Received: 25 January 2024 | Revised: 14 March 2024 | Accepted: 15 March 2024

DOI: 10.1002/psc.3600

REVIEW

Journal of PeptideScience WILEY

Genetically encoded libraries and spider venoms as emerging sources for crop protective peptides

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Funding information
Australian Research Council Future Fellowship, Grant/Award Number: FT190100482; EU Horizon 2020-EU.1.2 Future and Emerging Technologies (FET) open research and innovation action, Grant/Award Number: 828940; MIUR (Ministero dell'Università e della Ricerca)–PRIN (Progetti di Ricerca di Rilevante Interesse Nazionale) 2017, Grant/Award Number: 20173LBZM2_005

Agricultural crops are targeted by various pathogens (fungi, bacteria, and viruses) and pests (herbivorous arthropods). Antimicrobial and insecticidal peptides are increasingly recognized as eco-friendly tools for crop protection due to their low propensity for resistance development and the fact that they are fully biodegradable. However, historical challenges have hindered their development, including poor stability, limited availability, reproducibility issues, high production costs, and unwanted toxicity. Toxicity is a primary concern because crop-protective peptides interact with various organisms of environmental and economic significance. This review focuses on the potential of genetically encoded peptide libraries like the use of two-hybrid-based methods for antimicrobial peptides identification and insecticidal spider venom peptides as two main approaches for targeting plant pathogens and pests. We discuss some key findings and challenges regarding the practical application of each strategy. We conclude that genetically encoded peptide library- and spider venom-derived crop protective peptides offer a sustainable and environmentally responsible approach for addressing modern crop protection needs in the agricultural sector.

KEYWORDS
antimicrobial peptides, bioinsecticides, crop protection, genetically encoded peptide libraries, pesticides, spider venom peptides, two-hybrid assays

1 | INTRODUCTION

Various microorganisms like fungi, bacteria, and viruses but also herbivorous arthropods like insects and arachnids significantly reduce global crop yields. With limited arable land available, the need for plant protection products (PPPs) is inevitable to feed a growing global population. PPPs, also known as pesticides, are among the main pillars of modern agriculture and essential for counteracting pathogens and pests to promote plant health and reduce crop losses. However, while conventional PPPs remain the primary method for pest control, resistance insurgence is reducing the number of active compounds available for crop protection.[1,2] Most pesticide classes (like bactericides, fungicides, and insecticides) that have been registered in the

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last years suffer from a lack of new mechanisms of action (MOAs) and many marketed products act on the same molecular targets.[1] Thus, by acquiring resistance to one product, cross-resistance to others that share the same molecular target can be developed.[2,3] For instance, over 75% of current insecticides target only four molecular targets, that is, acetylcholinesterase, voltage-gated sodium channels, nicotinic acetylcholine receptors, and GABA-gated chloride channels. Furthermore, the massive application of pesticides often causes unwanted environmental effects and lacks specificity.[4] Therefore, restrictions on marketed PPPs have been increased over the years, and several compounds have been banned or included in a list of candidates for substitution, with growers now having access to only 35% of the active ingredients they had in 1991.[5] Developing new, acceptable PPPs is now more expensive and time-consuming for companies.[6] A more sustainable approach to crop protection is therefore crucial for the future of the global food system. In this context, antimicrobial and insecticidal peptides can represent a possible approach as they retain a low probability of resistance development, high potency, and low environmental persistence.[7] Peptides are one of the first strategies of defense that organisms possess to protect themselves from biotic stresses. They are generally cationic or amphipathic, displaying both broad- or narrow-spectrum specificity toward pathogens with a relatively low probability of resistance development.[8–12] In the clinic, they are considered a possible alternative to antibiotics, as they can exhibit activity against several multidrug-resistant pathogens[9,12] and they can be used alone or in combination with other therapeutics to obtain a synergistic effect.[13] This review will focus on two approaches: the rational identification of antimicrobial peptides (AMPs) using genetically encoded peptide libraries (GEPLs) and the utilization of insecticidal peptides from spider venoms (Figure 1).

