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In Silico Identification of Plant-Derived Antimicrobial Peptides

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1. Introduction

The widely available digital information about plant genomes and its products has triggered the use of bioinformatics and other *in silico* approaches over gene expression data, from genome to phenotype-based analysis methods. In such data universe, special attention has been given to a peptidic group of plant bioactive molecules, the antimicrobial peptides (AMP), usually small cysteine or glycine-rich peptides antagonistic to several pathogens and component of plant innate defense. Main classes of AMPs comprise defensins, thionins, lipid-transfer proteins, cyclotides, snakins and hevein-like, according to amino acid sequence homology.

Plant biodiversity for antimicrobial peptides search has led to increasing efforts on their identification and characterization, although such biodiversity still remains largely unexplored for drug development and other potential applications. As expected, crop species have been more frequently targeted for AMP research and application, mainly due to higher availability of molecular data (Pestana-Calsa et al., 2010). AMPs provide novel strategies not only in medicine but can potentially increase agricultural yields by phytopathogen or pest control. Those small peptides have wide-range inhibitory activity over phytopathogenic microorganisms (mostly fungi), as already comproved in enhanced crop resistance to pathogen attack through genetic breeding and transgenic manipulation (Terras et al., 1995).

Last decade advances in gene expression studies technology brought huge amounts of genomic, transcriptomic and proteomic datasets, a fruitful field for AMP prospection. Based on ethnobotanical or even laboratory information, literature concerning *in silico* analyses of plant antimicrobial peptides is much smaller than that focusing plant secondary metabolites. Additionally, bioinformatics tools are usually constrained to model species, whose "-omics" datasets are available, justifying many heterology-based studies for non-model species. Thus, data integration is highly desired and helpful to apply bioinformatics approaches aiming the *in silico* screening of genomic, transcriptional, proteomic and metabolomic datasets from cultivated or wild plant species. Preliminary analyses have been commonly linked to subsequent molecular techniques to identify and characterize AMP-coding genes, their products and their regulation, besides to validate putative functional aspects in a systems biology approach. For instance, in silico analyses of AMPs and respective coding genes determine the way by which different sequences can affect specific pathogens (Pestana-Calsa etal., 2010).

This chapter focuses on bioinformatics methods, usually associated to molecular biology tools, to prospect databases, identify and characterize putative or known AMPs, and

discusses procedures for testing them *in vitro* and *in vivo*. Regarding applications, molecular data must be generated in large scale for a comprehensive set of plant species which have not been addressed up to date in AMP research field. "-Omics" based experimental procedures will then be more efficient and reliable, making easier the application of scientific knowledge from molecular biology and bioinformatics on medicine, agriculture and industry needs.

2. Bioinformatics on AMP identification

The huge amount of available information over plant secondary metabolites effects on human health has demanded the usage of bioinformatics tools on transcriptomic, proteomic and metabolomic profiles from different plant species, concerning single as well as mixtures of phytochemicals (Ulrich-Merzenich et al., 2007). Indeed, most results experimentally obtained for bioactive antimicrobial secondary metabolites might be also derived from correlated AMPs sinergy (Verpoorte at al., 2005). Nowadays increasing number of described plant antimicrobial peptides, in parallel to exponential growth in nucleotide and protein data for public access, allows several possibilities of potential identification of novel AMP. Preliminary analyses have been commonly followed by molecular methods to characterize AMP-coding genes and its regulation. Here, *in silico* studies of AMP and respective coding genes contributed to unravel their functional aspects (Hammami et al., 2009).

2.1 Search by genomics and transcriptomics

Searches in genome and transcript sequences datasets have shown to be a comprehensive and higher yield initial step for seeking candidate AMP-coding genes, mostly due to the typical large-scale coverage of the organism genetic potential in such analyses. *In silico* starting approaches constitute a way to quickly achieve reliable AMP-coding potential of the studied plant species, even if it requires further biological validation.

Genomic analyses through DNA sequencing and mapping have been useful to AMP prospection. Considering model organisms, most green plant species genomes harbor 15-50 defensin-coding genes, even if this number is an under-estimation according to some authors and may grow by further studies on several other plant species genome-wide expressed sequence tag (EST) libraries (Silverstein et al., 2005; 2007; Manners, 2007). In Arabidopsis, 317 defensin-like sequences could be assigned in the genome by hidden Markov models of in silico search (Silverstein et al., 2005).

