely inhibitory concentrations (IC_{50} and MIC, respectively) in the range of 1 to 20 μM. Additional data such as those relative to inhibition of fungal spore germination or to killing activity have been also reported. These in vitro procedures, if conducted on nontarget microorganisms or a panel of different pathogens, can also be used as a means to evaluate peptide specificity or the activity profile (96). Although still limited, there are also examples of peptide screens that were targeted to specific pathogen functions (see below) (12, 111, 157, 158).

Peptide toxicity has been evaluated mostly by assaying the cytolysis toward human red blood cells. Although membrane-active peptides such as melittin are highly cytolytic in these assays, caution should be taken in interpreting the results, as the salt concentration of the buffers used to evaluate hemolysis is expected to block the electrostatic interactions that most peptides need to interact with microbes (see below). Toxicity evaluations should therefore be conducted under conditions mimicking the environment that the peptides will encounter in the host plants. In selected examples, assays have been used that are based on in vitro pollen or seed germination and protoplast viability in the presence of peptides (34, 65, 187).

Regarding peptide stability, extracts obtained from different plants or plant tissues (26, 133, 143), supernatants of cultures of pathogens (34, 127), or commercially available proteases (46) are well suited to determine peptide susceptibility to protease degradation and influence on peptide activity.

Modification of Natural Peptides

A primary strategy in the design of novel AMP is the modification of peptides found in nature. Numerous studies have focused on attempts to diminish nonspecific toxicity while retaining the desirable bioactivity through the design of sequence analogs or hybrid peptides.

Cecropins, magainins, and melittin (**Table 1**) are well-characterized membrane-active AMP (9) whose toxic properties have made their practical use difficult. Cecropins, a broad class of antibacterial peptides, were among the first in which attempts were made to modulate the ratio of activity against microbes vs toxicity to plant cells. Two sequence analogs of cecropin B with reduced toxicity to plant protoplasts, SB-37 and Shiva-1 (**Table 2**), were engineered that differed in their primary sequences. Each maintained the amphipathic properties of the parental molecule and showed differential activity against distinct plant pathogenic bacteria (66, 126).

Melittin is a cytolytic peptide from honey bee venom (**Table 1**). It has been widely studied as a model for the interaction of lytic peptides with biological membranes, and also to identify determinants of toxicity to human cells and selectivity to bacteria (147). It has also given rise to a significant number of AMP used in plant protection. The screening of nonhemolytic analogs of melittin resulted in the identification of peptides capable of inhibiting the infection of tobacco leaves by tobacco mosaic tobamovirus (TMV) (100). These peptides showed limited sequence homology with the TMV capsid protein and interacted abnormally with TMV RNA.

Fusions of fragments of natural AMP have been designed in an attempt to endow the resulting chimeras with desirable properties from the parentals. A well-documented instance is that of cecropin::melittin hybrids (**Table 2**). Examples are the peptides CEMA and its derivative MsrA1 (132); CAMEL with activity against different species of *Pectobacterium* (74); or Pep1 which is active against distinct phytopathogenic fungi (26). Likewise, cecropin A::magainin hybrids have been designed that show antibacterial activity but do not produce hemolysis (81).

Cecropins are not stable in the presence of plant extracts (107), and indolicidin or LL-37 is degraded by bacterial proteases (129, 159). The N terminus of cecropin A is cleaved

Table 2 Antimicrobial peptide analogs with activity against phytopathogens

Peptide[a]	Amino acid sequence[b]	Pathogen	References
SB-37 (Cec B)	MPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG	Bacteria	(126)
Shiva-1 (Cec B)	MPRWRLFRRIDRVGKQIKQGILRAGPAIALVGDARAVG	Bacteria	(66)
MrsA1 (Cec A::Mel)	MALEHMKWKLFKKI::GIGAVLKVLTTGLPALKLTK	*E. carotovora*, *F. solani*, *P. cactorum*	(132)
CAMEL (Cec A::Mel)	KWKLFKKI::GAVLKVL	Bacteria	(74)
Pep1 (Cec A::Mel)	KWKLLKKI::GAVLKVL	Fungi, *P. infestans*	(26)
P18 (Cec A::Mag)	KWKLFKKI::PKFLHLAKKF	Bacteria, *F. oxysporum*	(81)
Pep3 (Cec A)	WKLFKKILKVL	Fungi, *P. infestans*	(26)
BP76 (Cec A)	KKLFKKILKFL	Bacteria	(46)
MB39 (Cec B)	HQPKWKVFKKIEKVGRNIRNGIVKAGPAIAVLGEAKALG	Bacteria, fungi	(133)
Myp30 (Mag)	MGIGKFLREAGKFGKAFVGEIMKP	*E. carotovora*, *P. tabacina*	(84)
MSI-99 (Mag)	GIGKFLKSAKKFGKAFVKILNS	Bacteria, fungi	(2)
Rev4 (Ind)	RRWPWWPWKWPLI	*P. tabacina*	(83)
10R (Ind)	RRPWKWPWWPWRR	*E. carotovora*, fungi	(11, 42)
11R (Ind)	RWRRWPWWPWRRK	*E. carotovora*, fungi	(11, 42)
MsrA2 (Dermaseptin B1)	MAMWKDVLKKIGTVALHAGKAALGAVADTISQ	*E. carotovora*, fungi	(131)
MsrA3 (Temporin A)	MASRHMFLPLIGRVLSGIL	Bacteria, fungi	(130)
PV5 (Polyphemusin I)	MRRYCYRKCYKGYCYRKCR	*E. carotovora*, fungi	(10)
MBG01 (Rs-AFP1)	ARHGSCNYVFPAHKCICYF	*F. culmorum*	(158)
LfcinB17-31 (LF)	FKCRRWQWRMKKLGA	Fungi	(122)

[a]Peptide name and in parenthesis peptide parental. Cec: cecropin; Mag: magainin; Mel: melittin; Ind: indolicidin; LF: lactoferrin.
[b]Residues changed relative to the parental sequence are underlined. Other amino acid sequence details as in **Table 1**.

by extracellular proteases from the conidia of *A. flavus*, which may explain the lack of activity against the spores of this fungus but not against others (13). Distinct analogs of natural AMP with greater resistance to in vitro degradation have been described. Examples include MB39 from cecropin B, Pep3 from cecropin A, and Myp30 from magainin 2 (**Table 2**). A further derivative of Pep3 is BP76, which exhibits improved activity against phytopathogenic bacteria, reduced hemolysis, and lower susceptibility to proteinase K (46). Notably, the indolicidin reverse sequence Rev4 showed increased stability in the presence of plant extracts and commercial protease cocktails, as well as activity in vitro toward *Peronospora tabacina* (83).