2 | GEPLs AS A TOOL TO INCREASE AMPs SPECIFICITY

2.1 | AMPs identification through GEPLs

AMPs are widespread in nature—the Antimicrobial Peptide Database reports more than 3500 identified AMPs from six different life kingdoms[14,15]—and play a key role in defense mechanisms from pathogen attacks.[10] They are generally small (<50 amino acids) and can be classified based on their enrichment in specific amino acids (e.g., proline-, tryptophan-, arginine-, histidine-, and glycine-rich peptides) or according to their structure (e.g., α-helical peptides, β-sheet, or mixed).[10] AMPs may directly act on pathogen membranes leading to cell disruption. Alternatively, membrane inactive AMPs can affect the function of intracellular targets. Notably, many peptides display multiple MOAs resulting in a very low probability of resistant strain development.[10,11,16–23]

Besides their favorable characteristics, AMPs present some relevant drawbacks like poor stability, availability, and reproducibility among in vitro and in vivo conditions, high production costs, and unwanted toxicity.[11,12] Toxicity is one of the major unfavorable aspects to be considered because crop-protective AMPs will potentially come in contact with a plethora of different organisms, many of which are of environmental and/or economic relevance, and pervasive negative effects must be avoided. One option to increase the specificity of AMPs toward the target pathogen/pest is by selecting peptides interfering with proteins that are essential for the target organism, but absent (or highly dissimilar) in off-target organisms. In this scenario, GEPLs could be pivotal due to their widespread usage in identifying new peptide sequences that can effectively target specific proteins. In most cases, GEPLs combine randomized codons to create millions or billions of casual combinations of amino acids which can be screened at the same time either in vitro or in cell-based assays and selected for their binding to a target, their function, or other outputs.[24] Hits can be identified by sequencing DNA encoding for the peptide. Additionally, these isolation pipelines enable the identification of peptides without any or little information about the target protein.[24,25] GEPLs virtually do not have protein target limitations and could allow the exploitation of unprecedented drugging modalities when compared to classical small molecules[16,24]—like the inhibition of essential protein–protein interactions or the activity of transcription factors, thus providing the possibility to identify novel MOAs. Taking advantage of the continuously expanding genomic databases further allows for the identification of essential and pathogen-specific genes.[1]

Phage display, a well-known GEPL technique, functions by exposing peptides via fusion with a coat protein[26,27] enabling the screening of peptide libraries having billions of different combinations. Screenings are performed in vitro aiming at the identification of high-affinity ligands toward an immobilized target protein on a solid support or by incubating phages with the entire target pathogen, recovering bound phages after several washes. Phage display has been successfully used to identify peptides interacting with coat[28] and cytoskeletal[29] proteins of viruses implicated in plant diseases or unknown structures on the surface of infection-related structures.[30] Unfortunately, these studies did not rigorously investigate the antimicrobial activities of hit peptides, hampering the correct evaluation of phage-display suitability for identifying AMPs for crop protection. However, some successful examples were reported for human pathogens.[31–37] Two-hybrid (2H)-based assays are probably the most widely adopted GEPL methodologies to identify plant-protective AMPs.

2.2 | 2H-driven identification of peptide "aptamers"

Different studies reported the successful identification of plant-protective AMPs from 2H-based technologies by relying on peptide aptamers (PAs) libraries. PAs were first defined by Colas et al.[38] as target-specific peptides that are inserted in a biologically neutral scaffold protein, thus not possessing any activity or taking part in protein–protein interactions.[39] Additionally, scaffold embedding peptides induce conformational constraints and consequently tight binding to the target,[40] being also more stable than scaffold-free linear peptides.[41] Oligonucleotides encoding for the aptamers can be