Actually, a comprehensive search on standard publicly available gene expression databases for plant transcript sequences provides a relatively trustable picture of annotated and putative AMP- related sequences from plants (Table 1). Plant AMPs represent almost 16% of deposited AMP sequences and, considering all the organisms as well as just plants, lipid transfer proteins constitute the most abundant group, what may be an over-estimation (Pestana-Calsa et al., 2010) since they are not only involved in direct plant defense (Chen et al., 2008; Boutrot et al., 2008). Noteworthy, snakins and heveins appear as plant-representative peptides, while defensins and lipid transfer proteins have relatively similar distribution that when considering all organisms. In opposite, thionins accesses are about 4 times more abundant in plants than in all organisms dataset, and plant cycletides occur in about 3 times smaller frequency than for all organisms in GenBank, what may represent under-estimation.

AMP main group	All organisms	Green plants	(%)
Defensin	4,506	755	16.8
Thionin	1,318	780	59.2
Lipid transfer protein	54,485	13,139	24.1
Cycletides	10,497	591	5.6
Snakin-like	13	13	100.0
Hevein-like	1,434	1,043	72.7
Other	31,251	160	0.5
Total	103,504	16,481	15.9

Table 1. Number of AMP-related accesses in GenBank/NCBI/Entrez, identified as AMP main groups (from Pestana-Calsa et al., 2010).

Concerning reference available plant EST collection, the TIGR Plants Gene Indices (http://compbio.dfci.harvard.edu/tgi/plant.html) is useful to achieve distribution of AMP-related transcript sequences from crop and weed/pasture species (Figure 1). From the 14 botanical families represented by 34 species, ten are important commodities as soybean, maize, sugarcane, orange and coffee. It suggests grasses and solanaceous are snakins richer, while legumes (Graham et al., 2004) have less thionin coding genes.

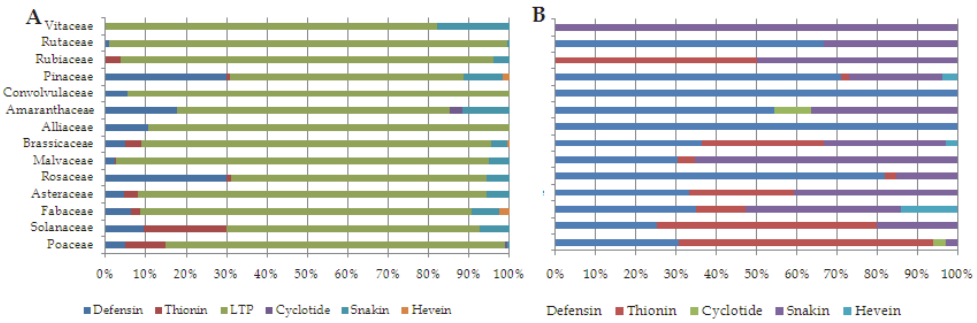

Fig. 1. Distribution of plant EST unigenes (clusters + singletons) from TIGR Gene Indices putatively annotated as AMPs. Frequencies were considered including (A) or not including (B) the putative lipid-transfer proteins (LTP) coding transcripts. Data in each botanical family derive from: Poaceae (*Hordeum vulgare, Zea mays, Oryza sativa, Secale cereale, Sorghum bicolor, Saccharum spp., Panicum virgatum, Festuca arundinacea, Triticum aestivum*); Solanaceae (*Capsicum annuum*, *Petunia hybrida*, *Solanum tuberosum*, *Nicotiana tabacum*, *Solanum lycopersicum*); Fabaceae (*Phaseolus vulgaris*, *Medicago truncatula*, *Phaseolus coccineus*, *Glycine max*); Asteraceae (*Lactuca sativa*, *Lactuca serriola*, *Helianthus annuus*); Rosaceae (*Malus_x_domestica*, *Prunus persica*); Malvaceae (*Theobroma cacao*, *Gossypium*); Brassicaceae (*Brassica napus*); Alliaceae (*Allium cepa*); Amaranthaceae (*Beta vulgaris*); Convolvulaceae (*Ipomoea nil*); Pinaceae (Pinus); Rubiaceae (*Coffea canephora*); Rutaceae (*Citrus sinensis*); and Vitaceae (*Vitis vinifera*). Original data from Pestana et al. (2010).

Such type of analysis usually constitutes an initial step in antimicrobial peptide research in plants. By allying *in silico* tools to predict potential AMP-coding genes with "wet"-bench procedures like PCR-based methods using specific designed oligonucleotides to amplify AMP-related regions from genomic DNA, novel grass and legume β-defensins have been

recently identified from sugarcane (*Saccharum* spp.; Padovan et al., 2009) and cowpea (*Vigna unguiculata*; Padovan et al., 2010a). These novel peptides expression was co-related to fungal infection and drought responses, suggesting biotic and abiotic responsiveness for defensin activation.