Sequence modification of natural peptides has also resulted in increased potency against (selected) pathogens. The magainin 2 derivative MSI-99 (**Table 2**) has more positive charge and antibacterial activity than the parental peptide, while activity against filamentous fungi and oomycetes was maintained (2). The indolicidin analog CP-11 (42) has increased positive charge and amphipathic properties that result in greater antifungal and antibacterial activities. Further derivatives of this are peptides 10R and 11R (**Table 2**).

Peptide cyclization is a remarkable modification of synthetic peptides that in some cases has a dual effect. It has resulted in increased stability and also selectivity against microbes due to conformational constraints that facilitate the amphipathic separation of residues in the molecule. Initially, this approach was applied mostly to cationic tryptophan-rich peptides (33, 156), but also has been extended to other peptide classes (112, 113). Although this type of modification precludes peptide production through biotechnological approaches, the increases in stability and antibacterial activity are remarkable and should be considered if peptides of this class are to be used as phytosanitary products for field or postharvest treatments.

Peptide library: an ordered collection of peptides or peptide mixtures that contains and represents the sequence diversity of peptides of a given size or property. The intrinsic order and organization of a peptide library permit individual bioactive sequences to be identified under appropriate assay conditions

Rational Design of Antimicrobial Peptides

The rational design of novel peptides is based on knowledge of the biophysical and structural properties of natural AMP. There are numerous examples in the biomedical field, but not many have been tested for their utility in plant protection. ESF1 and ESF12 (**Table 3**) are α-helical peptides that mimic the charge distribution and structure of magainins. They were developed in studies that showed that a reduction in positive charge in their structural scaffold decreases antimicrobial activity, whereas an increase in hydrophobic residues is correlated with unspecific cytotoxicity and therefore should be avoided (143, 144). Peptides GR7 and SA3 (**Table 3**) were derived from ESF1 and have higher amphipathicity and net charge, and reduced the inhibitory concentration toward *F. oxysporum* down to 0.1 μM (40).

A similar rational design approach was taken to develop D4E1 and D2A21 (**Table 3**). The activities of these peptides were compared with those of cecropin B and magainin II, showing that they are more active and have no adverse effect on pollen and seed germination (65). In contrast to most of the examples described above that are α-helical, D4E1 presents β-sheet folding. Interestingly, it is also one of the peptides that have been successfully expressed in a larger number of plant species (see below).

Synthetic Peptide Libraries

The tools of synthetic combinatorial chemistry approaches have been harnessed to synthesize and assay vast sources of molecular diversity for the identification of lead bioactive compounds (14). From hundreds to millions of compounds can be screened in much shorter times than with traditional methods. Despite the disadvantage of their high cost, reports of their use in agriculture and food applications are increasing (76, 86).

The so-called "nondefined" synthetic peptide libraries do not have sequence restrictions and represent all possible sequence combinations of a peptide of a given size. They are collections arranged as mixtures of peptides and a deconvolution procedure is needed to identify the individual active peptide(s) (14). There are two main deconvolution strategies: iterative and positional (see **Supplemental Figure 1**. Follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). A cell-based

Table 3 Rationally designed AMP against phytopathogens

Peptide	Amino acid sequence[a]	Pathogen	References
ESF1	MASRAAGLAARLARLALRAL	Bacteria, fungi	(143)
ESF12	MASRAAGLAARLARLALR	Bacteria, fungi	(143)
GR7	MASRAARLAARLARLALRAL	Bacteria, fungi	(40)
SA3	MAARAARLAARLARLALRAL	Bacteria, fungi	(40)
D4E1	FKLRAKIKVRLRAKIKL	Bacteria, fungi	(34)
D2A21	FAKKFAKKFKKFAKKFAKFAFAF	Bacteria, fungi	(155)
PAF26	RKKWFW	Fungi	(96, 120)
BM0	RFWWFRRR	Fungi	(111, 120)
PPD1	frlhf	Fungi	(154)
66-10	frlkfh	Fungi	(154)
77-3	frlkfhf	*F. sambucinum*	(53)
BP100	KKLFKKILKYL	Bacteria	(8)
ACHE-I-7.1	SINWRHH	Nematodes	(182)
Pc87	ADRPSMSPT	*P. capsici*	(12)

[a]Amino acid residues in lower case indicate D-estereoisomers. Other amino acid sequence details as in **Table 1**.

assay of in vitro growth inhibition toward four different phytopathogenic fungi was used on an iterative library to identify one pentapeptide (PPD1) and one hexapeptide (66-10) (**Table 3**). Likewise, a positional hexapeptide library was assayed against the postharvest fungal pathogen *Penicillium digitatum*, identifying PAF26 (**Table 3**). In this latter case peptides were also assayed against nontarget microorganisms (the bacteria *Escherichia coli* and the unicellular fungus *Saccharomyces cerevisiae*) to identify peptides with distinct spectra of antimicrobial activity.

In these approaches it is common to use peptides synthesized with the D-enantiomers of amino acids because of their resistance to degradation (14). The D- or L-versions of this short AMP do not present significant differences in terms of antimicrobial potency (111, 120, 181), which could be related to their mode of action and is also a prerequisite for production through biotechnology.