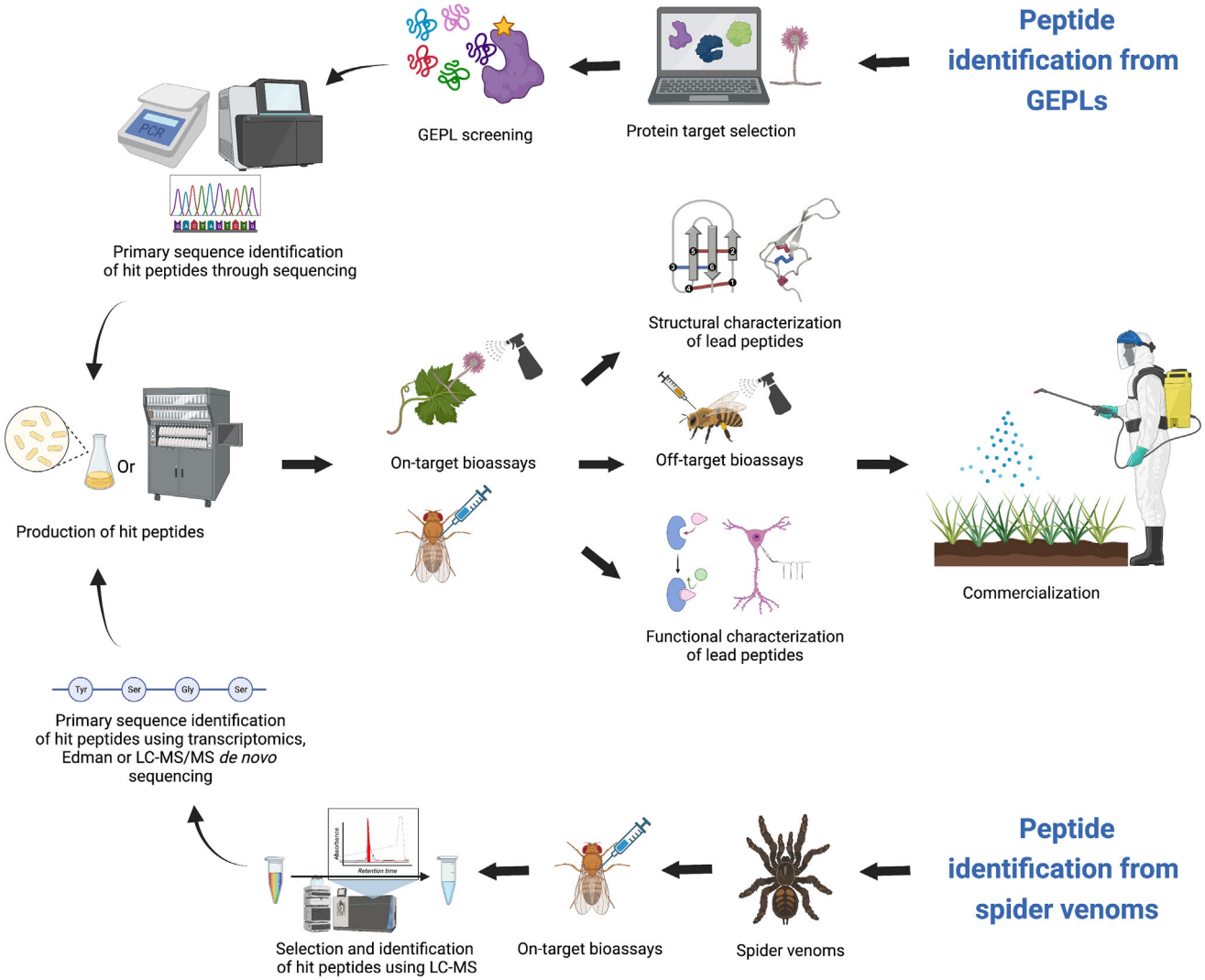

FIGURE 1 Similarities and differences in the process of peptide identification from genetically encoded peptide libraries (GEPLs) and spider venoms to develop new plant protection products. In GEPL-based pipelines (top of the panel), the target protein essential for the pathogen survival/infection is identified and then cloned into suitable vectors for the GEPL screening, using genetic databases, in silico methods, and/or published literature. Thereafter, the library is screened for peptides interacting with the chosen target protein. Hit peptide sequences are deducted by PCR amplification coupled to Sanger sequencing or next generation sequencing techniques. For spider venom peptides, a larger panel of venoms is initially screened against the target pest species to determine the most promising venoms. This is followed by the isolation of the pure insecticidal peptide(s) using chromatographic and mass spectrometric techniques and primary sequence determination by Edman degradation, de novo sequencing by mass spectrometry, venom gland transcriptomes, or a combination of these methods. The two pipelines converge at the production of hit peptides stage. Milligram amounts of hit peptides can be produced using recombinant techniques or chemical synthesis to enable their laboratory characterization. Lead peptides are identified by performing on-target bioassays using in vitro and in vivo techniques. This includes lead peptide application to infected leaves to address their ability to counteract pathogen disease symptom development or direct injection into target pests to confirm the insecticidal activity of the lead peptide. The lead peptide characterization process comprises elucidation of the 3-D peptide structure in the presence/absence of the molecular target, evaluation of undesired off-target activities, and a functional validation of the underlying mode of action, using biochemical or electrophysiological techniques. When moving toward commercial applications, the cost of production becomes a crucial factor, and therefore, recombinant production is usually the method of choice for economic large-scale production of peptide toxins to enable larger field trials. Created with BioRender.com.

