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Functional Peptides for Plant Disease Control

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Abstract

Plant disease control requires novel approaches to mitigate the spread of and losses caused by current, emerging, and re-emerging diseases and to adapt plant protection to global climate change and the restrictions on the use of conventional pesticides. Currently, disease management relies mainly on biopesticides, which are required for the sustainable use of plant-protection products. Functional peptides are candidate biopesticides because they originate from living organisms or are synthetic analogs and provide novel mechanisms of action against plant pathogens. Hundreds of compounds exist that cover an extensive range of activities against viruses, bacteria and phytoplasmas, fungi and oomycetes, and nematodes. Natural sources, chemical synthesis, and biotechnological platforms may provide peptides at large scale for the industry and growers. The main challenges for their use in plant disease protection are (*a*) the requirement of stability in the plant environment and counteracting resistance in pathogen populations, (*b*) the need to develop suitable formulations to increase their shelf life and methods of application, (*c*) the selection of compounds with acceptable toxicological profiles, and (*d*) the high cost of production for agricultural purposes. In the near future, it is expected that several functional peptides will be commercially available for plant disease control, but more effort is needed to validate their efficacy at the field level and fulfill the requirements of the regulatory framework.

INTRODUCTION

Crop losses in agriculture due to pests and diseases, excluding weeds, have been estimated to be one-third of the potential productivity, despite the protection measures taken, and 13% of crop losses are due to plant diseases (119). Diseases and pest control are of extreme importance for food security, especially under the adaptation that agricultural practices must adopt because of climate change (28).

Several strategies used for plant disease control depend on the crop and particular agricultural and climate conditions as well as on the regulatory framework. They include exclusion, eradication, cultural and other management practices, host genetic resistance, and chemical and biological control (147). Of these methods of control, host genetic resistance appears to be the most durable strategy, but obtaining resistant varieties via conventional approaches requires time, and the faster genetic modification technologies may not be well accepted by authorities and consumers.

Therefore, chemical and biological control are still the main pillars of crop protection. However, several countries have reduced the number and type of pesticides authorized because of the nontarget environmental effects of many pesticides and the need to produce safe food. For example, in recent years, the European Union has required a strong reduction in active substances for pesticides, and similar actions have been taken by governments around the world. This reduction in the use of conventional pesticides has not been compensated by sufficient novel compounds or biopesticides or by efficient cultural and management practices, and several diseases may now be insufficiently controlled or noncontrolled. The situation is more difficult in the case of bacterial diseases than fungal diseases because there have always been fewer bactericides than fungicides (155). In addition, emerging and re-emerging bacterial diseases of economic importance, e.g., fire blight of apple and pear (*Erwinia amylovora*), bacterial leaf blight of rice (*Xanthomonas oryzae* pv. *oryzae*), bacterial wilt of tomato and potato (*Ralstonia solanacearum*), Xanthomonas wilt of banana (*Xanthomonas vasicola* pv. *musacearum*), bacterial canker of kiwifruit (*Pseudomonas syringae* pv. *actinidiae*), and bacterial blight of cassava (*Xanthomonas axonopodis* pv. *manihotis*), have been established in several areas without effective means of control (147, 155). Particularly, the diseases caused by endophytic or systemic pathogens like fastidious bacteria (e.g., *Xylella fastidosa* and *Candidatus* Liberibacter), phytoplasms, and viruses remain difficult, if not impossible, to control with the measures and tools currently available (138, 164). In addition, the spread of resistance within plant-pathogen populations is particularly important in bacteria and fungi, especially resistance against pesticides with single-point mechanisms of action (99), and in pathogens with a high genetic variability and ecological fitness (94).

In spite of the research efforts to find and develop new plant-protection products, there has been little interest by pesticide companies in providing novel pesticides to growers because of the low return on the investment (market value) and the difficulties of obtaining registration approval due to strong requirements by the regulatory framework. Also, many novel approaches for disease control are still in development (e.g., RNAi, defense elicitors), have safety concerns (e.g., novel formulations in nanoparticles), or have not been sufficiently evaluated and validated at the field level.

Functional peptides have been the object of strong research efforts in the field of crop protection, as in the veterinary, medical, and food industry areas (93, 106, 129). Peptides are considered polypeptides of up to 50–60 amino acids (upper size limit considered as big peptides or small proteins) but also include pseudopeptides with peptide bonds and non-natural or modified amino acids. Most peptides originate in living organisms, with a mode of action of antagonism or antibiosis in microorganisms, and are responsible for the first immune defense barrier and important in stress mitigation in animals and plants (61).

The present review summarizes and describes the origin, structure, and mechanisms of action of functional peptides, the processes to search for and develop new compounds and discover their mechanisms of action, the current knowledge on peptides targeted to plant pathogens and the diseases they cause on crops, the production platforms for exploitation, and the challenges and prospects to be novel biopesticides.

PEPTIDES FROM LIVING ORGANISMS

Functional peptides are produced by microorganisms, plants, and animals and are classified according to several criteria that include their origin, structure, and amino acid composition or activity (61). The most frequent classification is based on the structure they can adopt in vivo like α-helix, β-sheet, β-hairpins, or looped configuration and linear peptides with unusual bias (129).

Peptides adopting an α-helical structure are commonly found in animals and are generally potent antimicrobials adopting helical cationic amphipathic structure (54). Several peptides have β-hairpins or a looped configuration, e.g., some bacteriocin lantibiotics, or are linear peptides rich in amino acids, e.g., Pro, Gly, His, or Trp. Some peptides have β-sheet structure and contain disulfide bonds between cysteines or are cyclic and are common in plants (86). Most of these peptides are synthesized by ribosomal protein synthesis; selected examples are shown in **Figure 1**.

Another group of peptides contains modifications in the amino acid sequence or nonproteinogenous amino acids and is produced by nonribosomal peptide synthases (NRPSs), such as peptaibols, cyclo-lipopeptides, and cyclo-depsipeptides, and pseudopeptides (**Figure 2**). Peptaibols are antimicrobial peptides of a short chain length that include unusual amino acids (α-amino isobutyric acid, isovaline, ethylnorvaline, or hydroxyproline), are acetylated at the N-terminus, and contain a C-terminal amino alcohol (60). Cyclic lipopeptides (CLPs) are amphiphilic compounds composed of a fatty acid tail linked to a short oligopeptide that form a macrocyclic ring structure (152), and cyclodepsipeptides (CLDPs) have a lactone ring (127). Pseudopeptides have peptide bonds but complex chemical modifications in their structure (171).

Peptides in Microorganisms

Microorganisms synthesize bacteriocins, CLPs, peptaibols, and pseudopeptides. Bacteriocins are a heterogeneous group of ribosomal synthesized peptides produced by bacteria that have activity against other related and unrelated bacteria (1). Examples of bacteriocins produced by Gram-positive bacteria against plant pathogens are Bac-GM17 from *Bacillus clausii* GM17, ericin S from *Bacillus subtilis* A1/3, and amylocyclicin from *Bacillus amyloliquefaciens* FZB42 (26, 111, 142, 153). In Gram-negative bacteria, bacteriocins effective against plant-pathogenic bacteria are generally small antimicrobial proteins and are not considered here.