To reduce costs and restrict the biological, sequence, or structural properties of peptides, one option is the use of "defined" libraries (15). For instance, a defined octapeptide library that included an amino acid motif for attachment to the yeast surface was screened for yeast growth inhibition and, secondarily, toward a *S. cerevisiae* plasma membrane ATPase as a target, to identify the octapeptide BM0 (**Table 3**) (111), which later showed activity against fungal phytopathogens (120).

In other cases, defined libraries are built upon lead peptides of known activity previously identified by nondefined approaches; this can be considered as a further step in the pipeline of combinatorial AMP identification (see **Supplemental Figure 1**). Heptapeptides 77-3 (**Table 3**) and 77-12 are derivatives of 66-10, and were identified against the potato pathogen *Fusarium sambucinum* (53). Likewise, a series of heptapeptide derivatives of PAF26 was assayed against a panel of phytopathogenic fungi to show distinct activity profiles of specific peptides (121). That *Magnaporthe grisea* was the fungus most dissimilar in terms of sensitivity to peptides indicates the existence of distinct fungal components that modulate the interaction with peptides. A remarkable recent example is the identification of BP100 (**Table 3**) from a 125-member library derived from BP76, which was assayed for antibacterial activity, low cytotoxicity and protease degradation, and in vivo protection in a detached flower assay (8).

In a distinct approach of a defined library use, scrambling of positions 1–4 of a cyclic decapeptide allowed the identification of improved AMP against *Erwinia amylovora* that showed low hemolysis (112).

Peptide Libraries Produced through Biotechnology

Peptide libraries can also be generated and produced in vivo through biotechnological methodologies. Peptide library production and activity may then be induced and identified either in *cis*, against the producer microorganism (suicide strategy) (30), or in *trans*, against a target microbe different from the producer (153). The strain producer of the bioactive peptide can be subsequently cloned and used to isolate the peptide sequence. Theoretically, these strategies enable the bioassay of more peptide complexity than the synthetic procedures (i.e., longer peptides), but to our knowledge no examples of use in plant protection have yet been reported. These strategies can also incorporate codon-shuffling methodologies for the directed evolution and improvement of antimicrobial proteins (151), a procedure for which their small size seems to be particularly well suited.

Biotechnological approaches for peptide library production also allow bioassays other than cell-based ones to be used for peptide identification. Phage-display techniques are broadly known for the selection of peptides and proteins with binding affinity toward biomolecules. The peptide ACHE-I-7.1 (**Table 3**) was identified from a phage library as an inhibitor of acetylcolinesterase, a target of pesticides that disrupt chemosensing in nematodes (182), and was successfully expressed in potato to suppress parasitism by cyst nematodes (87).

Peptides with affinity for zoospores of *Phytophthora capsici* were also isolated by phage display under the assumption that binding to spore envelopes would alter the normal interaction with plants and thus the life cycle of the pathogen (12) (**Table 3**), as was confirmed later (44).

The yeast two-hybrid system to identify interacting protein domains in vivo was used to isolate aptamers with affinity for a replication protein of tomato golden mosaic geminivirus (98). A rich diversity of peptides (around 20 residues in length) that successfully inhibited viral DNA accumulation in tobacco protoplasts was obtained. Their sequence comparison allows the identification of peptide motifs that would confer broad protection against geminivirus. In a similar but more defined strategy, a random peptide library was generated from fragments of the capsid protein of tomato spotted wilt tospovirus (TSWV), and the yeast system enabled the identification of a 29-residue aptamer that conferred resistance to tospoviruses (157).

Antimicrobial Peptides Derived from Proteins

Short AMP whose sequence belongs to plant antimicrobial proteins were identified in experiments to map the minimal active domains of the full-length proteins. Peptide MBG01 from a *Raphanus* defensin (**Table 2**) is as potent as the full protein and allowed the design of sequence modifications to improve its properties. A similar strategy was followed with a thionin and afterwards generated a series of rationally designed derivates with strong activity (178, 179). Most of these designed sequence modifications are nonnatural and thus probably of interest to the biomedical field, but their application to specific plant protection problems should not be discarded.

In recent years, food-related proteins and their hydrolysates have been intensively studied as source of AMP for their obvious practical implications (138). Lactoferricin (Lfcin) is the antimicrobial core of the milk protein lactoferrin (LF) (45). Distinct peptides derived from Lfcin have antimicrobial properties against bacteria, fungi, or viruses (45, 59), and have demonstrated antifungal activity against plant pathogens (**Table 2**). There are examples of recombinant LF-related proteins produced in plants, with the objective of generating transgenic plants either with resistance phenotypes or as production factories for molecular farming (82, 124, 192).

Novel Strategies

Although not yet applied to plant protection, alternative in silico strategies could be a novel source of AMP not found in nature. Virtual assay approaches, for example, are capable of predicting the antimicrobial activity of peptide sequences, thus reducing the number of bioassays to be conducted in the real world (153, 163). The rational design of new peptides has been also achieved by defining "grammar rules" that describe the presence of amino acid residues within an AMP (94). Both examples are bioinformatic tools that take advantage of the wealth of information in AMP public databases, regarding peptide sequences, structure activity relationships, and mode of action (see Related Resources).

The accompanying **Supplemental Figure 1** (Follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**) summarizes the strategies described to identify novel antimicrobial peptides (AMP) by rational design.

MODE OF ACTION AGAINST MICROBIAL CELLS

A detailed knowledge of the mode of action of AMP is essential if the goal of applying them to plant protection is ever to be reached. In some of the examples cited above, the activity screens conducted implicitly indicate the mechanism by which the peptides affect the pathogen. However, for most of the cases in which activity was determined as cell-based growth inhibition assays of bacteria or fungi, the peptide mode of

action needs further investigation. Of note is the elucidation of the mechanisms that confer peptide specificity toward phytopathogenic microorganisms and that avoid toxicity to animals or plants. Such information should aid further identification and design of novel peptides. Several excellent reviews address these questions in the biomedical field (21, 191); here we summarize only those specific examples that are relevant to phytopathology.

Interaction with Microorganisms

The first step in the AMP mode of action is the physical interaction with outer structures that surround the microbial cell. In general, it is assumed that such interaction is not stereospecific since it has been shown in selected examples that peptides synthesized with either the L- or the D-enantiomers of amino acids do not differ substantially in antimicrobial activity (181).