designed to generate fragment[42] or combinatorial[40] peptide libraries. Additionally, scaffold proteins can be fused to a transcriptional activator and used to perform yeast-2H (Y2H) or bacterial 2H (B2H) screens. Y2H and B2H are probably the most widely used methods to study binary protein–protein interactions and to perform interactomics.[43] 2H technologies rely on 2 hybrid proteins (i.e., fusion proteins), one is constituted by a DNA binding domain fused to a bait protein, and the other is a transcription activation domain fused to a prey protein. A successful bait–prey interaction promotes the transcription of some reporter genes of choice, allowing the growth of yeast or bacterial positive colonies on selective media. When the two fusion proteins interact, the activation domain promotes the

transcription of the marker gene(s) downstream to a regulatory sequence recognized and bound by the DNA binding domain. Generally, when screening PA libraries, the bait protein is the target of choice, and the prey is the peptide library.

During the last decades, the employment of 2Hs for bait-interacting PA identification has been reported. Noteworthy, the pioneering work of Rudolph et al.[42] used a Y2H approach to identify peptides capable of counteracting infections promoted by different tospoviruses. The multifunctional nucleocapsid protein (N) of the tomato spotted wilt virus (TSWV) is involved in several relevant interactions, including homodimerization.[44,45] Therefore, the authors[42] generated a library of peptides constituted by fragments derived from the N protein of TSWV which was screened against the TSWV N proteins itself, used as bait. The screening yielded overlapping peptides to a C-terminal region of the bait protein, which was previously characterized as an essential interaction domain.[45] The shortest (29 amino acids) of these overlapping peptides also bound to tomato chlorotic spot virus, groundnut ring spot virus, and chrysanthemum stem necrosis virus N proteins upon its fusion to the β-Glucuronidase tag and subsequent expression in *Nicotiana benthamiana* provided resistance to all these viruses. This indicated that peptides can be used for the targeting of essential and evolutionary conserved functional domains to protect from different but related plant pathogens.

Similarly, a 2.9×10^9 20-mer PA library inserted in the active site of Thioredoxin A (TrxA) scaffold was screened (using Y2H) against the highly conserved Replication initiator Protein (ReP/AL1) belonging to the tomato golden mosaic virus.[46] More than 35% of identified TrxA PAs were able to interfere with virus replication once transfected in tobacco protoplasts and to interact with another ReP/AL1 belonging to another germivirus (cabbage leaf curl virus). A22 and A64 TrxA-peptide fusions, targeting ReP/AL1, were successfully used in a more recent study[47] to produce transgenic tomato plants resistant to two unrelated germiviruses, tomato yellow leaf curl virus and tomato mottle virus.

Using B2H, a 16-mer random PA library has been embedded in an exposed loop of the *Staphylococcus aureus* nuclease scaffold (SN) and screened toward the calmodulin of *Magnaporthe oryzae* (causal agent of rice blast).[48] Among the SN-PA fusions, the SN-PA-D4 demonstrated the capacity to inhibit spore development via interference with the calmodulin N-terminus, which was used as bait protein in the assay.

In all the mentioned examples, PAs have been maintained in a scaffold protein to perform antimicrobial assays, by being genetically expressed from plants susceptible to target pathogens attack[42,46,47] or directly incubated with the target pathogen.[48] In 2020, Colombo et al.[49] identified NoPv1, a linear octameric PA isolated by a Y2H screen toward the *Plasmopara viticola* cellulose synthase A2 (PvCesA2) bait protein. Once identified, NoPv1 amino acid sequence was chemically synthesized as an unconstrained peptide, therefore, without being embedded in the TrxA scaffold protein, used in the 2H assays. The synthetic NoPv1 significantly reduced symptoms related to *P. viticola* infections, the causal agent of grapevine downy mildew, in leaf disc bioassays and greenhouse conditions. This demonstrated that PA sequences can be used to fight crop diseases without needing a scaffold protein, which could ease their chemical formulation and bypass the need for transgenic crop production. Furthermore, NoPv1 does not negatively affect some tested organisms like plants, bacteria, eukaryotes, and human cells, meanwhile being able to target *Phytophthora infestans*, an oomycete closely related to *P. viticola*. Authors suggested that this oomycete-specific spectrum of action can be attributed to the CesA2 enzymes being almost identical among oomycetes but very dissimilar to all the other known CesA and CesA-like enzymes.