CLPs and CLDPs are predominantly produced by *Bacillus*, *Paenibacillus*, and *Pseudomonas* (127, 152). *Bacillus* species synthesize a wide range of CLPs grouped into iturins, fengycins, and surfactins. The iturins and surfactins are cyclic heptapeptides (e.g., iturin A, iturin C, iturin D, iturin E, bacillomycin D, bacillomycin F, bacillomycin Lc, mojaversin A, mycosubtilin, and mixirin), and fengycins and plipastatins are cyclic decapeptides. Bacillomycin, fengycin, iturin, and surfactin are typically produced by *Bacillus* strains of the *subtilis*, *amyloliquefaciens*, or *velezensis* species (26, 110). *Pseudomonas* spp. produce CLPs grouped into viscosins, syringomycins, syringopeptins, amphisins, putisolvins, and tolaasins (48). For example, tensin is produced in *Pseudomonas fluorescens* 96.578 (117), poaeamide in *Pseudomonas poae* RE1-1-14 (169), massetolide that belongs to the viscosin family (159), orfamide A synthesized by *Pseudomonas protegens* CHA0 (91), or nunamycin and nunapeptin in *Pseudomonas* ln5 (97).

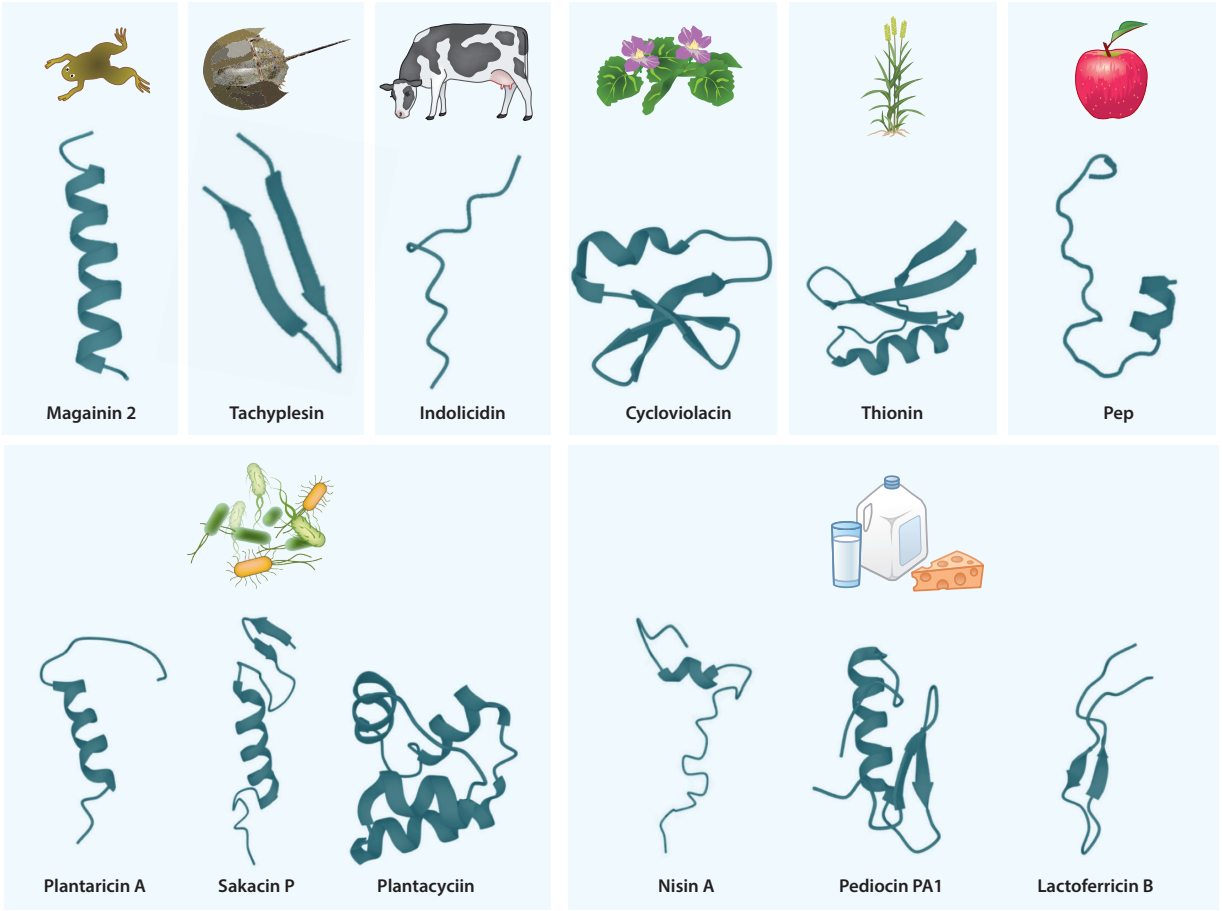

Figure 1

3D structure of selected peptides from natural sources. Magainin 2 (*Xenopus laevis*), tachyplesin (horseshoe crab; *Limulus polyphemus*), indolicidin (cow's blood neutrophils), cycloviolacin (*Viola odorata*), thionin (*Triticum turgidum*), Pep (*Malus domestica*), plantaricin A (*Lactobacillus plantarum*), sakacin P (*Lactobacillus sake*), plantacyclin (*Lactiplantobacillus plantarum*), nisin A (*Lactococcus lactis*), pediocin PA1 (*Pediococcus acidilactici*), and lactoferricin (cow's lactoferrin hydrolysate). The structures were made with the amino acid sequences of the peptides using PEPFOLD3, UNIPROT, APD3, and RCSB-PDB servers and databases. S-bonds are not indicated for the plant defensins and lantibiotic bacteriocins.

Peptaibols are a large family of compounds produced by fungi, e.g., the genera *Trichoderma*, *Emericellopsis*, and *Gliocladium*, of which some examples with activity against plant pathogens are trichokonins, trichorzianines, and peptaivirins (35, 68). Several fungi produce cyclic dipeptides or diketopiperazines, such as chaetomin from *Chaetomium globosum* (39), defensin-like peptides PAF or PgAFP from *Penicillium chrysogenum*, and AcAFP and AcAMP from *Aspergillus* (56).

Pseudopeptides are common among actinomycetes, such as the peptidyl nucleoside antibiotics polyoxins and nikkomycins produced by *Streptomyces cacaoi* and *Streptomyces tendae* (122, 171), the neopeptins from *Streptomyces* sp. KNF2047 (70), the cyclic thiopeptide cyclothiazomycin from *Streptomyces* sp. HA 125–40, cyclo (L-leucyl-L-prolyl) from *Streptomyces* sp. KH-614 (131), coronamycin from *Streptomyces* sp. MSU-2110, glomecidin from *Streptomyces lavendulae* H698 SY2 (79), and kutznerides from *Kutzneria* sp. 744 (17).

