Heterologous Expression Systems for Plant Defensin Expression: Examples of Success and Pitfalls

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Abstract: Defensins are a superfamily of antimicrobial peptides, present in vertebrates, invertebrates, fungi and plants, suggesting that they appeared prior to the divergence in eukaryotes. The destitution of toxicity to mammalian cells of plant defensins has led to a new research ground, i.e., their potential medical use against human infectious diseases. Isolating defensins from natural sources, like plant tissues, can be time-consuming, labor intensive and usually present low yields. Strategies for large-scale production of purified active defensins have been employed using heterologous expression systems (HES) for defensin production, usually based in *E. coli* system. Like any other technology, HES present limitations and drawbacks demanding a careful experimental design prior the system selection. This review is proposed to discuss some of the major concerns when choosing to heterologously express plant defensins, with special attention on bacterial expression systems.

Keywords: *E. coli,* fusion, carrier protein, secretion signal, inclusion bodies, antimicrobial peptides.

1. INTRODUCTION

Organisms throughout all kingdoms (including prokaryotes, lower and higher eukaryotes) present an ancient defense mechanism based on the secretion of small proteins called Antimicrobial Peptides (AMPs) that act by inhibiting the growth of bacteria, fungi, parasites and viruses. Together with other defense mechanism, they form the innate immune system, protecting the host against microbial attacks [1].

AMPs are gene-encoded and they are either constitutively expressed or rapidly transcribed upon induction by the presence of invading microbes or their products. AMPs are classified according their function and structure, which are determined by the amino acids residues on the primary sequence of the protein (glycine, cysteine, histidine, proline, tyrosine, arginine, lysine and serine) [2]. Defensins are a superfamily of the AMP, and can be found in vertebrates, invertebrates, plant and fungi suggesting that it appeared prior to the evolutionary divergence in eukaryotes [3]. Although defensins present a low sequence similarity, they share highly conserved α/γ c-core primary sequence motifs [4] and a cysteine-stabilized α/β (CSα/β) tertiary structure [5]. There are eight typical cysteine residues that form four disulfide bonds providing stability to the defensin [6].

Isolating defensins from natural sources, like plant tissue can be time-consuming, labor intensive and usually do not result in high yields [7]. Therefore, other strategies for large-scale production of satisfactory amounts of purified active defensins have been employed, such as the use of recombinant DNA technology [8], which has become a promising and rapidly expanding area. A growing number of studies have reported the use of different heterologous expression systems (HES) for defensin production. However, like any other technology, HES has also been target of practical limitations and drawbacks (in both plant bacteria or fungi platforms) demanding a careful experimental design prior to the system selection. In this context, the present review aims to discuss the important elements involved in the production of recombinant plant defensins with a special focus on the *Escherichia coli* heterologous expression system. The presented data include steps for obtaining the peptide coding sequence until its isolation and purification.

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2. PLANT DEFENSINS

The first plant defensins were discovered in 1990, in wheat and barley seeds [9], initially termed δ-thionin due to their similar size and number of disulfide bonds to α-thionins and β-thionins. Mostly, defensins present three to five disulfide bonds, which stabilize an antiparallel beta-sheet flanked by an alpha-helix thus conferring resistance against extreme pH and temperatures [10].