Altogether, exploiting 2H methods for AMPs identification can provide an example of how in vivo GEPLs can be used to isolate functional peptides, which can be genetically expressed or exogenously applied to counteract disease symptoms in crops.

3 | WEAVING A GREENER FUTURE: TURNING SPIDER VENOM PEPTIDES INTO ECO-FRIENDLY INSECTICIDES

3.1 | Why spider venom peptides?

In recent years, there has been a growing preference for bioinsecticides over classical, small molecule-based insecticides.[50] Bioinsecticides are native chemicals or compounds derived from organisms such as plants, bacteria, fungi, or animals.[51] The global bioinsecticides market has grown significantly from $1.6 billion in 2009 to $8.2 billion in 2022 and is expected to match the value of currently available insecticides by 2040 to 2050.[51] Microbial biopesticides are generally derived from bacteria, fungi, viruses, or protozoa, among which ~75% are based on δ-endotoxins derived from the bacterium *Bacillus thuringiensis*.[52] These toxins have been genetically engineered into crops, such as cotton, for controlling insect pests.[53] *B. thuringiensis* δ-endotoxins are considered the gold standard for bioinsecticides, offering advantages such as high biodegradability, potency, and selectivity and no adverse effects on vertebrates.[50] Other popular bioinsecticides are plant-based extracts and essential oils which contain bioactive chemicals for defense and signaling purposes. In recent years, an increasing number of essential oils have been approved for use in agriculture as bioinsecticides.[54,55]

Spiders have been natural predators of arthropods for ~400 million years and possess the ability to quickly paralyze or kill insects by injecting venom. Previous research indicates that spider venom is composed of a variety of compounds including low molecular mass compounds, cytolytic peptides, disulfide-rich neurotoxic peptides, and high molecular mass proteins and enzymes.[56–58] Disulfide-rich peptides, which have been identified in most spider venoms, are the most abundant and are considered a promising candidate for insecticide development.

Spider venom peptides in the 3–8 kDa range possess ideal characteristics for insecticide use, particularly when compared with traditional small-molecule insecticides (<500 Da) such as neonicotinoids. The larger surface area and structural complexity of spider venom

peptides provide them with exceptional target selectivity and fewer side effects on nontarget organisms.[59–61] Furthermore, the unique 3-D structure of disulfide-bridged venom peptides confers them with remarkable stability. This distinguishes them from large peptides (>10 kDa) that are also recognized for their specificity and potency but are characterized by other disadvantages like low metabolic stability, intricate mode of action, or production challenges.[4,62] In addition, insecticidal spider venom peptides are primarily fast-acting neurotoxins.[63] This rapid action of venom peptides is advantageous for the treatment of herbivorous pests because it can stop crop damage shortly after the application.

Despite the significant advantages of spider venom peptides as insecticides, there are still some concerns regarding their use. For example, it remains unclear whether most spider venom peptides have topical or oral activity because spiders deliver their venom via injection. Additionally, most spiders are polyphagous predators, suggesting that their venoms might be broad-spectrum weapons that lack the selectivity toward pest species.[64]

3.2 | Turning spider peptides into bioinsecticides: Current challenges and future prospects

Given the strong opposition to genetically modified crops that still exists in the general public, insecticides in large-scale agricultural settings are preferably applied via spraying. This poses a challenge for insecticidal peptides regarding their mode of uptake. For insects that are present at the time of spraying, the peptides could be taken up topically or as aerosols through the spiracles. For targeting any insects that are shielded from or not present during the spraying, either contact or oral activity by the peptide covering the crops would be required. As far as insecticidal spider venom peptides are concerned, there has so far been evidence for oral activity and uptake via the spiracles.[57,63,65] Especially the oral route can be harsh on peptides due to the insect midgut boasting with digestive enzymes and exhibiting extremes in pH.[66,67]

Thus, another challenge for insecticidal peptides is the need to exhibit sufficient physicochemical stability to survive the harsh environment of the insect digestive system. Fortunately, many spider venom peptides conform to a three-dimensional structure known as the inhibitor cystine knot (ICK) motif.[59,68] This structural fold endows peptides with extreme physicochemical stability[69,70] that makes insecticidal ICK peptides promising bioinsecticide candidates. Unsurprisingly, the only spider venom peptide (ω/κ-hexatoxin-Hv1a) that has so far reached the market as a bioinsecticide also conforms to the ICK motif.[71]