Computational search matches have provided growing helpfulness for AMP genes identification in concluded and current DNA sequencing projects, as in large transcript profiling datasets, however the predicted defense-related genes must be functionally tested (Pestana-Calsa et a., 2010; Belarmino et al., 2010). In spite of rare use, in situ hybridization methods for AMP studies in plants keep very promising to analyze the spatial and temporal expression of such peptides in several organs and tissues, under a wide range of treatments (Tavares et al., 2008).

Genomics and transcriptomics platforms allow the development of strategies to identify distinct classes of antimicrobial peptides. In pepper (*Capsicum annuum*), an AMP was identified and named CaAMP1, which was isolated in a cDNA library from leaves inoculated by *Xanthomonas campestris* pv. *vesicatoria*. CaAMP1 expression was induced in leaves during pathogen infection and after abiotic stressing (Lee & Hwang, 2009). A cDNA sequence, named VvAMP1, was isolated from grape (*Vitis vinifera*) berries and coded for a 77 amino acid peptide homologous to defensins (de Beer & Vivier, 2008); also in grape, comprehensive *in silico* searches have successfully resulted in AMP candidates (Zamyatnin & Voronina, 2010).

Transcripts tag sequences from different large-scale gene expression analyses, like cDNA microarray and SAGE, have been assigned to plant AMP. For instance, cDNA microarray was used to validate regulation of known AMP genes after hormone signalling in defense responses (Wan et al., 2002). On the other hand, SAGE-derived approaches have resulted in suitable databases for AMP detection, as like in sugarcane (Calsa Jr. & Figueira, 2007) and soybean; in both, tens of transcripts have been putatively annotated as AMP-like and are under experimental validation (data not shown).

2.2 Search by proteomics

Plant genomic and post-genomic researches have generated large amounts of information that contributes to understand gene and protein expression profiles, besides their connection to biological processes. Proteomics results in important data over biological systems, because it produces information about proteins and peptides, the major functional and structural determinants of cells (Baginsky, 2009). Conventional two-dimensional gel electrophoresis (2-DE) to separate and visualize proteins, and mass spectrometry (MS) to identify proteins and peptides of interest, have been applied. Some 2-DE limitations concerning AMP analysis refer to retention and visualization on gel of target proteins with molecular weight lower than 10 KDa (Baggerman et al., 2004) and frequent production in small quantities by plant cells (Antunes, 2008).

Small protein and native peptide component of plant tissues is a still neglected proteomic area, and relatively few studies are available (Zhang et al., 2006). Peptidomics have improved the study of such small polypeptides, by coupling bi or multidimensional liquid chromatography to mass spectrometry, where high complexity or low concentrated samples can be efficiently separated via multidimensional liquid chromatography (MDLC), whose advantage over 2-DE is separation of complex mixtures by using multiple columns (Barbosa, 2008). Even so, conventional 2-DE followed by MS has also provided important antimicrobial subproteome/subpeptidome information associated to specific plant samples,

as soybean xylem sap (Djordjevic et al., 2007), and to plant-virus interaction, as from *Capsicum annuum* cv. Bungang (hot-pepper) infected by tobacco mosaic virus (TMV) (Lee e al., 2006). Differentially expressed pathogenesis-related proteins and peptides, as defensin, from sugar beet infected by beet necrotic yellow vein virus (BNYVV) were separated by MDLC, identified by MALDI-TOF/MS and annotated through homology-based search using amino acid sequence deduced from the MS/MS spectra (Larson et al., 2008).
An interesting perspective on antimicrobial peptides was developed to study the conservation of structural motifs in AMPs from phylogenetically distant organisms using proteomic bioinformatics datasets and tools, like PROSITE (ExPASy Proteomics Server, 2011), for multidimensional signature model for AMPs (Yount & Yeaman, 2004). Modeling is an attempt to unify structural domains in distinct classes of AMP.
Additionally, proteomics approaches have been successful in the identification of signalling elements that regulates defensins in plants (Widjaja et al., 2010).