Defensins are small (<10 kDa, 45-54 amino acids), often highly basic, cationic, cysteine-rich peptides and participate of the innate immune system of plants, as an ancient strategy of protein secretion for protection against invading microorganisms [11]. Defensins exhibit a variety of biological activities, especially antifungal and antibacterial activity [12] requiring a low minimal inhibitory concentration [13]. Seeds are energetic substances storage organs, becoming target to a wide variety of heterotrophic organisms, especially fungi. Therefore, defense mechanisms based on cysteine-rich antimicrobial peptides are highly represented in seeds. Recent studies have found new *in vivo* roles for plant defensins: protective role in seeds germination by creating a microenvironment around the seed in which fungal growth is suppressed [14]; protective role in embryo formation by local expression in the embryo-surrounding right after pollination [15]; protective role against biotic stress, including fungi [16], bacteria [17], herbivore insects [18], nematodes [19] and parasitic plants [20] as well as role in symbiotic interactions (e.g., down-regulation in root when in the presence of nitrogen fixing bacteria [21]). A recent study using *Prunus persica* (peach) defensin, PpDFN1, found that the addition of a neutral sphingolipid ceramide β-D-galactoside to a lipid monolayer of egg-phosphatidylcholine (ePC) increased significantly the defensin affinity to the artificial membrane. Nevertheless, when the same experiment was made using lipids isolated from phytopathogenic fungi, the affinity presented was even higher, demonstrating that the binding of a plant defensin to target pathogen membrane is dependent on its lipid concentration and composition [5]. The same study showed no PpDFN1 defensin activity against human erythrocytes, in accordance with Wong and Ng [22] findings on vulgarin (a defensin isolated from *Phaseolus vulgaris*), which is known to be devoid of toxicity to mammalian spleen cells and erythrocytes. Also, in Gonçalves *et al.* [23] it is shown that *Pisum sativum* defensin, Psd1, presents no affinity to cholesterol-enriched lipid bilayers, just like the membranes found in mammalian cells. Some recent discoveries on plant defensin antifungal activity shows that such activity might be mediated by electrostatic interaction with anionic lipid components of fungal membranes [24].

Since their discovery, studies on defensins have been conducted regarding (i) activation pathway [25, 26], (ii) mode of action [13], and (iii) potential for improving plant resistance against phytopathogens [27]. Therefore, the knowledge about the lack of plant defensins toxicity to mammalian cells has led to a new research ground, that is, its potential medical use against human infectious diseases [22, 28, 29].

2.1. Plant Defensin Prospection

Obtaining a mature plant defensin peptide sequence can be basically achieved by two main strategies: reverse genetics or database prospection. The reverse genetics strategy consists basically in the extraction and purification of native plant defensins from tissues followed by amino acid sequencing. Defensin sequence prospection (data mining) is based on the use of bioinformatic tools to find sequences with similar defensin motifs in genomic plant databases. In both cases, the knowledge of the defensin sequence will provide the information needed to design primer for cDNA amplification and/or artificial plasmids baring the mature defensin sequence.

2.1.1. Reverse Genetics Strategy

The conserved structure and biochemical features of plant defensins allow the purification of previously unknown peptides from plants for which the genome sequences are still not available. Although seeds are the most commonly used tissue for defensin isolation [30-32], defensins have also being isolated from other plant organs. Lay *et al.* [33] isolated one defensin from ornamental tobacco and two from *Petunia*, in both cases using floral tissue. Through immunoblot analysis, they discovered that defensins were more concentrated at the ovaries, petals and pistils during the early stages of flower development. Defensins isolation was also reported from *Capsicum annuum* mesocarp of ripe fruits [17], of *Ipomoea batatas* (L.) Lam. 'Tainong 57' storage roots [34], on injured *Arabidopsis thaliana* [25] and also on intact *Spinacia oleracea* [35] leaves.

The process of extraction often consists in powdering the tissue, producing seed flour or macerating other tissues with liquid nitrogen, followed by the addition of a saline extraction buffer at low temperature (4°C). The supernatant is then collected and exposed to different concentrations of ammonium sulfate [36] causing the precipitation of groups of proteins based on their charges. The pellets precipitated at each ammonium sulfate concentration must be resuspended and submitted to chromatographic methods in order to obtain a purified sample of the target peptide [31]. Due to its cationic profile the sample passes through an affinity chromatography column (weak anion exchange column) and the fraction containing the defensins is then applied to a size-exclusion chromatography column (a process also referred as gel-filtration chromatography) [37].