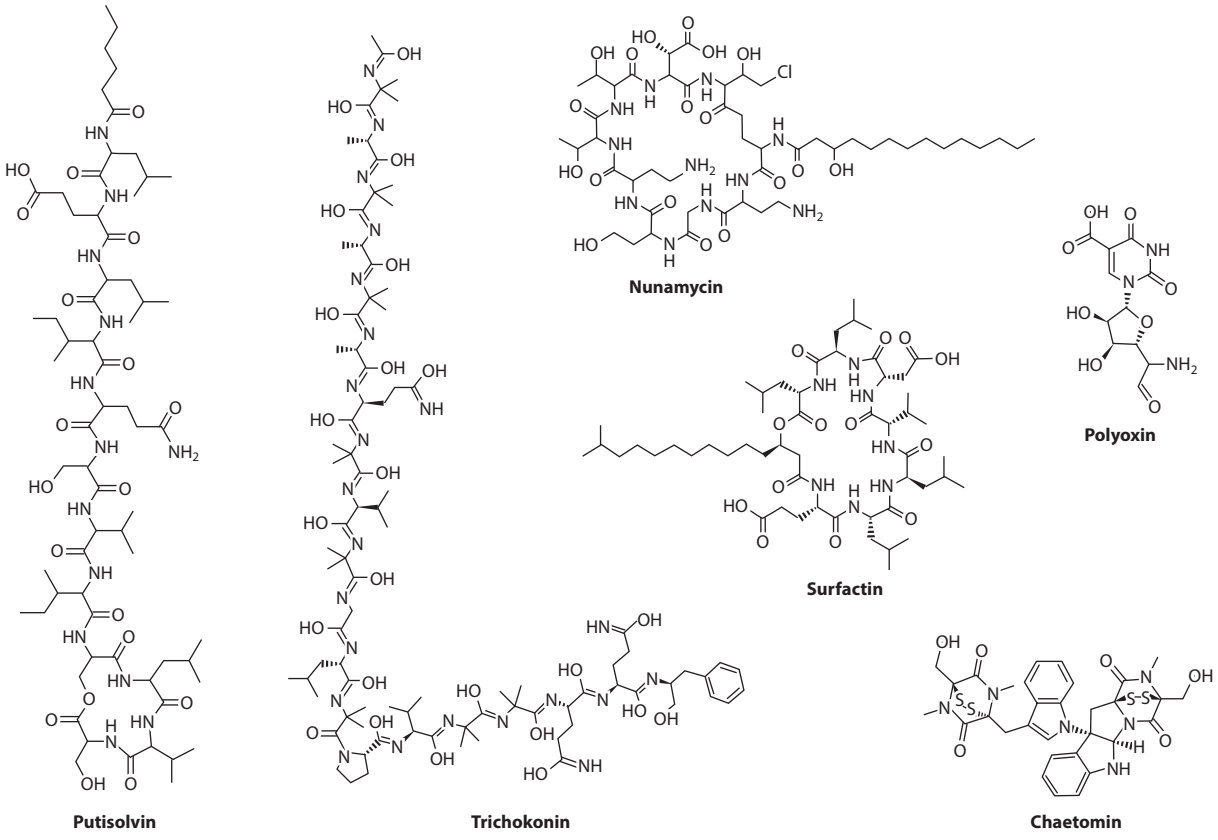

Figure 2

Chemical structures of peptide secondary metabolites from microorganisms. Putisolvin (*Pseudomonas putida*), trichokonin (*Trichoderma pseudokoningii*), nunamycin (*Pseudomonas fluorescens* ln5), surfactin (*Bacillus amyloliquefaciens*), polyoxin (*Streptomyces cacaoi*), and chaetomin (*Chaetomium cochliodes*).

Peptides in Animals

Peptides in animals have an essential role in defense against pathogens due to their antimicrobial activity; in some cases, they modulate the immunological response or the symbiotic interactions with microorganisms (54, 92). Peptides are present in poriphers, cnidarians and mollusks (discodermin A, defensins, mytilins, and myticins), arthropods (cecropins, mellitin, thanatin, and apidaecins), fishes (cathelicidins, β-defensins, hepcidins, and piscidins), amphibians (bombinins, maximins, cathelicidins, esculentins, dermaseptins, phylloseptins, palustrin, magainin, pseudins, and ranaturerins), birds and reptiles (fowlicidins and defensins), and mammals (cathelicidins, indolicidin, protegrins, bactenecins, and diverse defensins, dermicidin, hepcidin, and hepticin) (3).

The most relevant antimicrobial peptides of animal origin are the cecropins, melittins, magainins, defensins, and cathelicidins. Cecropins are antimicrobial peptides (AMPs) first identified in the hemolymph of the giant silk moth as cecropin A (84) or in the nematode *Ascaris suum*, a parasite of the pig intestine, as cecropin P1 (18). Melittin from bee venom (167), magainins from amphibians such as the African clawed frog skin (95), and β-defensins and cathelicidins from diverse animals are among the most studied (76).

Several antimicrobial peptides of animal origin have been tested against plant-pathogenic bacteria and fungi, originating in insects (cecropins A and B, apidaecin IA, drosocin, melittin pyrrhocoricin, gomesin, metchnikowin, sarcotoxin), crustaceans (tachyplesin I, penaeidin), amphibians (magainin, dermaseptin, hylin), sheep (SMAP-29), cow (indolicidin, lactoferricin, tripticin), and human (histatin-5, cathelicidin, lactoferricin) (6, 33, 46, 47, 67, 71, 74, 154, 172).

Peptides in Plants

Plants have developed complex mechanisms to defend themselves by perceiving molecular patterns associated with pathogens [PAMPs (pathogen-associated molecular patterns); e.g., bacterial flagella] and nonpathogenic microorganisms [MAMPs (microbe-associated molecular patterns)] or with damage in their injured cells [DAMPs (damage-associated molecular patterns); e.g., breakdown products of plant extracellular matrix] (24, 52) via effector-triggered immunity (ETI) [e.g., cell death, antimicrobial pathogenesis-related (PR) proteins, and reactive oxygen species (ROS)] (34, 86). During the ETI response plants produce antimicrobial peptides or small antimicrobial proteins like thionins, defensins, heveins, knottins, cyclotides, lipid transfer proteins, snakins, and hairpinins (86). Several examples of these antimicrobials have been reported as active against plant pathogens like the defensins from petunia, the peptide MaSAMP (*Microcitrus australasica* stable antimicrobial peptide) from citrus, thionins from cereals, Rs-AFP1 and Rs-AFP2 from raphanus, LTP and snakins, hevein or γ-thionin-SE60, and *Mirabilis jalapa* defensin Mj-AMP1 (16, 31, 41, 49, 62, 66, 75, 81, 102, 141). Plants also produce short endogenous elicitor peptides (PROPEPs and Peps) in response to pathogen attacks that have been identified in Brassicaceae, Poaceae, Rosaceae, Fabaceae, and Solanaceae (15, 90, 134).

MECHANISMS OF ACTION

There are several mechanisms that have been reported in functional peptides that affect plant pathogens (**Figure 3**). The mechanisms in antiviral and nematicidal peptides are less known than those in antibacterial or antifungal peptides and are not described in detail in this review.

Among antimicrobial peptides, lytic peptides cause cell membrane damage that results in its lysis or inactivation. Most lytic peptides are amphipathic cationic and interact with the negatively charged phospholipid layer and lipopolysaccharides (LPSs) of the bacterial membrane, mainly in Gram-negative bacteria, via postulated toroidal, barrel-shape or carpet-like mechanisms, causing pores and membrane destabilization (13, 61). Lysis is not only restricted to peptides with α-helix structure, but it is also reported in β-sheet (e.g., BPC194) or linear (e.g., indolicidin) peptides (98, 105).

A group of antimicrobial peptides affect internal cell processes (82) because they can penetrate the target cell, in some cases breaking the membrane and in others not, and interfere with protein or nucleic acid synthesis or cell division or inhibit proteinases. This is the case in magainins, which affect DNA synthesis and metabolic processes in bacteria and fungi (95); cathelicidins, which inhibit replication, translation, and ion channels; and the antifungal PAF26 (113). However, some cell-penetrating peptides (CPPs) do not disrupt cell membranes or affect intracellular processes and have been used to deliver cargo molecules to the cytoplasm of eukaryotic cells (e.g., plant, fungus, human), e.g., in the case of BP100 to BY2 tobacco cells (40) and BP16 to human tumor cells (150), or improve the uptake of RNAi in plant cell transformation (118). Other CPP, such as peptide nucleic acid (PNA) conjugates, penetrate plant pathogen cells and target selected genes as, for example, a 10-nucleotide oligomer that targets the regulator gene *acpP* involved in the fatty acid biosynthesis in *E. amylovora* (124).