The cationic properties of many AMP and proteins result in an electrostatic attraction to the negative-charged microbial envelopes, such as lipopolysaccharide (LPS) of gram-negative bacteria, which is a plausible explanation for peptide specificity to microbes and lower toxicity to animal and plant cells (41, 191). Distinct experimental data support such an electrostatic attraction. The peptide CEMA (**Table 2**) binds LPS, and this binding is correlated with the differential antimicrobial activity of an analog with lower affinity for LPS (141). Mutants of *Ralstonia solanacearum* with increased sensitivity to thionins and LTP present alterations in a LPS biosynthetic gene (172). Regarding amino acid sequence, an increase in net positive charge is linked with increased antimicrobial activity (121, 143). Likewise, a set of alanine substitution analogues of PAF26 showed that the higher contribution to its antifungal activity results from its three cationic residues (121), thus reinforcing the importance of ionic attraction.

In general, the electrostatic attraction is perturbed by the increase of ionic strength and, as occurs with plant antimicrobial proteins (19, 50), peptide antimicrobial activity diminishes with the addition of salt ions to the in vitro assay medium. However, the activity of selected AMP also depends on an ionic milieu comparable to that of mammalian body fluids (38, 140). This issue must be taken into account in relation to the composition of the microenvironment, cell, or tissue location in which the peptides are expected to act.

However, there are examples of antimicrobial peptides and proteins whose interaction with microbes either does not rely primarily on electrostatic attraction or is aided by binding to specific components. The use of the model fungus *S. cerevisiae* has allowed the identification of specific cell wall or membrane components that mediate the action of antimicrobial proteins. Thus, the plant antimicrobial protein PR-5 (osmotin) effect on yeast is influenced by cell wall glycoproteins (189). Also, the sensitivity of *S. cerevisiae* to distinct plant defensins is determined by glycolipids specific to fungal membranes (168, 170), and similar conclusions were drawn from a study with the filamentous fungus *Neurospora crassa* (135). Mutants of *F. oxysporum* and *Aspergillus oryzae* in chitin biosynthesis had lowered sensitivity to the AFP from *Aspergillus giganteus* and its membrane permeabilizing effect (54).

Regarding AMP, the case of nisin is significant inasmuch as it shares the membrane disrupting capabilities of many AMP (see below) that are promoted by a specific interaction of nisin with a Lipid II precursor (18). Also, peptide D4E1 (**Table 3**) preferentially binds ergosterol, an esterol that is a hallmark of fungal membranes (34). Taken together, all these findings indicate that although surface net charge is undoubtedly relevant to cationic AMP action, other factors can also modulate peptide interaction with microbes.

Interaction with Synthetic Membranes and Membrane Mimetics

Most AMP have amphipathic properties that enable their insertion into and disruption of model lipid membranes, as determined by

structural and biophysical techniques (93, 145). Many studies correlate peptide antimicrobial activity with effective interaction with artificial lipid bilayers that would mimic those of target microorganisms. Topological models have been proposed to explain the interaction of AMP with membranes (21). In summary, the propensity of a given peptide to interact and disrupt a lipid membrane and the concrete topology of the membrane disruption depend on the size and structure of the peptide, membrane lipid composition, a ratio of peptide to lipid concentration, and folding capabilities of the peptide, among others.

The conclusions derived from such studies and models have allowed the rational design of some AMP (34, 143, 155). However, the implicit rationale that peptide activity is a direct consequence of membrane-disturbing capabilities and thus microorganism cell permeation is a generalization that cannot always be used to explain antimicrobial action (see below). This view stems from the fact that among the first AMP characterized are examples of peptides with a high propensity to disrupt lipid bilayers. Peptides that have such lytic properties clearly also have a higher probability of being toxic to nontarget cells.

In any case, it should be considered that the first line of interaction between a given AMP and the target microorganism is not the lipid bilayer but rather the outside structures and cell envelopes, which can vary from one type of organism to another.

Morphological Alterations

A number of studies have described distinct physical alterations in the microbial cells after exposure to antimicrobial peptides. Generally, the two most relevant and broadly documented are alterations of shape and growth, and cell permeation.

Distinct microscopy techniques have allowed the visualization of morphological and growth alterations in bacterial cell shape after exposure to distinct AMP (21), and in the mycelium of phytopathogenic fungi exposed to short AMP (3, 4, 26, 89, 119, 149, 155). In selected examples, alterations described at the ultrastructural level are indicative of cell degradation (79, 127, 155).

At concentrations above those that are completely inhibitory, collapsed fungal hyphae were also indicative of cell death. At lower peptide concentrations, partial fungal growth inhibition was reflected in shorter interseptum distances, thick hyphae, cell enlargement, mycelial aggregates, and/or abnormal branching patterns. The latter could be either hyperbranching (3, 4, 89), or abnormal tip dichotomous branching and aborted lateral branching (89, 119). In some cases, fungal mycelium reacts with alterations in the deposition of chitin (116, 119). Of note is that some of these alterations are phenocopies of mutations in genes that are involved in controlling polar growth or biosynthesis of cell wall components (58, 101).

It is difficult to draw comparative conclusions among studies from different laboratories and thus it is open to debate whether distinctive alterations are indicative of distinct modes of action. Microscopic analyses have shown that different AMP have different effects on *Pseudomonas aeruginosa* cells, which indicates that they would have different targets or mechanisms of activity (21, 73). In an analogous study conducted in fungi, two sequence-related plant defensins induced distinctive alterations and molecular responses in *F. graminearum* (150).

Cell Permeabilization

Permeation of synthetic membranous vesicles can be easily measured through the release of (fluorescent) probes. For a substantial number of AMP, their capability to insert into and destabilize lipid bilayers has been shown to correlate with permeation of artificial vesicles as well as with antimicrobial activity (75, 169).