2.1.2. Databases Prospection Strategy

The increasing drug-resistance of microbial pathogens has created a necessity for the discovery of new antimicrobial drugs. Antimicrobial peptides, especially plant defensins, have proven to be effective against many bacterial and fungal pathogens and to present no toxicity against mammalian cells. Therefore, the development of AMPs and defensins databases has helped many researchers in their studies, providing not only sequences, but tools to enhance the way the information is presented leading to a better understanding of their protein of interest. Some databases provide programs to perform homology searches, multiple sequence alignments, phylogenies, physicochemical profiles, and other calculations and predictions [38].

An example of such a database is the PhyAMP - a plant specific data repository for natural plant antimicrobial peptides. Records for 55 plant defensins can be found in their

database, along with very useful diagrams and charts concerning defensins phylogenetic trees, base and acid amino acid distribution and activity. Tools to make the prospection easier or to provide further data for study, as similarity search, hidden Markov Models, physicochemical profiles and sequence alignments are available at PhyAMP [39]. In some cases, if a researcher is acquainted to the protein he is looking for, some non-specific databases such as GenBank (www.ncbi.nlm.nih.gov/genbank), can be also helpful [40].

As an example, Karri and Bharadwaja [40] searched GenBank to find the sequences of Tfgd2 (*Trigonella foenum - graecum* defensin 2; GenBank accession number AY227192) and RsAFP2 (*Raphanus sativus* antifungal protein 2; GenBank accession number U18556) to construct the fusion gene Tfgd2-RsAFP2 (that was later deposited at GenBank under the accession number KF498667). The constructed Tfgd2-RaAFP2 proved to be three times more efficient against *Phaeoisariopsis personata* conidia germination. A list presenting some of the available AMPs and defensins databases is provided in Table **1**.

3. HETEROLOGOUS SYSTEMS FOR PLANT DEFENSIN PRODUCTION

Advances in recombinant DNA technology have provided an opportunity to produce high levels of plant defensins. This technology enables cloning of foreign genes in specific vectors for expression in prokaryotic and/or eukaryotic systems, and is considered to be the most effective method regarding time and production costs [41]. Proteins have several features that should be carefully observed when choosing a host system for heterologous production, such as size, intracellular localization or secretion, proper folding, and glycosylation pattern [42].

The main hosts used for AMPs production are bacteria and yeasts, representing 97.4% of heterologous-expressed AMPs [42, 43]. More recently, plants have emerged as a promising host for AMPs production since transgenic plants can be directly used for microbial control expressing the peptide in the desired crop [42, 44].

Different hosts have been used for the production of plant defensins in prokaryotic and eukaryotic systems. Plant defensins have already been produced in bacteria [5, 45, 46], yeasts [47-49] and transgenic plants [50-52] with variable degrees of success and protein yield.

3.1. *Escherichia coli* Expression System

Escherichia coli is the most employed microorganism for heterologous protein production. This system is the first choice as host for plant defensin production and so far the one with highest yields reported. There are many reasons for that: *E. coli* has proven to be the most cost-effective method of recombinant protein production due to its rapid growth, large availability of commercial expression vectors, well-established DNA manipulation protocols and extensive knowledge regarding its genetics, biochemistry and physiology [53]. However, there are some pitfalls that need to be overcome in order to achieve effective production in bacteria.

Plant defensins have already been produced heterologously in *E. coli* expression systems. Nanni *et al.* [5] achieved a yield of 0.5 mg.L-1 of the cloned PpDFN1 using the pET-32 vector (Novagen) and *E. coli* BL21 (DE3) Origami pLys cells (Novagen). The produced defensin was functional and displayed antifungal activity against *Botrytis cinerea*, *Monilinia laxa* and *Penicillium expansum*, with IC50 values of 15.1, 9.9 and 1.1 μg.mL-1, respectively.

Similarly Kovaleva *et al.* [54] had already expressed the *Pinus silvestris* defensin 1 (*Ps*Def1) in the pET system (pET42a) and BL21 (DE3) *E. coli* strains, although with a fusion protein glutathione-S-transferase (GST). They were able to produce a full length defensin which was not biologically active when fused to GST but strongly functional against a panel of pathogenic fungi when separated from the fusion protein.