2.3 AMP databases

Hundreds of known AMP sequences are available at UniProtKB/Swiss-Prot, while other repository collections are in other accessible links (Table 2). The major ones are ANTIMIC (Brahmachary et al., 2004), Antimicrobial Sequence database AMSDb (eukaryotic), Peptaibol (fungal, Whitmore & Wallace, 2004), APD (Wang & Wang, 2004) and APD2 (Wang et al., 2009). Among them, the PhytAMP (http://phytamp.pfba-lab.org), a database specific for plant AMPs, was developed providing access to informations regarding studies and applications for these peptides (Hammami et al., 2009), and organizing sequences and corresponding taxonomic, physicochemical, structural, taxonomical and publications over each deposited AMP.
Bioinformatics tools have been developed in agreement to "–omics" databases requirements. An example was the alignment of known antibacterial peptides aiming to detect preferential residues by artificial neural network, since certain amino acids are more frequent in some positions, particularly at the N or C terminus (Lata et al., 2007). Other successful advances resulted from enlargement of AMP catalogs, basically through clustering and alignment to previously annotated sequences; this approach has been quite useful on putative AMPs identification in comprehensive datasets (Fjell et al., 2207). Statistical modeling is applied over genomic, transcriptional and proteomic data in the aim to identify peptides, also domains from already functionally annotated proteins, which present significant motifs and/or structure match to AMP (Nagarajan et al., 2006).

Database	Web site (http://)	Organisms (Reference)
AMSDb	www.bbcm.units.it~tossi/pag1.htm	Eukaryotes (Tossi & Sandri, 2002)
ANTIMIC	research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC	General (Brahmachary et al., 2004)
APD	aps.unmc.edu/AP/main.html	General (Wang & Wang, 2004)
APD2	aps.unmc.edu/AP/main.php	General (Wang et al., 2009)
APPDb	ercbinfo1.ucd.ie/APPDb/	general
Defensins	defensins.bii.a-star.edu.sg/	general
PenBase	penbase.immunaqua.com/	general (Gueguen et al., 2006)
Peptaibols	www.cryst.bbk.ac.uk/peptaibol	Fungi (Whitmore & Wallace, 2004)
PhytAMP	http://phytamp.pfba-lab.org	Plants (Hammami et al., 2009)
SAPD	oma.terkko.helsinki.fi:8080/~SAPD	General (Wade & Englund, 2002)

Table 2. URLs for main available databases for characterized antimicrobial peptides (modified from ExPASy Proteomics Server and Pestana-Calsa et al., 2010).

As expected, economically relevant crop plant species have commonly been the main target for antimicrobial peptides research and biotechnological application, due to associated agricultural and social demand and impact, but also because the higher availability of molecular data. In spite of such trend, several research efforts have pointed a huge potential for wild plant species prospection to achieve discovery of novel AMPs, as well as the identification of novel biological functions to known AMPs. Hence, plant biodiversity in different biomes and ecosystems is an outstanding promising focus on antimicrobial peptides investigation (Pieters & Vlietinck, 2005).
A very recent revised list of experimentally confirmed plant AMP, with physicochemical and antibacterial specificity is also available, comprising members of the main families: 23 defensins, eight hevein-like, six vicilin-like, five knottins, four cyclotides, two Impatiens-like, two sepherins, two snakins, one MBP-1 (from maize), one glutamate-rich, one glycine-rich, and other eight peptides not classified in these structural families (Pelegrini et al., 2011).

3. Structural analyses and function

An example of the lack in plant AMP cataloguing is the organized database for antibacterial phytochemical compounds hosted and managed by Kioto Encyclopedia of Genes and Genomes (KEGG Compound, Plant Secondary Metabolites), where the related biosynthetic enzymes and molecular targets in human organism are fully described. However, no plant-derived AMP or coding gene sequence is set, since the database allows the access only to human microbicidal and cytotoxic peptides.
Bioinformatics have been essential on plant AMP prospection and establishment of catalogues, which have increased in last decade (Hammami et al., 2009). Several sequence aligment tools, usually BLAST-based (www.ncbi.nlm.nih.gov/blast) are available, but other main technical approaches have included *in silico* structural peptide prediction and putative analyses of AMP-target molecule interactions. Last but not least, direct chemical isolation have also depended of *in silico* tools to achive more complete structural and functional characterization of isolated AMP.