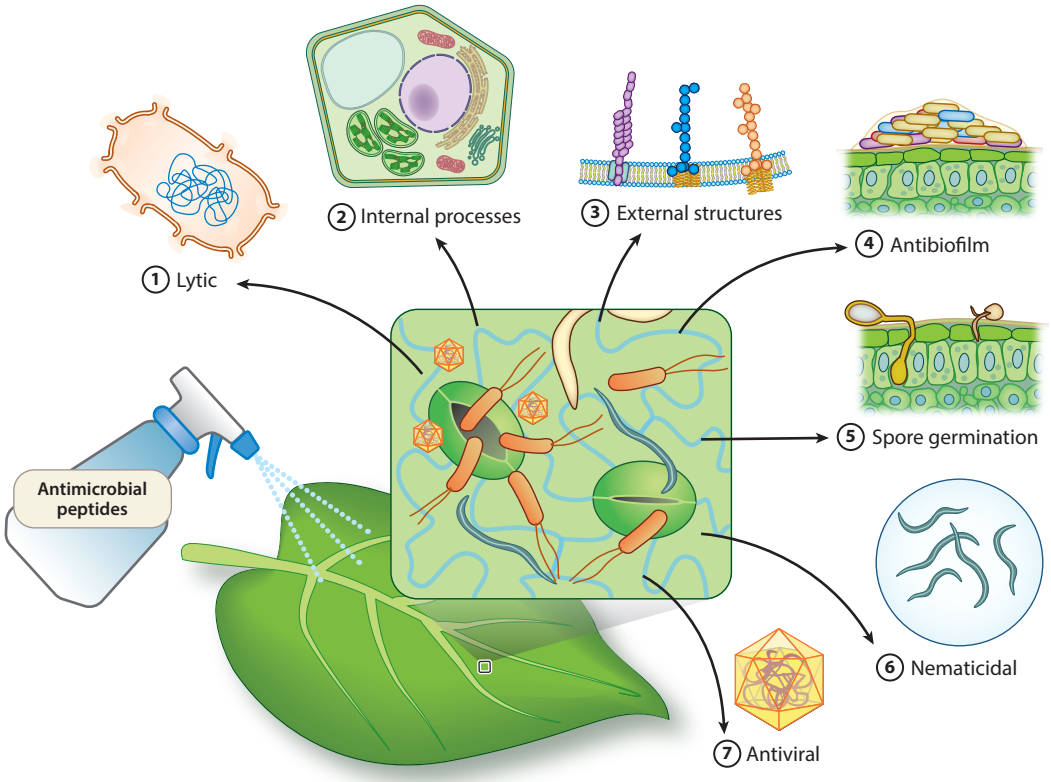

Figure 3

Mechanisms of action of antimicrobial peptides. ① Produce pores and disorganize cell membranes, which causes cell lysis. ② Interfere with internal cellular processes like in cell-penetrating peptides or peptide–nucleic acid conjugates. ③ Interact with external cell structures such as lipopolysaccharides, fimbriae, or flagella in bacteria or chitin in fungi. ④ Inhibit biofilm formation and other colonization structures in bacteria. ⑤ Inhibit the spore germination or germ tube elongation in fungi. ⑥ Modify the behavior or the external coat of plant-pathogenic nematodes. ⑦ Inhibit virus attachment or replication.

There are peptides that interact with external cell structures. For example, lipid transfer proteins bind fungal membrane lipids to inhibit pathogen penetration into the host cell, heveins bind chitin in fungi (53), and some defensins interact with eukaryotic receptor proteins to target intracellular functions (75). Antibiofilm peptides affect biofilm formation on plant surfaces or in the vascular system, a key process in the pathogenesis of bacteria (103). Some peptides inhibit spore germination in fungi (e.g., peptide BP15) (7, 125).

Another mechanism of action is priming the plant by inducing defense responses against pathogens (**Figure 4**). This activity is generally due to the modulation of different cascades in their immune system via interaction with cell membrane receptors [PEPs/PROPEPs, leucine-rich repeat (LRR)-receptors] and triggering a cellular/tissue response (168). Apoplastic mature secondary DAMPs are perceived by specific pattern recognition receptors, typically LRR receptor-like kinases (LRR-RLKs), and upon binding, they further interact with LRR-RLK coreceptors (e.g., Bak1) to initiate downstream signaling. The responses of plants to treatment with elicitor peptides include phosphorylation events, Ca^{2+}-dependent signaling, activation of mitogen-activated protein kinases (MAPKs), production of nitric oxide and ROS, coactivation of salicylic acid (SA),

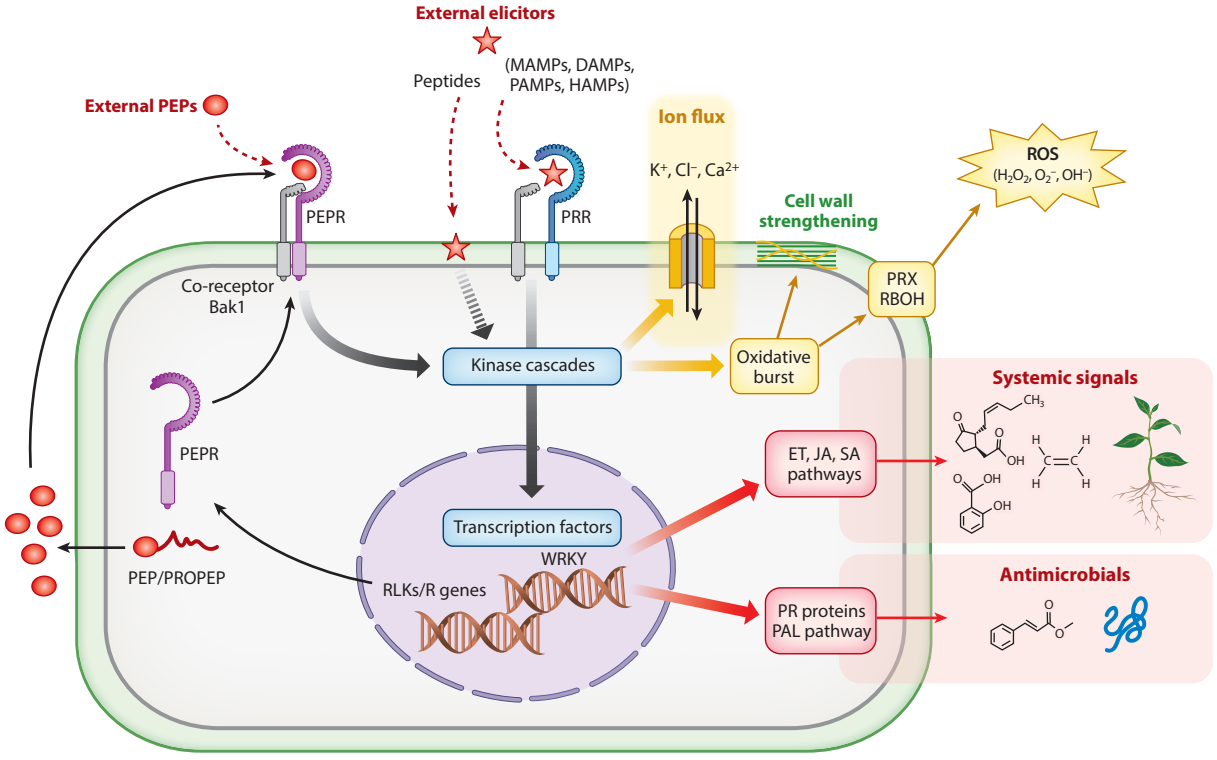

Figure 4

Mechanisms of action of plant defense elicitor peptides. Several peptides induce plant cell responses through an effector-triggered immunity (ETI). Endogenous elicitor peptides interact with endogenous elicitor peptide receptors (PEPR), and other peptides can interact with pattern recognition receptors (PRRs), as is done by microbe-associated molecular patterns (MAMPs), damage-associated molecular patterns (DAMPS), pathogen-associated molecular patterns (PAMPs), and herbivore-associated molecular patterns (HAMPs), or through unknown mechanisms of interaction (peptides). The PEP/PROPEP cycle generates the receptors and effectors that act in a self-induction process. The signaling processes activate kinase cascades and transcription factors, leading to the overexpression of genes involved in the ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) pathways (systemic signals), the phenyl-alanine ammonium lyase (PAL) pathway (phenylpropanoids), or the synthesis of pathogenesis-related (PR) proteins (antimicrobials). During activation, ROS reactions, cell wall reinforcement, and changes in ion flux are also produced. Abbreviations: PRX, peroxiredoxins; RBOH, respiratory burst oxidase homolog; RLKs, receptor-like kinases; ROS, reactive oxygen species; WRKY, W-box transcription factors.

jasmonic acid (JA), and ethylene (ET) hormone pathways, and production of antimicrobial PR proteins and phenylpropanoids (52, 59).