In the following sections some approaches that have to be considered for *E. coli* heterologous expression will be discussed in detail.

3.1.1. Escherichia coli Strains

The lack of comparative studies of recombinant plant defensin expressed in different strains makes it difficult to predict which strain will provide better results. Choosing the right expression system may be crucial to achieve a satisfactory yield of a recombinant defensin. Sometimes, a preliminary assay using different strains can help making such decision, as recombinant protein yield can vary from a discreet to a discrepant 10-fold difference among strains [55].

Defensins present eight cysteine residues that interact creating four disulfide bonds, therefore, it is important that the chosen expression strain is able to form such fold. The disulfide bond formation is inhibited in the *E. coli* cytoplasm, because of the presence of thioredoxins, and it is dependent on an enzyme alkaline phosphatase, which is only active in the periplasm [56]. Cytoplasmic expression and correct folding of defensins in *E. coli* has become possible through the use of thioredoxin reductase (trxB) mutant strains and even more if periplasmic isomerases, such as the enzyme disulfide-bond isomerase (DsbC, catalyze the formation and correction of disulfide bonds; also called foldases) were engineered to be expressed in the cytoplasm as well [57]. Some strains present plasmids encoding for rare tRNAs attenuating the codon bias, making codon optimization less crucial for a successful expression [58]; others provide a more oxidized environment in the cytoplasm enhancing the recombinant defensin disulfide bond formation due to mutations in the trxB and glutathione reductase (gor) genes [59]. There are also strains available in the market that combine these two modifications, for a better expression control and cytoplasmic disulfide binding formation of heterologous proteins expressed in *E. coli* [60].

Protein degradation during purification steps can be minimized when using strains mutated for the Ion protease and the ompT outer membrane protease [55]. It is important to highlight that even with optimal experimental design it is not possible to predict the outcome of a successful soluble recombinant defensin production. Kovalskaya and Hammod [61] constructed cassettes using the pET26b expression vec-

Table 1. Antimicrobial peptides and defensins databases.

Database	Year*	Summary	URL
LAMP	2013	LAMP: a Database Linking Antimicrobial Peptides.	http://biotechlab.fudan.edu.cn/database/lamp
DAMPD	2011	DAMPD: an update and a replacement of the ANTIMIC database.	http://apps.sanbi.ac.za/dampd
CAMP	2009	Collection of Antimicrobial Peptides, India.	http://www.bicnirrh.res.in/antimicrobial
RAPD	2008/09	A database of recombinantly-produced antimicrobial peptides, USA.	http://faculty.ist.unomaha.edu/chen/rapd/
APD2	2004/09	The Antimicrobial Peptide Database, USA	http://aps.unmc.edu/AP/main.html
PhyAMP	2008	A database dedicated to plant antimicrobial peptides, Tunisia.	http://phytamp.pfba-lab-tun.org/
Defens-ins	2007	Defensins Knowledgebase, Singapore.	http://defensins.bii.a-star.edu.sg/
AMPer	2007	A database and discovery tool for antimicrobial peptides, based on hidden Markov models and the SwissProt databank, Canada.	http://marray.cmdr.ubc.ca/cgi-bin/amp.pl
AMSDb	2002/04	Antimicrobial sequence Database, Italy.	http://www.bbcm.univ.trieste.it/~tossi/amsdb.html
* Creation and/or update of the database			

tor and two plant AMP's, a snakin (SN1) and a defensin (PTH1), for heterologous production in BL21 (DE3) strains. The strategy to produce soluble molecules failed and the recombinant proteins were found to be concentrated in the Inclusion Bodies (IBs).

There is a myriad of expression strains available, each one with different combination of strategic modifications: RNAse E mutated strains for a longer intracellular RNA lifespan, T7 Lysozyme for better control of toxic peptides, salt-induced promoters for better solubility, lacY mutants for homogeneous induction levels in each cell and accurate expression control and addition of DsbC chaperone for better cytoplasmic folding (Table **2**).