The use of specific fluorescent probes allows the visualization of cell permeation of microorganisms after exposure to peptides. Assays are based on coincubation of the microbe with the peptide and the probe, which can penetrate the cell only if the plasma membrane is disturbed,

and once inside binds to specific cell components and emits fluorescence. This emission can be quantified and visualized. Data of this type have been obtained with plant antimicrobial proteins (169), AFP proteins from *Penicillium* (115, 167), and with synthetic AMP (53, 119, 122, 127, 154, 155), acting on phytopathogenic fungi. In most of these examples, the observation of cell permeation is interpreted as the direct and primary effect of peptides that leads to cell killing.

However, other studies have argued that there is not always a complete correlation between cell permeation and antimicrobial activity (41, 183). Significant conclusions can be drawn when permeation is evaluated at different peptide concentrations, since it becomes clear that at the minimal lethal concentration not all peptides kill through membrane disruption (105). For instance, the cationic tryptophan-rich peptide BM2 did not cause yeast permeation at low concentrations that partially inhibit growth (111), and areas of fungal mycelium that show morphological alterations and growth inhibition due to peptide exposure do not have detectable permeation (119, 122). Specific peptides kill bacteria, but they either do not permeate bacterial membranes or have additional modes of action (16, 25, 164).

These and other results (see below) suggest that specific AMP could have more subtle mechanisms of action not necessarily or primarily linked to microbe permeation (21, 56, 93, 185). A hallmark property in this regard is the ability of some AMP to cross membranes and have an intracellular mode of action (21, 55).

CPP: cell-penetrating peptides

Intracellular Modes of Action

Specific cationic peptides rich in arginine or aromatic residues have the propensity to cross biological membranes in a nondestructive manner, and translocate to the cell inside; they have been named penetratins or cell-penetrating peptides (CPP) (61, 69). Initially, they were described as part of viral or homeotic intercellular signaling proteins. Their biotechnological use as shuttles to deliver bioactive proteins to cells has been proposed (160).

This translocation capability to either fungal or bacterial cells is being demonstrated for an increasing number of antimicrobial peptides, some of which with activity against plant pathogens (**Table 4**). In selected examples, internalization to fungal mycelium has been shown at low concentrations at which no effect on growth inhibition or cell permeation is detected (119). Cell-penetrating properties have been also shown for AFP in phytopathogenic fungi (116) and also for plant defense proteins (27, 92).

The reverse direction has also been taken, and recent studies have derived antimicrobial properties from peptides previously known only as CPP (72, 134, 193). These relationships

Table 4 Cell-penetrating antimicrobial peptides

Peptide	Amino acid sequence	AMP[a]	CPP[b]
Penetratin	RQIKIWFQNRRMKWKK	(134)	(37)
Pep-1	KETWWETWWTEWSQPKKKRKV	(193)	(118)
Tat (47-58)	GRKKRRQRRRPPQ	(72)	(180)
Apidaecin	GNNRPVYIPQPRPPHPRI	(24, 24a)	(25)
Buforin II	TRSSRAGLQFPVGRVHRLLRK	(136)	(78)
Indolicidin	ILPWKWPWWPWRR	(43, 80, 120)	(165)
LfcinB	FKCRRWQWRMKKLGAPSITCVRRAF	(122, 173)	(60)
Magainin 2	GIGKFLHSAKKFGKAFVALKAL	(2, 80, 190)	(103)
PAF26	RKKWFW	(96)	(119)
Polyphemusin I	RRWCFRVCYRGFCYRKCR	(109)	(146)

[a]AMP: antimicrobial peptides. Literature references in parentheses report antimicrobial activity.
[b]CPP: cell-penetrating peptides. Literature references in parentheses report cell translocation activity.

CP-AMP: cell-penetrating antimicrobial peptides

raise questions about the real differences between specific groups of antimicrobial and cell-penetrating peptides (61), which in fact would belong to a unique class for which the proposed name of cell-penetrating antimicrobial peptides (CP-AMP) seems appropriate. This question deserves to be explored in the near future since it perhaps holds the key to a novel source of AMP sequences and information on the design of peptides with improved activity and new properties against (plant) pathogens.

Once inside the cell, some AMP may act as antimicrobials through specific mechanisms that include binding to nucleic acids or the inhibition of enzymatic activity or synthesis of macromolecules (proteins, nucleic acids, or cell wall components) (21, 52). Relevant examples of peptides active against phytopathogens are those of indolicidin and lactoferricin (165, 174). Due to the cationic nature of many antimicrobial peptides and proteins, their affinity for anionic nucleic acids is expected and in vitro binding has been shown in a number of cases (62, 116, 119). Although such activity in vivo could alter cell homeostasis severely, it remains to be determined to what extent this property mediates peptide antimicrobial action.

The steps reviewed above in the interaction of AMP with microorganisms and mechanisms of action are modeled in **Figure 1**. Although interaction of AMP with lipid bilayers and cell (cytoplasmic) membranes is an essential property and step in the killing process, other questions might be more descriptive of peptide specificity toward microbes, such as the interaction with outer cell structures, ability to translocate across membranes, and whether putative intracellular targets exist, and should receive more attention in the future development of novel peptides. For simplicity but also because detailed knowledge is lacking, the model does not differentiate among bacterial or fungal microorganisms. Selected micrographs with examples of some of the steps described in the model are shown in **Supplemental Figure 2** (Follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**) for the interaction between the synthetic peptide PAF26 (**Table 3**) and the phytopathogenic fungus *P. digitatum*.

RESPONSE TO ANTIMICROBIAL PEPTIDES

It was initially assumed that the lytic mode of AMP action, significantly nonspecific, would prevent the development of resistance among microbes, as occurs with other more specific therapeutics. However, this is not the case (137, 140). In fact, it has been proposed that the great diversity of AMP in nature results from the coevolution of hosts and microbes in a continuous process in which microorganisms mount resistance responses as a countermeasure to peptide action and hosts diversify peptide structures to circumvent resistance mechanisms (140, 185). Microbial resistance mechanisms to AMP can be summarized as the secretion of extracellular proteases or proteins capable of binding to and inactivating AMP, alteration of microbial surface composition and net charge to prevent attachment, and induction of active membrane transporters capable of extruding peptides from cells. Conceptually, each of these mechanisms can be envisioned as blocking the different steps leading to peptide interaction and action as described in **Figure 1**. Pathogenic bacteria could actively induce these responses because they have specific sensor systems that are able to recognize the presence of antimicrobial peptides and activate signaling cascades with the potential to change gene expression (7, 91, 104).