Plant-elicitor peptide receptor (Pep-PEPR) complexes and the plant response to Peps have been thoroughly analyzed in *Arabidopsis* and *Prunus*, and PROPEPs and Peps have been identified in most angiosperm plant families, including Brassicaceae, Poaceae, Rosaceae, Fabaceae, and Solanaceae (15, 45, 132, 135). In addition to Peps, several synthetic or microbial-derived peptides induce plant defenses, thus acting as MAMPS or PAMPs, such as linear lipopeptides derived from BP100 (120), the bifunctional peptide BP178 and flagellins 15 and 20 (104, 109), peptaibols (163), ultrashort cationic lipopeptides (19), hexapeptide PIP1 (101), and the MaSAMP peptide derived from *Citrus* (62). Interestingly, peptide BP178 when applied to tomato by spray or in almond by endotherapy and spray induced the overexpression of nearly 100 genes, some involved in the SA, JA, and ET pathways or in the synthesis of PR defense proteins (104, 109). However, except for the plant endogenous peptides that use specific receptors (45, 63), in other elicitor peptides, the

mechanism of signal transduction and the existence of receptors that induce the plant response are not known.

SEARCHING FOR AND DEVELOPING PEPTIDES FOR PLANT DISEASE CONTROL

Living organisms offer great possibilities to discover peptides, such as native compounds obtained by further hydrolysis from functional proteins or lactoferricin B obtained by acidic-pepsin hydrolysis of the bovine milk lactoferrin (157). The knowledge of the chemical structure and physico-chemical and biological properties of natural peptides provides the basis to develop analogs or newly designed compounds that can be chemically synthesized to build peptide libraries. The approaches to obtaining analogs include chemical modification (e.g., halogenation, cyclization, capping, conjugation) of existing compounds and the use of particular motifs (at the end, in chain, or repeated seqs) such as the ATCUN, Rana box, and LPS binding gamma-core motifs (112, 156).

The de novo design of peptides may include tandem repeated sequences, cyclization, or addition of particular end sequences or amino acids (e.g., D-amino acids). An extensive variety of de novo designed cyclic peptides have been developed for plant disease control (83), such as the cyclic peptide BPC194, a member of a cyclic decapeptide library, which exhibited a potent antibacterial activity (105). A further library of analogs of BPC194 was developed by combinatorial chemistry with several bactericidal members (32), and recently another library was obtained from BPC194 to develop CLPs that were selective against fungal, bacterial, animal, or plant cells (162). Another example of de novo designed peptides is the bactericidal antisense PNA-CPP, which was targeted to a regulatory gene and was effective against infections by *E. amylovora* in vitro and in flowers (124).

After this stage of building peptide libraries, the compounds are submitted to an in vitro screening platform that tests antimicrobial activity (growth inhibition, killing assays), susceptibility to protease hydrolysis, and stability under extreme physico-chemical conditions and preliminary toxicity (hemolytic activity, phytotoxicity). Inhibition assays that target plant-pathogenic bacteria or fungi can be done using microbial growth analyzers or by studying fungicidal or bactericidal properties using viability methods (e.g., SYTOX green, resazurin, v-qPCR) (12, 23). Many plant pathogens, as well as bacterial epiphytes and endophytes, form biofilms as part of their mechanism of pathogenesis. Thus, inhibition of biofilm formation is an objective in the screening of functional peptides (103). To study the effect of peptides on cell behavior, microfluidic chambers can be used independently of the type of motility of the target cell (e.g., flagellar, twitching) (36). Some assays are addressed to detect peptides that interact with the LPSs using an LPS binding assay or a chitin-binding assay for antifungal peptides (53). Also, stability in front of protease hydrolysis or high temperature is important for the performance of the peptides (11, 62).

Toxicity is also an important property that is used during in vitro screening to retain or discard compounds for further development. Cytotoxicity is currently studied using the hemolysis assay, but other methods are better at assessing the phytotoxicity of peptides developed for plant protection, such as pollen germination, tobacco leaf infiltration, and seed germination assays (162). In these assays, melittin, which is a highly hemolytic and toxic peptide, is used as a reference peptide (37).

Because some peptides can have defense elicitor properties in plants, the screening platform can use in vitro approaches like tobacco BY2 cell cultures to measure pH or electrolyte leakage as a plant cell response to peptide treatment (10, 19). The members that have been selected from the peptide library with high activity and stability and minimal toxicity are then submitted for structural analysis. Further studies involve monitoring the peptide within the cell structures of the target pathogen, generally using a reporter conjugated to a peptide, and the complex is viewed

by confocal microscopy (113, 125). The selection of the reporter is critical because it can modify the properties of the native peptide (e.g., DSRed or GFP reporters are not adequate).

In planta assays are performed to identify the peptides with plant defense elicitor activity. They consist of the topical application of selected compounds to a model plant (e.g., tobacco, *Arabidopsis*, tomato) and the study of the expression of a selection of genes involved in defense (e.g., phenylalanine ammonium lyase, PR proteins, lipoxygenase) or stress response (e.g., *trans*-acting siRNA genes), using reverse transcription quantitative PCR or, more exhaustively, cDNA microarrays or RNAseq (104, 109).

The last stages in searching for and developing peptides for plant disease control consist of proofs-of-concept that are performed using plant hosts to control infections by a selected range of plant pathogens, either under controlled environment conditions or in the greenhouse, lower toxicity in mammals, lessen environmental impact, and shape the development of suitable production and formulation platforms (discussed below). Further studies include field assays with batches of good manufacturing practice products.

Multifunctional Peptides

Peptides that have simultaneous mechanisms of action are interesting in plant protection because they counteract possible resistance in the pathogen and improve its activity. There are several examples of multifunctional peptides developed in different ways, e.g., by engineering from sequences of other peptides or searching in the genome/transcriptome/proteome of a disease-resistant plant.

Peptide BP178 (KKLFKKILKYLAGPAGIGKFLHSAKKDEL) was engineered for expression in plants and consists of a BP100 moiety (1–11), an AGPA hinge (12–15), a magainin sequence (16–25) (GIGKFLHSAK), and a terminal end KDEL sequence (26–29) for secretion through the endoplasmic reticulum of plant cells (9). The predicted structure of the peptide is two amphipathic α-helices connected by a hinge. The BP100 undecapeptide (KKLFKKILKYL) originated from a combinatorial library of 125 members obtained from the BP76 peptide (KKLFKKILKFL), which is an analog of the Pep3 peptide (WKLFKKILKVL), a cecropin A melittin hybrid peptide (27). BP100 has a potent bactericidal activity but does not induce plant defense responses (109), whereas peptide BP178 retained the bactericidal activity of BP100 but induces defenses in tomato and almond, and probably other plants, and can be produced heterologously in plants (104, 109).

Another example of a multifunctional peptide is the engineered peptide DS01-THA, which is a chimera of dermaseptin and thanatin that sticks to the wax layer of soybean, barley, and corn, inhibits spore germination, and controls infections by *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust (144).