But even for peptides with sufficient stability to survive in the insect midgut, another challenge is imposed by the need to cross the epithelial barrier that is lining the midgut to reach the hemolymph and subsequently their molecular targets in the insect's nervous system.[72] Translocating the midgut barrier can either occur via the transcellular or paracellular (i.e., via the septate junctions) route and it can either be through passive diffusion or by active transport.[72,73] While it has been shown that spider venom peptides can cross the midgut epithelium,[74] the mechanism remains to be determined. Even for peptide candidates that do not exhibit oral toxicity, a range of strategies exists for their delivery such as employing entomopathogenic fungi or baculoviruses as vectors or using lectins or Cry proteins to enhance their midgut translocation.[72,75]

Another desired characteristic of insecticidal peptides is their phyletic specificity. Ideally, a peptide-based bioinsecticide should only affect the targeted pest species, while not having any negative effects on beneficial species or the environment. Nentwig argued that spider venoms are not suitable insecticides, because spiders are generalist predators and therefore their venoms are unlikely to be specific toward pest insects.[64] While this argument seems logical at first sight, a closer look into the venom composition of spiders reveals extremely complex mixtures of smaller inorganic and organic molecules, peptides, and proteins,[58] and synergistic activities between different components are well documented.[76] Thus, the insecticidal activity of spider venoms is usually not attributed to individual components but rather to a combination of different toxins that act in a concerted effort to exert the desired insecticidal effect. While the combined effect of multiple toxins enables the spiders to target a wide range of insect prey, it does not exclude that individual toxins can exhibit a certain degree of phyletic specificity. This can be exemplified by the venom of Australian funnel-web spiders, which contains a range of insecticidal toxins from the omega, kappa, and delta toxin families, which are all insecticidal but each of these toxins acts on different molecular targets and affects only specific insect taxa.[77–82] Another example of a spider toxin with a narrow phyletic specificity is Dc1a, which is insecticidal to German, but not American cockroaches.[83] But even insecticidal candidate peptides that do not exhibit the desired phyletic specificity could still be utilized as bioinsecticides. By using host-specific entomopathogens as delivery vectors, phyletic specificity can be added via the chosen entomopathogen species.[75]

Another argument that is often used for stating the disadvantages of peptides as drug candidates in comparison with their small molecule rivals is their cost of production.[62] In the agricultural industries, this becomes even more of a challenge due to the lower margins to be gained from selling insecticides as compared with therapeutic drugs. Another reason that makes it even harder for peptide-based insecticides to become profitable is their need for distribution across extensive acreages of crops, which requires larger quantities in comparison with therapeutic drugs that are confined to the body of treated patients. On the other hand, peptides are often orders of magnitude more potent than small molecules, thereby reducing the overall quantities required in comparison with small molecule insecticides. The final proof of concept that insecticidal spider venom peptides can be commercially viable as bioinsecticides has been provided by the US-based Vestaron Corporation. In 2014, they obtained approval from the US Environmental Protection Agency for their insecticidal product Spear T®, containing the funnel-web spider venom peptide ω/κ-hexatoxin-Hv1a as active ingredient.[71] Spear T® targets a wide range of insect pest orders, while being safe for mammals and honeybees.

4 | CONCLUDING REMARKS

AMPs identified from GEPLs and insecticidal spider venom peptides are promising alternatives to traditionally used pesticides displaying a low probability of resistance development, high potency, and low environmental persistence. Their biological degradation into amino acids, the building blocks of life, prevents accumulation of their active molecular form or any harmful degradation products in the food chain and therefore makes peptide-based products safer for the environment. In contrast, many marketed PPPs accumulate and spread into the environment, causing diffused pollution and unwanted side and long-term effects. Notably, major off-target effects for current peptide-based PPPs have not yet been reported.[7] There is widespread agreement in the scientific community that peptide-based approaches represent a good alternative to several more harmful PPPs currently on the market. At least alternating or combined applications of peptide- and small molecule-based PPPs could be considered an integrative strategy to help reducing environmental impact and resistance insurgence. These highlighted advantages cannot be considered a general "rule of thumb" for all antimicrobial and insecticidal peptides as several examples of toxicity are reported in the literature.[11,12] Therefore, toxicity and biosafety assessment remains a crucial requirement for bringing these peptide-based PPPs to the market. Nowadays, peptide-based product registration might be subjected to the same pipelines as for new small molecule-based PPPs, although Vestaron received expedited approval through the US Environmental Protection Agency for their biologically based spider venom peptide insecticide Spear T®.[71] Similarly, ProBlad® Verde (Sim-Agro) (a wide spectrum AMP-based fungicide) was exempted from the European Union's Maximum Residue Limit—that is, maximum residual pesticide amount remaining in food products to avoid human health concerns—thanks to its safety profile.[7] These two examples could pave the way for easing registration pipelines for peptide-based PPPs in future, without relying on regulatory exceptions.