There are numerous reports related to human pathogen resistance and response to AMP, although examples on microbial phytopathogens are still scarce. However, evidence indicates that both plant and animal pathogens deal in a similar way with host AMP (51). It has been suggested that the microorganism response could be specific to an antimicrobial peptide type, which might be relevant in the study of plant-pathogen interactions (51). Cecropin A is degraded by conidial extracellular proteases from certain plant pathogenic fungi but not from others (13, 35). The ABC class of

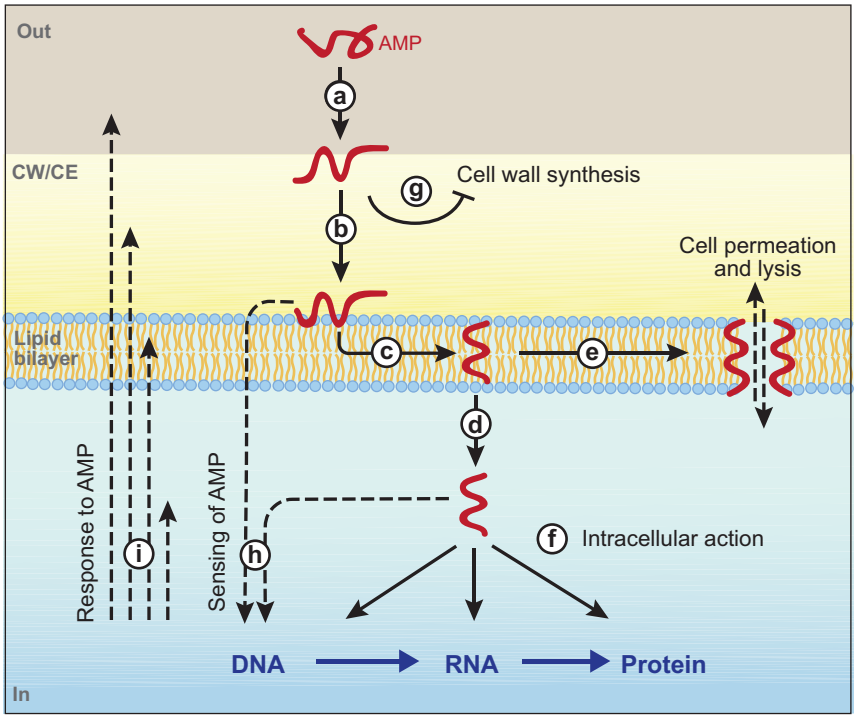

Figure 1

Model of peptide interaction with microorganisms and mode of antimicrobial action. The proposal is based on previous reports and considers recent findings summarized in the text. As presented, it can accommodate prokaryotic and eukaryotic cells. Steps involved are outlined as follows. (*a*) AMP (in *red*, may be unstructured) interact with the outer cell envelope, being the electrostatic attraction the initial driving force of the interaction. (*b*) The AMP can diffuse toward and bind to deeper lipid bilayers, where the biophysical properties of most AMP enable insertion into the bilayer (*c*), a process that is dependent of lipid composition. During this progression, the AMP folds owing to changes in the microenvironment. Folded AMP might enhance their amphipathicity and thus their antimicrobial properties. At this point, AMP can either translocate the bilayer and penetrate to the cytosol (*d*), disrupt the membrane architecture and lead to cell permeation (*e*), or both; the balance among these alternatives is dependent on time and peptide concentration, as well as on properties of peptide and cell components. (*f*) Internalized AMP can bind to DNA, RNA, and/or proteins, and disrupt DNA replication, RNA synthesis, or enzyme activity, depending on the peptide. Intracellular action can lead to cell killing and subsequent cell permeation. (*g*) Other modes of AMP action are disruption of cell wall synthesis, architecture, or cell morphology due to abnormal interaction with outer cell components. Microorganisms have sensing mechanisms to detect and signal peptide presence (*h*), a process that is required to change gene expression and protein activities to counteract peptide action at the cytosol, membrane, cell envelope, or cell environment (*i*).

membrane proteins is involved in the detoxification of compounds by active extrusion from cells. Some of the corresponding bacterial genes are related to susceptibility to AMP and in addition involved in bacterial virulence to plant tissues (99). Also, as mentioned above, modification of the net surface charge can affect sensitivity to peptides (139, 172). A more general approach has been taken to screen a collection of mutants from the alfalfa symbiont *Sinorhizobium meliloti* for their increased sensitivity to an antimicrobial peptide (125). Of the seven genes identified, three are involved in the biosynthesis of an exopolysaccharide accumulated on the cell surface, and one is, again, an ABC membrane transporter.

The findings that microorganisms can develop resistance to AMP, as they do to other antimicrobials, could diminish the interest in their use. However, knowledge of the mechanisms affecting sensitivity/resistance to AMP will surely help in designing more specific and effective peptides, which could exploit multiple modes of action within a single molecule; for instance, membrane-permeating properties combined with binding to specific targets (140) (**Figure 1**). It must also be borne in mind that AMP and proteins are an ancient and effective defense mechanism that has persisted during evolution, most likely because hosts can deplete an arsenal of different peptides with distinct modes of action acting on a given microorganism (140). Similarly, multiple peptides or multidomain AMP could be used or engineered to mimic nature's solution to this question.

The genomic characterization of the transcriptional changes of selected microorganisms should increase our knowledge on the specific microbial response to the action of peptides (38, 48). Likewise, the isolation of mutants with altered sensitivity to peptides will continue to provide information on peptide targets and interacting counterparts, and thus will help to evaluate the feasibility of selection of resistant phenotypes.