A third example is the peptide MaSAMP, which was identified from the huanglongbing (HLB)-tolerant *Microcitrus australasica* among several natural defense genes potentially responsible for HLB tolerance (62). One of the candidate gene product regulators was the antimicrobial peptide MaSAMP, found in the phloem of the plant. The predicted structure of the peptide is, similar to BP178, two amphipathic α-helices connected by a hinge, where the helix-2 domain is the bactericidal motif. MaSAMP is bactericidal and induces defenses in the *Citrus* host and inhibits infections by *Ca.* Liberibacter asiaticus.

PEPTIDES TARGETING PLANT PATHOGENS FOR DISEASE CONTROL

Owing to the interest in developing new compounds for plant protection, an extensive number of peptides have been discovered or developed to work against plant viruses, bacteria, fungi, and nematodes, covering a wide range of mechanisms of action. In spite of the importance of

diseases caused by viruses and nematodes, few reports involve research on peptides targeted at plant-pathogenic viruses and nematodes and are not considered in the present review. In addition, most studies with plant-pathogenic prokaryotes, fungi, and oomycetes deal with activity in vitro and less frequently involve infectivity assays on plant pathosystems. Many of these studies consist of the heterologous expression of the peptide for self-protection in plants (65).

Peptides Targeting Plant-Pathogenic Prokaryotes

Selected examples of peptides that inhibit bacteria and phytoplasms are presented in **Table 1**. Many antimicrobial peptides mediate the mechanism of action of microbial biocontrol agents, and some compounds have been tested in vitro or in planta. *Bacillus* spp. bacteriocins such as amylocyclicin (142) and ericin S (153) have been reported with activity in vitro. CLPs such as iturin and surfactin also have antibacterial activity (139). Several peptides, e.g., those from crustaceans (tachyplesin), sheep (SMAP-29), human (histatin-5, LL37), cow (indolicidin), insects (cecropins A and B, apidaecin, drosocin, pyrrhocoricin), frog (magainin and melittin) (154), cow lactoferricin (46), or the plant-derived snakins (16), also inhibit plant-pathogenic bacteria. Among the synthetic peptides, the cecropin analogs BP100 and BP178 (8, 9, 109) and the de novo designed cyclo-decapeptide BPC194 from the CYCLO10 library (105) have also been reported to show activity against plant-pathogenic bacteria. Interestingly, a group of synthetic peptides inhibits biofilm formation (103).

Table 1 Selection of functional peptides active against plant-pathogenic prokaryotes or associated diseases

Peptide name	Origin	Target	Assay	Activity	Reference(s)
Amylocyclicin	*Bacillus* FZB42	Cm	IV	AB	142
BP100	Synthetic	Ps, Ea, Xv, CaP	IV, SP	LY	10, 133
BP178	Synthetic	Psto, Xv, Dd, Xf	IV, SP, ET, HE rice	DE/LY	9, 13, 104, 107
BPC194	Synthetic	Ps, Ea, Xv	IV	LY	105
BP528	Synthetic	Xf	IV	ABF	103
Cecropin B	Moth	Xf	HE grapevine scion	LY	33
D2A21	Synthetic	Psta, Xc, CaLa	HE tobacco/citrus	LY	55
Dermaseptin	*Phyllomedusa* sp.	Xc	HE sweet orange	LY	47
γ-Thionin	Soybean	Ps	HE soybean	AM	31
Gomesin	*Acanthoscurria* sp.	Xf	Tobacco	LY	44
Hylin-A1	Synthetic	Xc	Sweet orange	AM	71
Lactoferricin	Cow milk lactoferrin	Ps, Xc, Rs, Psta	IV, HE tobacco	LY	46
Melittin	Bee venom	Xf	HE Pa-Hv grapevine	LY	6
MaSAMP	*Microcitrus australasica*	CaLa	ET sweet orange	LY/DE	62
PpPep1/2	*Prunus persica*	Xap	SP peach	DE	135
AtPep1/2	*Arabidopsis thaliana*	Ps	SP/HE tomato	DE	63
PIP1/2	*A. thaliana*	Psto	SP	DE	59
Sarcotoxin	Fly	Xc	HE sweet orange	LY	74
Tachyplesin	Synthetic	At, Xc	IV	LY	154
Snakin	Solanaceae/soybean	Dd, Rs, Cm	HE potato/tomato	AM	16, 102

Abbreviations: AB, antibacterial; ABF, antibiofilm; AM, antimicrobial; At, *Agrobacterium tumefaciens*; CaLa, *Candidatus* Liberibacter asiaticus; CaP, *Candidatus* Phytoplasma; Cm, *Clavibacter michiganensis*; Dd, *Dickeia dadantii*; DE, defense elicitor; Ea, *Erwinia amylovora*; ET, endotherapy; HE, heterologous expression; IV, in vitro; LY, lytic; MaSAMP, *Microcitrus australasica* stable antimicrobial peptide; Pa-Hv, *Pantoea agglomerans–Homalodisca vitripennis*; Ps, *Pseudomonas syringae*; Psta, *P. syringae* pv. *tabaci*; Psto, *P. syringae* pv. *tomato*; Rs, *Ralstonia solanacearum*; SP, spray; Xap, *Xanthomonas arboricola* pv. *pruni*; Xc, *Xanthomonas citri*; Xv, *Xanthomonas vesicatoria*; Xf, *Xylella fastidiosa*.

Plant assays are more limited, and some peptides, such as gomesin in tobacco (44), hylin-a1 and tripticin in *Citrus* (71), MaSAMP for Citrus HLB control on potted plants (62), PpPep1 and PpPep2 endogenous peptides in peach plants (135), and AtPep, AtPROPEP1, AtPROPEP2 (63), and PIP1 and PIP2 (59) in *Arabidopsis*, have been topically applied. Synthetic peptides such as BP100 are effective against infection by Stolbur phytoplasm in periwinkle (133) and by several plant-pathogenic bacteria on pear, pepper, and tomato, and peptide BP178 is effective in controlling infections by *Xylella fastidiosa* on almond and by other bacteria in tomato under greenhouse conditions (10, 104, 109). Interestingly, transient expression of BP178 in *Nicotiana benthamiana* using a PVX virus construct was effective against infection by *X. fastidiosa* (14).

Many transgenic plants have been modified to express AMPs of plant origin against plant-pathogenic bacteria (24, 81), as in tobacco (31) and potato (102). Also, expression of peptides from animals was effective in controlling bacterial infections in tobacco (46), sweet orange (47, 74), and the scion of grapevines (33).

An innovative strategy, the paratransgenic approach, has been applied to bacterial diseases transmitted by insect vectors, e.g., the diseases caused by *X. fastidiosa*. The method consists of the genetic modification of the bacterium *Pantoea agglomerans*, which is an endogenous inhabitant of *Homalodisca vitripennis*, one of the disease vectors (6). The genetic modification consisted of the heterologous expression of scorpion and magainin peptides in the bacterium, which inhibited the acquisition of *X. fastidiosa* by the vector to prevent disease spread.

Peptides Targeting Plant-Pathogenic Fungi and Oomycetes

A selection of antifungal peptides that have been reported to be active against plant-pathogenic fungi and oomycetes is presented in **Table 2**. Purified or enriched cell-free extracts containing peptides from bacteria or fungi have been studied. Strong antifungal activity was shown by surfactin A (139), peptidic antibiotics like tensin (117), poaeamide (169), nunamycin and nunapeptin (97), neopeptins (70), cyclic dipeptides or diketopiperazines such as chaetomin (39), the cysteine-rich defensin-like peptides antifungal protein (AFP) (161), PAF or PgAFP, ANAFP, NAF, AcAFP, AcAMP, and NFAP (56), peptaibols such as trichokonins and trichorzianines (68), and polyoxins (122).