AMPs selection through GEPLs can fulfill different needs for the development of new PPPs as they can be used to target a protein of choice, also exploiting drugging modalities difficult to be attained with classical small molecules,[24] thus providing the possibility to broaden the number of antimicrobial targets and finely tailor their selection, raising the possibility to register products with new MOAs. Such pipelines can also enable the identification of peptides designed to work in synergy with already marketed single-target pesticides, to reduce doses and resistance insurgence.[1] These AMPs can reduce the off-target effects when choosing pest-specific target proteins or mechanisms,[84] and theoretically, any GEPL methodology can be applied to crop protection. Currently, no GEPL-derived active ingredients are present among 18 registered peptide-based PPPs,[7] presumably due to the relatively young age of this research field applied to crop protection.[31] The existing lack of GEPL-based peptides in the PPPs market hampers a more precise analysis of their limitations and challenges for their industrial scalability and commercialization.

Bioinsecticides and peptide-based PPPs are a rapidly growing market,[7,51,85] and Vestaron has already proven their scalability and economic feasibility by commercializing the spider venom peptide ω/κ-hexatoxin-Hv1a, with more peptide-based insecticides waiting in their pipeline to be rolled out to the market within the next few years. If their business model of selling environmentally friendly bioinsecticides proves to be a continuous success, it seems likely that other larger global players in the agrochemical industry will increase their investments and efforts in this space. With over 51,000 species of known spider species[86] and each of them containing hundreds of different peptide toxins on average, there is certainly no shortage of potential insecticidal peptides to be discovered from spider venoms.

The main challenge for these peptide-based approaches for crop protection will be to identify those synthetic or venom-derived peptides that fulfill all the criteria required for a successful bioinsecticide candidate, in particular, the need to be bioavailable in and selective toward the targeted pest insect species. Another challenge comprises the large-scale production via heterologous methodologies, which lacks a generalizable approach. However, most of the commercialized peptides currently on the peptide-based PPP agricultural market are produced using recombinant strategies.[7] We hope that collective research efforts in this and related fields will lead to the establishment of reliable and streamlined pipelines capable of bringing peptides from early discovery to commercialization in shorter timeframes, while ensuring safety for the environment and global food chains.

Future directions and challenges for GEPL-derived peptides might be the establishment of solid screening platforms for faster hit-to-lead transitions, enabling to deal earlier with safety assessments, formulations, field trials, and product registration, which is probably the most time-consuming aspect of this process. Future challenges for insecticidal spider venom peptides are the exploration of previously understudied spider taxa[87] for sourcing novel insecticidal peptides, examining potential effects of sublethal doses on insect memory as reported from commercial insecticides,[88,89] as well as streamlining methods and species used for determining insect toxicity.[65,90]

ACKNOWLEDGMENTS

We thank Cátia Teixeira for her comments and support of this manuscript. V. H. was funded by an Australian Research Council Future Fellowship (FT190100482). S. R. and E. M. F. want to acknowledge EU Horizon 2020-EU.1.2 Future and Emerging Technologies (FET) open research and innovation action under grant agreement 828940 granted to Paolo Pesaresi (Department of Bioscience, University of Milan) and MIUR (Ministero dell'Università e della Ricerca)–PRIN (Progetti di Ricerca di Rilevante Interesse Nazionale) 2017 (protocol 20173LBZM2_005) granted to Simona Masiero (Department of Biosciences, University of Milan). Open access publishing facilitated by University of the Sunshine Coast, as part of the Wiley - University of the Sunshine Coast agreement via the Council of Australian University Librarians.

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How to cite this article: Fassolo EM, Guo S, Wang Y, Rosa S, Herzig V. Genetically encoded libraries and spider venoms as emerging sources for crop protective peptides. *J Pept Sci*. 2024;e3600. doi:10.1002/psc.3600