USE OF ANTIMICROBIAL PEPTIDES IN PLANT PROTECTION

Most of the initial work on AMP is based on in vitro data showing the activity of synthetic peptides in growth media. These approaches offer obvious advantages since they allow high-throughput formats to screen complex collections of peptides quantitatively, with a minimum amount of peptide use, and thus exploiting one of the advantages of the work with short peptides, i.e., the capacity to analyze high diversities and defined sequence modifications. As such, these strategies are needed to identify candidates and lead compounds, which subsequently must be tested in plants to complete the identification of potentially useful peptides.

Control of Plant Diseases through Addition of Synthetic AMP

Interaction between peptides and pathogens in the plant microenvironment is thought to be modulated by complex factors that must affect peptide activity. A first step in introducing these factors in peptide selection and evaluating the control of plant diseases through AMP is to conduct experimental coinoculations in which peptides and pathogen are applied to susceptible tissues. Such experiments can be carried out on different candidate peptides of potentially useful in vitro properties.

Assays based on detached leaves or leak disks (2, 4), flowers (8), fruits (90, 95, 96), or potato tubers (4, 74) have been conducted with positive results. The protective effect reported is limited in most of these examples, likely due to the fact that peptides are applied locally onto the plant surface or the inoculation point and, therefore, the pathogen escapes the AMP action if it grows outside this area. In general, in all these reports the reduction of disease incidence or progression correlates with the in vitro activity of a given peptide. However, a recent comparative study showed that in vitro inhibitory activity of a set of eight distinct AMP is not correlated with their capacity to retard fruit decay caused by fungi (120).

There are examples in which these experimental bioassays are close to the real plant pathology application. A case study is postharvest fruit diseases, for which AMP could be used as an additional postharvest additive (89, 90, 95), and synthetic peptides that incorporate nonnatural peptide modifications to increase their properties (as noted above) could be considered provided that the production costs are minimized.

Combined Action of AMP with Other Antimicrobials

Some AMP are active against natural isolates of fungi that have developed resistance against common commercial fungicides (53, 97), a property that reinforces their potential use as an alternative to the phytochemicals

currently used. Moreover, the synergistic activity reported for synthetic peptides and thiabendazole against *Fusarium* suggests that AMP could be tools to lower the dosage and thus the residue level of fungicides (53). Although still unexplored, the combined action of distinct antimicrobial strategies to arrive at more environmentally friendly control practices could incorporate the use of AMP at sublethal concentrations.

A significant example is the combination of AMP with biocontrol microorganisms, a prerequisite being the absence of toxicity of the peptide against them (95). In one case, a genetically modified biocontrol agent could be even used to produce and release the AMP (see below). The model yeast *S. cerevisiae* transformed with a gene fusion that secreted peptide Pep3 (**Table 2**) behaved as an antagonistic organism that protected tomato fruits against *Colletotrichum* decay (71).

Transgenic Expression of AMP in Plants and Disease Resistance

An obvious advantage to the use of peptides as antimicrobials is their in situ production through biotechnology. The overexpression of transgenes encoding plant antimicrobial proteins has been demonstrated as a successful approach to protect plants against diseases caused by microorganisms (22, 49, 110). Likewise, genes from nonplant origins have been transferred, including fungal AFP (128) and LF from human or bovine origin (166, 192).

To date, the structure and properties of many AMP have been studied, and a number of them have been analyzed for the effects of heterologous expression on plant resistance to bacterial and fungal phytopathogens. Transgenic plants expressing AMP found in nature have been shown to exhibit broad-spectrum disease resistance (5, 47, 67, 142). Likewise, rationally designed AMP also showed stable expression and antimicrobial activity in plant tissues (**Table 5**).

Different synthetic peptides provided resistance against *E. carotovora* in potato (130, 131, 132). In the case of the chimera MrsA1, absence of toxicity was inferred by feeding mice with transgenic potato tubers (132). Two significant examples of peptides that have been expressed in different plants and conferred protection against distinct microbial pathogens are D4E1 and MSI-99 (see **Table 5**). Also remarkable are indolicidin and polyphemusin variants that showed broad-spectrum enhanced resistance to different bacterial, fungal, and viral pathogens when expressed in tobacco (10, 11, 126).

One potential problem with transgenic expression of peptides is low stability due to small size and susceptibility to protease degradation. Some contradictory published data on the protective effect of peptide transgenes in plants may be attributable to different rates of peptide degradation by endogenous peptidases (133). Differences of stability are likely in relation to peptide structure and plant species. Therefore, strategies are needed to optimize production through stability of expressed peptides in transgenic plants. Rational design and molecular modeling of peptides are effective methods to increase peptide stability without compromising activity (see above) (130–132). Another alternative is directing the peptide to specific locations where protease activity is presumed to be low (31, 84, 161). An additional interesting approach is to mimic natural plant systems for the delivery of small-sized peptides, as engineered with the peptide sequence Pep11, which replaced systemin in the prosystemin polypeptide and was successfully expressed in tomato (70).

In vitro data does not have to be completely definitive in order to evaluate the potential utility of a given AMP *in planta*. The peptide MsrA3 had only a modest potency in vitro but gave positive results in transgenic potatoes that allowed prolonged tuber storage (130). By contrast, in the case of highly toxic (unspecific) peptides, the use of plant gene promoters that induce expression only under pathogen attack has produced positive results in different pathosystems (88, 108, 186–188). In fact, nonconstitutive production at specific times or plant tissues could