Several CLPs produced by *Bacillus* spp., such as fengycins and surfactins, which induce significant protection in bean and tomato leaves, trigger defense responses in plants against infection by plant-pathogenic fungi (121). Some CLDPs produced by pseudomonads, such as massetolide of the viscosin family (159) and orfamide A, which trigger induced systemic resistance in rice, also induce defense-related responses in plants (91). Peptaibols like trichokonin VI induce resistance in tobacco and Chinese cabbage (98) as well as apoptotic programmed cell death (149).

Many plant-derived peptides have strong activity against a wide range of plant-pathogenic fungi, such as Rs-AFP1 and Rs-AFP2 that showed protection of *Arabidopsis* plants against several fungal pathogens and the defensins protecting soybean, banana, tomato, and potato from fungal infections (16, 49, 81).

Heterologous expression in plant hosts is also a strategy frequently reported to provide protection from fungal pathogens (4, 24, 81), such as those found in rice and wheat (66), tobacco (75), and other Solanaceae (141) as well as aflatoxinogenic fungi in maize (143). The most frequent strategy used with peptides from animals has been heterologous expression in transgenic plants, and a series of studies reported that this method provided some protection from fungal infections in Pearl millet and tomato (130), barley (128), grass (172), and tobacco (46).

Although many approaches have demonstrated the potential of peptides against fungal pathogens, there are only a few studies done under field or postharvest conditions. The most

Table 2 Selection of functional peptides and small proteins active against plant-pathogenic fungi and oomycetes, and associated diseases

Peptide name	Origin	Target	Assay	Activity	Reference(s)
AFP	*Aspergillus giganteus*	Mg, Fm, Pi	IV	AF	161
BnPRP1	*Brassica napus*	Ss, Bc, Mo	IV	AM	25
BP15	Synthetic	Pe, Sv	SP, postharvest, field	AF	7, 126
Chaetomin	*Chaetomium globosum*	Pyt	IV	AM	39
Defensin	*Mirabilis jalapa*	As	SP tomato	AF	141
Depsipeptides	*Pseudomonas* spp.	Rs, Pyt	IV	AF	97
DS01-THA	Thanatin-dermaseptin	Ppa	SP soybean	LY	144
Glomecidin	*Streptomyces lavendulae*	Gc	IV	AF	79
Hevein	*Pharbitis nil*	Pp	SP tobacco	AF	75, 164
Neopeptins	*Streptomyces* KNF2047	Px	SP cucumber	AF	70
Orfamide (CLP)	*Pseudomonas protegens*	Mo, Rs	IV, SP	AF	91
Polyoxins	*Streptomyces cacaoi*	Bc, Mo	SP grapevine, rice	AF	122
PAF series	Synthetic	Pe	SP postharvest orange	AF	113
Rs-AFP1–2	*Raphanus sativus*	Fo, Ft, Mo, Rs	HE soybean/tobacco/banana	AF	49, 80
Snakin SN1	Wheat	Bg	HE potato/wheat	AF	41
Surfactin A	*Bacillus* NH100	Fo	IV	AF	138
Thanatin	*Podisus maculiventris*	Mo	HE rice	AF	64
Thionin	Barley	Cfi	SP sweet potato	AF	114
Trichokonin VI	*Trichoderma pseudokoningii*	Fo	SP tobacco, Chinese cabbage	AF	149
Trichorzianins	*Trichoderma harzianum*/*Trichoderma virens*	Ss, Rs	SP cucumber	AF	68

Abbreviations: AF, antifungal; AM, antimicrobial; As, *Alternaria solani*; Bc, *Botrytis cinerea*; Bg, *Blumeria graminis*; Cfi, *Ceratocystis fimbriata*; CLP, cyclic lipopeptide; Fm, *Fusarium moniliforme*; Fo, *Fusarium oxysporum*; Ft, *Fusarium tucumaniae*; Gc, *Glomerella cingulata*; HE, heterologous expression; IV, in vitro; LY, lytic; Mg, *Magnaporthe grisea*; Mo, *Magnaporthe oryzae*; Pe, *Penicillium expansum*; Pi, *Phytophthora infestans*; Ppa, *Phakopsora pachyrhizi*; Pp, *Phytophthora parasitica*; Px, *Podosphaera xanthii*; Pyt, *Pythium* sp.; Rs, *Rhizoctonia solani*; SP, spray; Ss, *Sclerotinia sclerotiorum*; Sv, *Stemphylium vesicarium*.

relevant field study reported that undecapeptide BP15 provided significant control levels of brown spot disease of pear (*Stemphylium vesicarium*) in orchard plots (126). In postharvest assays, blue mold (*Penicillium expansum*) was controlled by the peptide PAF26 on orange fruits (113) and by several CECMEL11 peptides in apples (7).

PRODUCTION OF FUNCTIONAL PEPTIDES AT LARGE SCALE

Small amounts (e.g., milligrams) of peptides are required for in vitro screening, but for plant assays or even field testing, moderate-to-high quantities (e.g., grams) are necessary. The future of functional peptides as plant-protection products depends mainly on the capacity to produce large quantities using industrial platforms. Peptides can be obtained directly from natural sources, by chemical synthesis, or through heterologous expression in living biofactories.

Natural Sources

Peptides are generally at low concentrations in natural sources. The food industry can be a good source of peptides because it generates important amounts of by-products (e.g., blood, whey, etc.) containing peptides and proteins that can be processed, either directly or by enzymatic digestion,

to leave functional peptides (96, 136, 140, 160). In the case of microbial fermentations, peptides can be more abundant, e.g., in the production of nisin by improved strains of *Lactococcus lactis* at 100–300 mg/L in batch or fed-batch fermentation reactors (30, 73) or of surfactin in *B. subtilis* 3NA, which provided very high yields of 26.5 g/L (72). Also, the production of trichokonins by *Trichoderma longibrachiatum* SMF2 achieved levels of nearly 200 mg/L in liquid culture or 146 mg/kg in solid-state fermentation (151, 173). Therefore, companies producing microbial pesticides may valorize their biomass production process by obtaining functional peptides from the cell-free spent-growth media rich in bacteriocins, CLPs/CLDPs, or peptaibols.

Chemical Synthesis

Large-scale chemical synthesis based on O-ring solid phase or liquid phase synthesis has been developed for many peptides for medical use (5, 112, 156). Chemical synthesis is more suitable in the pharma sector, where high-added-value products are more reliable, than in agriculture for plant protection, which requires less expensive products. The random mixtures synthesis, which generates a range of peptides with different sequences and activities but does not require complex purification steps, is one attempt to decrease the costs of synthesis (158).

Biotechnological Production

Production of peptides by heterologous expression in living systems (biofactories) has been extensively used in the pharma sector, and this approach is relatively well developed and produces linear peptides composed of proteinogenic amino acids by ribosomal synthesis (123). However, in nonribosomal synthesized peptides (e.g., CLPs, peptaibols), biotechnological production is less developed, although advances have been made in cloning biosynthetic gene clusters (165). For example, the bacillomycin NRPS cluster from *B. amyloliquefaciens* FZB42 has been cloned and introduced for heterologous expression in *B. subtilis* (89).

The more extended strategy for ribosomally synthesized peptides often requires the redesign of the peptide due to the need to include sequences to adapt the gene construct to intra- and extracellular trafficking in the genetically modified organism cells, which can modify the properties of the resulting peptide (9). In addition, it requires solving problems related to the small size of certain peptides, degradation due to intracellular proteases, toxicity toward host cells (e.g., an antimicrobial activity in a microbial platform), low yield, and the complexity of downstream processes needed for the extraction and purification of the peptide.