3.1.2. Vectors

It is important to highlight that one must try to combine strains and expression vectors strategies to make the expression more prone to success. For example, the use of expression vectors baring a N-terminal *pelB* signal is recommended when working with lon protease and the ompT outer membrane protease mutated strains, providing a periplasmic location for the recombinant protein, for better folding and solubilizing conditions [62].

Expression vectors can add binding molecules to the recombinant protein making protein purification less time consuming and laboring. The pMAL expression vectors (commercialized by New England BioLabs), for instance, are divided in two major groups: the pMAL-c and the pMAL-p vectors. Both groups add a maltose binding protein to the recombinant defensin in a single-step purification process. The pMAL-c vectors (as the pMAL-c2x, pMAL-c5x, etc.) present the deletion of the *malE* signal sequence resulting in cytoplasmic expression of the fusion protein. It leads to better yields (recombinant protein levels of 20-40% of total cell protein), but it is not recommended for peptides that require disulfide bond formation [17]. The pMAL-p series bares the intact signal sequence of the *malE* gene, leading to the secretion of the recombinant peptide to the periplasm. The pMAL-p series shows lower yields (recombinant protein levels of 1-20% of total cell protein), but is more prone to form the correct disulfide bond folding.

For plant defensin heterologous expression, the most widely used expression system is the pET system, initially developed by W. F. Studier and B. A. Moffatt in 1968, that created an RNA polymerase expression system which was highly selective for bacteriophage T7 RNA polymerase [63]. These vectors have as main feature a strong bacteriophage transcription signal, leading to a high level of heterologous expression, with the recombinant protein comprising up to 50% of total cell proteins a few hours after induction. These vectors must be cloned into strains that have the T7 Polymerase gene for expression. Strains, like BL21 (DE3) were engineered to present the T7 polymerase gene under the control of a *lac* promoter derivate L8-UV5 lac [64]. The derivate L8-UV5 *lac* promoter contains three point mutations, which increase promoter strength, decrease its dependence on cyclic AMP and creates a stronger promoter that is less sensitive to glucose. These modifications permit a strong induction of T7 RNA polymerase using IPTG as inducer [65].

3.1.3. Carrier Proteins and Secretion Signals

It is not surprising that expressing an antimicrobial peptide in a prokaryotic system can lead to the death of the hosts strains themselves. The use of a carrier protein helps minimizing the toxic effects of the expressed defensin in the host cells. Because of its reduced size, defensins are prone to be degraded by intracellular proteases. Such degradation can be overcome by fusion of a carrier protein to the heterologous peptide. The carrier protein mimics the *E. coli* native proteins pro-segments protecting the fused protein from intracellular proteases attack.

The two most often used carriers for soluble expression in *E. coli* are the thioredoxin (TRX) and the glutathione-S-

Table 2. ***Escherichia coli*** **strains used in plant defensin heterologous expression.**