Table 5 Rational-designed antimicrobial peptides expressed in plants

Peptide	Host	Pathogen(s)	References
Shiva-1	Tobacco (*N. tabacum*)	*P. solanacearum*	(66)
	Potato (*S. tuberosum*)	*E. carotovora*	(188)
	Paulownia tomentosa	Phytoplasma	(39)
MB39	Tobacco (*N. tabacum*)	*P. syringae*	(63)
	Apple (*M. domestica*)	*E. amylovora*	(88)
SB-37	Potato (*S. tuberosum*)	*E. carotovora*	(6)
MsrA1	Potato (*S. tuberosum*)	*E. carotovora*, *P. cactorum*, *F. solani*	(132)
D4E1	Tobacco (*N. tabacum*)	*C. destructivum*	(23)
	Poplar (*Populus* sp.)	*A. tumefaciens*, *X. populi*	(106)
	Cotton (*G. hirsutum*)	*T. basicota*	(148)
Myp30	Tobacco (*N. tabacum*)	*E. carotovora*, *P. tabacina*	(84)
ESF12	Poplar (*Populus* sp.)	*S. musiva*	(85)
MSI-99	Tobacco (*N. tabacum*)	Bacteria, fungi	(28, 36)
	Grapevine (*V. vinifera*)	Bacteria, fungi	(177)
	Banana (*Musa* sp.)	*F. oxysporum*, *M. musicota*	(28)
	Tomato (*L. esculentum*)	*P. syringae*	(1)
MsrA3	Potato (*S. tuberosum*)	*E. carotovora*, *P. infestans*, *P. erythroseptica*	(130)
Pep11	Tomato (*L. esculentum*)	*P. infestans*	(70)
CEMA	Tobacco (*N. tabacum*)	*F. solani*	(187)
MsrA2	Potato (*S. tuberosum*)	*E. carotovora*, fungi	(131)
	Tobacco (*N. tabacum*)	Bacteria, fungi, oomycetes	(186)
ACHE-I-7.1	Potato (*S. tuberosum*)	*G. pallida*	(87)
Rev4	Tobacco (*N. tabacum*)	Bacteria, oomycetes	(184)
	Arabidopsis thaliana		
10R, 11R	Tobacco (*N. tabacum*)	*E. carotovora*, fungi, TMV	(11)
PV5	Tobacco (*N. tabacum*)	*E. carotovora*, fungi, TMV	(10)

minimize the risk for the emergence of new strains of microorganisms that are resistant to these candidate peptides.

Future Alternatives to the Production of AMP

To exploit the activity of AMP for the treatment of human and animal diseases, large amounts of peptides must be produced efficiently and at reasonable cost (57). To reduce the high production costs of synthetic AMP researchers have turned to recombinant expression in heterologous systems such as microorganism as cell factories. Genetically modified microorganisms can be engineered to produce and obtain suitable amounts of AMP (64), including peptides that have demonstrated activity against plant pathogens (77, 117). In the plant protection scenario, peptides obtained in this way could be part of formulates of phytosanitary products used in the field during plant cultivation or postharvest additives used on harvested commodities.

AMP-producing transgenic plants not only are protected against pathogen infection but also could be used as production platforms of antimicrobial proteins in molecular farming of crops. Data obtained from AMP transgenic expression for plant disease resistance can also be exploited for this purpose. Several factors responsible for functional peptide expression in transgenic plants have been tested including promoter sequences (186, 187), codon usage

(49, 132), fusion to signal peptide sequences (10, 11), and toxicity to the expressing plants (11). As a result, significant progress is being made in overcoming the problems involved during the heterologous production of AMP in plants, and yields in the range of 1 to 10 μg of peptide per gram of fresh leaf tissue have been achieved. Future options for improvement exist. Many plant antimicrobial proteins are produced and stored in seeds (171), which have obvious advantages as plant-based production systems (17). A significant example is that of the antimicrobial LF, which was produced in rice grains from which it can be extracted with significant purity and yield (124).

The incorporation of AMP into plants through genetic engineering offers a means to prevent disease-associated losses as well as to protect the environment. However, public resistance to the production of genetically modified plants is still an important factor. Selected AMP have been expressed in the chloroplast genome in order to confine the synthetic gene within the transgenic plant (36). Although the potential undesirable toxic effects of AMP, if any, need to be further investigated, it is emphasized that the small size of this class of compounds and the knowledge generated in the studies reviewed herein and in future contributions should help to modulate this and other peptide properties through peptide sequence modification. The successful application of AMP to plant protection will likely help eradicate certain plant diseases, reduce the environmental degradation of intensive agriculture, and improve the quality and safety of our food.

SUMMARY POINTS

1. Antimicrobial peptides are promising alternative strategies for use in plant disease control.
2. Peptide libraries and the rational design of peptide analogs are tools that can exploit peptide sequence diversity to identify novel and improved peptides specific against plant pathogens.
3. Transmembrane pore formation and cell permeation are not the sole mechanisms of microbial killing exerted by antimicrobial peptides. Interaction with cell envelopes and more subtle intracellular modes of action could be equally important in explaining peptide specificity and potency.
4. Antimicrobial peptides have been demonstrated to confer disease protection when produced by transgenic plants.

FUTURE ISSUES

1. Isolation, identification, and improved design by rational methods of novel AMP targeted to plant protection, with higher specificity toward plant pathogens, greater stability in plant microenvironments, and reduced nonspecific toxicity.
2. The increased use of suitable high-throughput technologies to boost the number and diversity of peptides that can be analyzed.
3. A shift from cell-based growth inhibition toward more specific screenings using molecular approaches to enhance the properties of new AMP.

4. Elucidation of the mode of action of selected AMP to facilitate the knowledge-based design of peptides.
5. Application of functional genomics tools such as array technology to monitor gene expression changes, the isolation of mutants, or screens of genome scale collections of gene deletions, to aid in the identification of the modes of action, cell targets, and microbe responses to AMP.
6. Identification in phytopathogenic microorganisms of biochemical/molecular targets that are related with their virulence or pathogenicity to plants and also amenable to screens of peptide collections.
7. Increased number and diversity of experiments to achieve the safe transgenic expression of AMP in plants.
8. Heterologous production of AMP using microorganisms as cell factories or in plants by molecular farming.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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RELATED RESOURCES

Antimicrobial Sequences Database (**http://www.bbcm.units.it/~tossi/amsdb.html**)
Antimicrobial Peptide Database (**http://aps.unmc.edu/AP/main.html**)
ANTIMIC (**http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/**).
AMPer (**http://www.cnbi2.com/cgi-bin/amp.pl**).

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Errata

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