Many recombinant functional peptides have been produced using microbial systems such as *Escherichia coli*, *Pichia pastoris*, and *Saccharomyces cerevisiae* (38). In the yeast *Pichia*, cecropin D was produced at a yield of 0.5 g/L (51), and penaedin, snakin, and LL-37 were produced at a yield below 1 g/L (78, 87). In *E. coli*, yields of cecropin A close to 300 mg/L were reported (77, 166). An interesting example is the NUMAtag system, which uses a secretion strategy based on the *E. coli* hemolysin A (HlyA) type 1 secretion system to produce heterologous peptides and proteins with elevated secretion levels (69). In the medical field, the production of the hormone theraparathide (34-aa length) with a Numaswitch system yielded as much as 2 g/L (116).

A few recombinant proteins and peptides have been expressed in microalgae systems such as *Chlamydomonas reinhardtii* and *Chlorella ellipsoidea* as cAFLPm3 in *C. reinhardtii* (85) and defensin mNP-1 in *C. ellipsoidea* (44) at yields near 1% total protein.

Transgenic plants used as biofactories offer several advantages for large-scale production (148) and are potentially one of the most economical systems (146). Different tissues and several promoters and strategies can be chosen to reduce product degradation and toxicity while increasing stability. In the *Nicotiana* platform, yields of 6–7 mg/kg fw of the peptides MsrA2 and DrsB1 have been reported, whereas transient expression enables the production of AfpB and SP1–1 at yields

of 225 and 25 mg/kg fw, respectively (170). In cereal seeds, the accumulation of chimeric rh-LL37 peptide was 0.55 mg/kg of seed, the accumulation of MBP-rhLL-37 was 250 mg/kg of spike tissue in barley (58, 100), and the accumulation of BP178, CecA, and PAF102 was 20–38 mg/kg of rice seeds (20, 21, 107, 108). Interestingly, using tandem multimeric expression (29 multimers), a yield of 2 g/kg of seed for lactostatin peptide was reported in rice seeds (22).

THE CHALLENGES OF FUNCTIONAL PEPTIDES AS NOVEL BIOPESTICIDES

Several challenges are associated with the success of functional peptides as plant-protection products. Resistance to antimicrobial peptides in populations of plant pathogens is an important issue. Several mechanisms could interfere with the interaction of the peptide with the target plant-pathogen cell, e.g., by limiting the access of the peptide to the cell due to adsorption by envelopes or external structures (e.g., biofilm barriers, exopolysaccharides, capsules), active elimination from cells (e.g., efflux pumps, outer membrane vesicles secretion), degradation by proteases, or enzymatic chemical modification (88). Several physicochemical conditions and compounds can reduce activity (e.g., cations, pH, phenolics) and are of particular importance for cationic amphipathic peptides. This reduction in activity is important in plant tissues or the vascular system (13), where in addition most peptides can be degraded by proteases due to the presence of recognition sequences of the proteinogenic amino acids (50). Certainly, these problems can be mitigated by modification of the peptide, with non-natural amino acids (e.g., including D-amino acids) (115), or an adequate formulation (e.g., nanoencapsulation), but these mitigations always add complexity to the development and production process.

The toxicological profile of some peptides may be an issue of particular importance because of nontarget effects in animals, plants, and the environment. For example, some peptides are practically nontoxic, e.g., piscidin (29); CLPs such as surfactin, with biosurfactant activity, have low toxicity (43); and iturin and fengycin are less toxic than the detergent sodium dodecyl sulfate (137); however, peptaibols are generally toxic (42). In some cases, e.g., synthetic peptides, it is possible to improve the toxicological profile by developing less-toxic analogs.

The method of delivering or expressing the peptides into the plants is another important challenge. Given the extensive number of reports dealing with heterologous expression of peptides in plant crops, it seems that this approach is more reliable than topical treatments but requires more research in terms of food safety and environmental impact, and some restrictions may exist in several countries to use this in genetically modified self-protected plants. The conventional methods of application of plant-protection products in agriculture based on spray or soil drench require high amounts of peptides (e.g., kg/ha). Endotherapy may be an alternative for trees, especially in the case of diseases affecting the vascular system, such as those caused by *Candidatus* Liberibacter asiaticus (Citrus HLB) and *Xylella fastidiosa* (citrus variegated chlorosis, sudden death syndrome of olive, leaf scorch of almond), because it is the most effective way to access the vascular system (2, 62, 145). However, endotherapy needs to be further developed and evaluated from both a technological perspective, e.g., to understand the kinetics and movement of the peptides into the plant, and the perspective of growers that are prone to using conventional methods.

Peptides must be incorporated into formulations to improve their shelf life and performance, as in other plant-protection products, but coformulants (e.g., surfactants, wetting agents, inerts, buffers, etc.) have to be selected carefully to not interfere with their activity, especially in the case of amphipathic peptides. Significant improvement in the efficacy of some peptides can be obtained by adding certain coadjuvants. For example, the bactericidal activity of the peptide BP100 was improved by adding lysozyme (23). Also, the use of mixtures of peptides with different mechanisms of action, or with other antimicrobial compounds, could be of interest. Advanced systems used with

conventional pesticides based on flow, polymers, or nano-encapsulation formulation techniques need to be adapted specifically for peptides.

The cost of production of peptides for plant protection is one of the main issues. Chemical synthesis by solid-phase methods, a common approach for research purposes or high-value peptides (e.g., pharmaceutical), is too expensive. An attempt to reduce the cost of chemical synthesis for agriculture has been proposed using mixtures of randomly synthesized peptides (158), but this approach provides mixtures in which not all the components may be active. Currently, in chemical synthesis, a crude preparation of an undecapeptide costs several hundred dollars per gram, and the cost increases for large peptides. For bactericidal or fungicidal use, based on the spray doses effective on in planta assays, a generally required concentration is 0.10–0.20 g/L, which means rates of 50–200 g/ha (8, 126). However, for defense elicitor peptides, the concentration needed is considerably lower, i.e., 1–2 mg/L (135). The use of natural sources of peptides like microbial fermentation with improved strains or biotechnological biofactories may be more sustainable, but information on costs is not generally available. The challenge of high costs may be counteracted by scaling up production.

Finally, the last challenge for peptides to be novel active substances for preparing plant-protection products is evaluation under the regulatory framework (e.g., EFSA in the European Union, FDA in the United States). Attending to the current knowledge that we have on peptides, it can be expected that some of the most advanced can meet the criteria of low-risk compounds, as happens with some insecticidal peptides already authorized in the United States (57).

The prospects are that in the near future, several functional peptides would be commercially available for plant disease protection, but more field work is needed to demonstrate their efficacy in control of the most relevant diseases of importance or in strategic crops.

SUMMARY POINTS

1. Functional peptides are potential biopesticides for future plant-protection products.
2. Several mechanisms of action are involved in the activity of peptides against plant pathogens and diseases, including antimicrobial activity by several mechanisms and plant defense elicitation.
3. Functional peptides can be developed that simultaneously exhibit several mechanisms of action or can be used as cell-penetrating peptides to facilitate access to intracellular targets in both the pathogens and the plant cells.
4. Plants are effectively protected from pathogen infections by heterologous expression of ribosomally synthesized functional peptides.
5. Peptides can be obtained at large scale by chemical synthesis or from natural sources (e.g., food industry by-products), microbial fermentations, and heterologous expression in living biofactories (microorganisms, algae, and plants).

FUTURE ISSUES

1. Develop new functional peptides using target-oriented approaches to improve selectivity against plant pathogens, optimize stability in the plant environment, and provide low toxicity.

2. Design and validate peptide formulations to increase efficacy and shelf life of the peptides.
3. Provide suitable methods for delivery of the peptide formulations to the plant host, especially with endotherapy devices, to protect trees against endophytic bacterial plant pathogens.
4. Set up cost-improved and sustainable, large-scale production processes to fulfill the needs of the plant-protection sector and growers.
5. Perform field tests with the most relevant diseases and crops for the evaluation and validation of the efficacy and performance of plant-protection products composed of functional peptides.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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