Bacterial Strain	Features	Manufacturer	References
BL21 (DE3)	High Transformation efficiency, IPTG induction, deficient of Lon and OmpT proteases. Suitable for non-toxic products expression.	Novagen/ Stratagene	[69, 70]
BL21 (DE3)-pLysS	Same as BL21 (DE3) with the addition of a plasmid, pLysS, which lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following IPTG induction. It is therefore suitable for expression of toxic genes.	Novagen/ Stratagene	[35, 58, 73, 74]
Origami 2	Origami™ 2 host strains are K-12 derivatives that have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, which greatly enhance disulfide bond formation in the *E. coli* cytoplasm and are recommended only for the expression of proteins that require disulfide bond formation for proper folding.	Novagen	[8, 19, 75]
Origami B	Origami B host strains carry the same trxB/gor mutations as the original Origami strains, except that they are derived from a lacZY mutant of BL21.	Novagen	[76]
Origami B (DE3)pLysS	Same as Origami B with the addition of a plasmid, pLysS, which lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. Thus it is suitable for expression of toxic genes.	Novagen	[5, 77]
Rosetta (DE3)	Rosetta host strains are BL21 lacZY (Tuner) derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA on a compatible chloramphenicol resistant plasmid. The tRNA genes are driven by their native promoters.	Novagen	[51, 78]
Rosetta (DE3)pLysS	In Rosetta (DE3)-pLysS, the rare tRNA genes are presented on the same plasmids that carry the T7 lysozyme.	Novagen	[55]
Rosetta-gami(DE3)LysS	Rosetta-gami host strains are Origami derivatives that combine the enhanced disulfide bond formation resulting from trxB/gor mutations with enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli.* These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, GGA on a compatible chloramphenicol-resistant plasmid. The tRNA genes are driven by their native promoters. In Rosetta-gami(DE3)-pLysS, the rare tRNA genes are presented on the same plasmids that carry the T7 lysozyme.	Novagen	[55, 79]
BL21 CodonPlus	BL21-CodonPlus-RIL chemically competent cells carry extra copies of the argU, ileY, and leuW tRNA genes. The tRNAs encoded by these genes recognize the AGA/AGG (arginine), AUA (isoleucine), and CUA (leucine) codons, respectively.	Stratagene	[8, 80, 81]
AD494	AD494 strains are thioredoxin reductase (trxB) mutants of the K12 strain that enable disulfide bond formation in the cytoplasm, providing the potential to produce properly folded active proteins.	Novagen	[82]
BL21trxB	BL21trxB strains possess the same thioredoxin reductase mutation (trxB) as the AD494 strains in the protease deficient BL21 background. The trxB mutation enables cytoplasmic disulfide bond formation.	Novagen	[8]

transferase (GST). Bogomolovas *et al.* [66] demonstrated that among 13 tested fusion partners, TRX presented the highest absolute yield. Thioredoxin is a resident *E. coli* protein that when fused to a heterologous peptide enhances the cytoplasmic solubility of the fusion protein keeping it from precipitating as inclusion bodies. The increase of the solubility is required for the proper formation of native disulfide bonds, also improving significantly the fused protein yield [67]. Fusing the thioredoxin to a sunflower defensin (HaDEF1), Zélicout *et al.* [20] were able to recover soluble and active peptides from *E. coli* cell lysate.

Despite many successful results with other antimicrobial peptides [68, 69], GST fusions are highly susceptible to proteolytic degradation resulting in inefficient protein production in *E. coli* [70]. To date, no carrier protein has been directly correlated with a higher yield of recombinant peptides. It has been reported that the effect of carrier proteins may vary according to the specific protein fused to it [71].

Some secretion signals can be added to the recombinant defensin to direct its allocation in the *E. coli* periplasm, or even direct the recombinant peptide migration and biological activity to the cytoplasm of a target cell [70, 72], but carrier

Table 3. Heterologous expression in *Pichia pastoris* Vs. *Escherichia coli*.

Organism	Pichia pastoris	*Escherichia coli*
Transcription regulation	Using the AOX promoter, the transcription of the foreign gene can be tightly regulated.	Most of the vectors present a detectable basal transcription of the recombinant gene.
Protein purification	Proteins can be easily secreted in the culture medium, there for; it can be purified in a single step.	Proteins usually need to be purified from cell lysate or inclusion bodies, which can be hard laboring.
Protein folding	Eukaryotic proteins with disulfide bonds can be correctly formed with no extra treatment.	A refolding procedure may be necessary, especially when the expressed protein is in the IBs.
Strains	*Pichia* still present a limited variety of expression strains.	There is a great list of engineered E. coli strains to choose accordingly to your goal.
Expression vectors	There is a single commonly used expression vector (integrative plasmid).	Many options of expression vectors available.

proteins and secretion signal are not infallible. Kovalskaya and Hammod [61] constructed cassettes using the pET26b expression vector and two plant AMPs, a snakin (SN1) and a defensin (PTH1) for heterologous production in *E. coli*. The pET26b vector was chosen due to its N-terminal *pelB* signal, which was supposed to provide a periplasmic location for the recombinant protein, for better folding and solubilizing condition. The strategy failed and all the recombinant protein was found to be concentrated in the IBs.

3.1.4. Inclusion Bodies

The recombinant defensin aggregated in inclusion bodies can be another strategy to concentrate all the expressed peptides and shorten the purification steps. Applying the appropriate washing condition allows the isolation of the IBs containing more than 90% pure recombinant protein [73]. Some works purposely add aggregation-inducer carriers to drive the formation of IBs, achieving a recombinant protein level of 30% of the total cell protein [74]. Kovalskaya and Hammond [61] solubilized and refolded the recombinant defensin stored in IBs using the reducing agent DTT (Dithiothreitol) and obtained an active molecule, able to inhibit bacterial (*Clavibacter michiganensis*) at 7 μM and fungal (*Colletotrichum coccoides*) growth at 14 μM concentration.

Some peptides can be fused to your construction to enhance IBs formation. Expressing a peptide fused to a hexahistidine tagged ketosteroid isomerase (to be removed enzymatically later) increases the insolubility of the produced peptide, leading its allocation in IBs, which can be purified later, achieving a high productivity of more than 30 mg of purified peptide per gram of dry cell weight. These results are suitable for the production of peptides for scientific research, as it achieves the required scale and purity, even for biological assays used in therapeutic peptides research [72].

CONCLUDING REMARKS

All efforts to develop novel or improved techniques that successfully achieve high levels of protein strongly favor bacteria as a host for the large scale production. However, it is known that if the peptide to be expressed requires certain types of post-translational modifications, which are not performed in prokaryotes, different hosts for defensin production have to be considered. Moreover, there are some challenges that need to be overcome in *E. coli* recombinant protein production, such as protein insolubility and purification steps.

In some cases, yeasts such as *Pichia pastoris* showed some advantages over *E. coli*, particularly in regard to post-translational modifications (specially, glycosylation), increasing the solubility and producing large quantities of heterologous protein. In addition, *P. pastoris* allows secretion of the recombinant protein, which results in less purification steps; did not require a carrier protein as reported for *E. coli*, which may imply facilitated scale-up processes and, also, can be cultivated at a high cell density with no toxic products or ethanol production using cost-effective culture media. Table **3** shows some important points on plant defensin heterologous expression in *Pichia pastoris* and *Escherichia coli*.

Nevertheless, there is a limited variety of plant AMPs produced in *P. pastoris* with the amount of recombinant peptide produced varying from 55 μg to 748 mg.L−1 of culture media [75]. Thus, *P. pastoris* is definitely a good choice for host expression, when the *E. coli* expression system was not satisfactory, although it still requires the establishment of a variety of promoters and strains in association with process development in order to achieve higher yield of protein.

CONTRIBUTION OF AUTHORS

LRSG, did the literature search and wrote the manuscript. VP and ALSJ, helped in the literature search and reviewed the draft manuscript. SC and AMBI edited the manuscript and assisted as consultants on defensins and plant defensins, respectively. ACF, mastermind of the manuscript, structured the review, revised the draft and final manuscript. All authors approved the final version of the manuscript.

LIST OF ABBREVIATIONS

HES = heterologous expression systems

AMPs = Antimicrobial Peptides

PpDFN1 = Prunus persica defensin

ePC	=	egg-phosphatidylcholine
Psd1	=	Pisum sativum defensin
Tfgd2	=	*Trigonella foenum-graecum* defensin 2
RsAFP2	=	*Raphanus sativus* antifungal protein 2
DsbC	=	disulfide-bond isomerase
GST	=	protein glutathione-S-transferase
trxB	=	thioredoxin reductase
gor	=	glutathione reductase
IBs	=	inclusion bodies
TRX	=	thioredoxin
Ha-DEF1	=	sunflower defensin
DTT	=	Dithiothreitol.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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