3.1 Isolation

Complementarily to the several classes of chemical compounds synthesized by plants secondary metabolism, with proved antimicrobial effects, the characterization of plant AMPs function correlated to such effects is extremely useful to improve research in this area. Although very likely, supposing that AMPs are co-responsible or even responsible for antimicrobial activity of complex plant extracts relies on sub-fractioning and experimental evidence (Moreira et al., 2011). Direct isolation of plant AMPs has succeeded in several species (Kovaleva et al., 2009; Rogozhin et al., 2011), associated to further cDNA cloning and characterization as defensin and lipid transfer protein. Also, extraction and purification of candidate peptides, followed by sequencing and match to AMP databases, allowed the identification of novel promising antimicrobial molecules in wheat (Odintsova et al., 2009).
Efforts to prospect AMPs in plant crop species and many examples of direct isolation followed by functional assays and characterization are available. As alternative to fungicide usage to pathogen control in agriculture, several studies have focused on searching for plant proteins and peptides with antifungal activities (AFPs). Recently, two novel 10 and 15 kDa

AFPs were isolated from rosemary pepper (*Lippia sidoides* Cham.) flowers through Octyl-Sepharose hydrophobic column separation, and were able to inhibit the development of *Botrytis cinerea*, an economically harmful phytopathogen for many crops (Moreira et al., 2011). The N-termini sequences of these AFPs have homology with NBS-LRR R proteins, well known plant defense elements.

A 11,500 heterodimeric antifungal protein, named Pa-AFP1, highly similar to 2S albumin family, was purified by anionic exchange Q-Sepharose chromatography associated with HPLC reversed-phase C4 chromatography and structurally confirmed as dimer by MALDI-TOF spectra analyses (Ribeiro et al., 2011). It inhibits the growth of fungus *Colletotrichum gloeosporioides*, but no antibacterial nor anti-yeast activity was observed. an antiviral 2 KDa peptide was purified from sorghum seeds by gel filtration, ion exchange and high-performance liquid chromatography (HPLC), and showed strong inhibition of herpes simplex virus type 1 (HSV-1) and bovine herpes virus (BHV) replication (Camargo-Filho et al., 2007). On the other hand, two anti-yeast peptides were isolated from seeds of a phytobacterial-resistance pepper (*Capsicum annuum*) genotype and identified by amino acid sequencing (Ribeiro et al., 2007). Another research identified peptides with bactericidal activity from sesame (*Sesamum indicum*) kernel flour; one of them, with 5.8 KDa, showed activity only against *Klebsiella* sp., a Gram-negative bacterium causal of human urinary infection (Costa et al., 2007). More detailed structural and functional results were achieved for a cowpea seed γ-thionin/defensin, a wide-spectrum bactericide whose primary structure, mechanism of action and tissue localization during germination provided the understanding of these bioactive peptides in plant defense responses (Franco et al., 2006).

An example of how native or introduced plant biodiversity may be a fruitful option on AMP research is the recent growing number of such peptides identified in Brazilian species. Direct purification was achieved in originally African legume *Crotalaria pallida*, a widely dispersed weed in South America and abundant in drought and warm "caatinga" biome. A novel peptide structurally similar to defensin/2S-albumin was isolated from seeds and presented inhibitory effects over bacteria (Pelegrini et al., 2008). From seeds of guava (*Psidium guajava*) and passion fruit (*Passiflora edulis*), the antifungal and antibacterial peptides Pg-AMP1, passiflin and a 2S albumin-like were isolated. PgAMP1 comprises approximately 6 KDa of molecular mass and small amounts of a homodimer; amino acid sequencing indicated it belongs to glycine-rich plant protein family, being the first one having activity towards Gram-negative bacteria; instead passiflin and the 2S albumin-like peptide show high antifungal properties (Pelegrini et al., 2006; Lam & Ng, 2009).

Several plant species from Atlantic rainforest and "cerrado" biomes have been studied to confirm and explain their antimicrobial activity as transmitted popular medicine (reviewed in Pestana-Calsa et al., 2010). For instance, out from 32 plant species selected after field survey, extracts from 13 species presented antimicrobial activity against *Staphylococcus aureus*, but further analyses to identify potential peptides involved in such activity keep lacking (Brasileiro et al., 2006; Silva Jr. et al., 2009). Molecular and bioinformatics approaches could start from applied phytochemical researches similar to these and is very likely that known and novel AMPs could be found.

3.2 Structural and functional analysis

As any peptide, AMP function is strictly dependent of strucuture. Specifically for relatively abundant α-helical AMPs, structural features and physicochemical properties have been

targeted to increase antimicrobial activity, usually by changing molecular size and charge, residues arrangement, hydrophobicity, amphipathicity and helix folding probability (Tossi et al., 2000; Tian et al., 2009). Antimicrobial peptides structure/activity ratio results in their molecular diversity, but they do share some common features, as the low molecular weight and the variable number of disulphide bond-cysteines residues stabilizing conserved scaffolds (Padovan et al., 2010c), which is used to group them into different structural classes, as depicted in Figure 2.

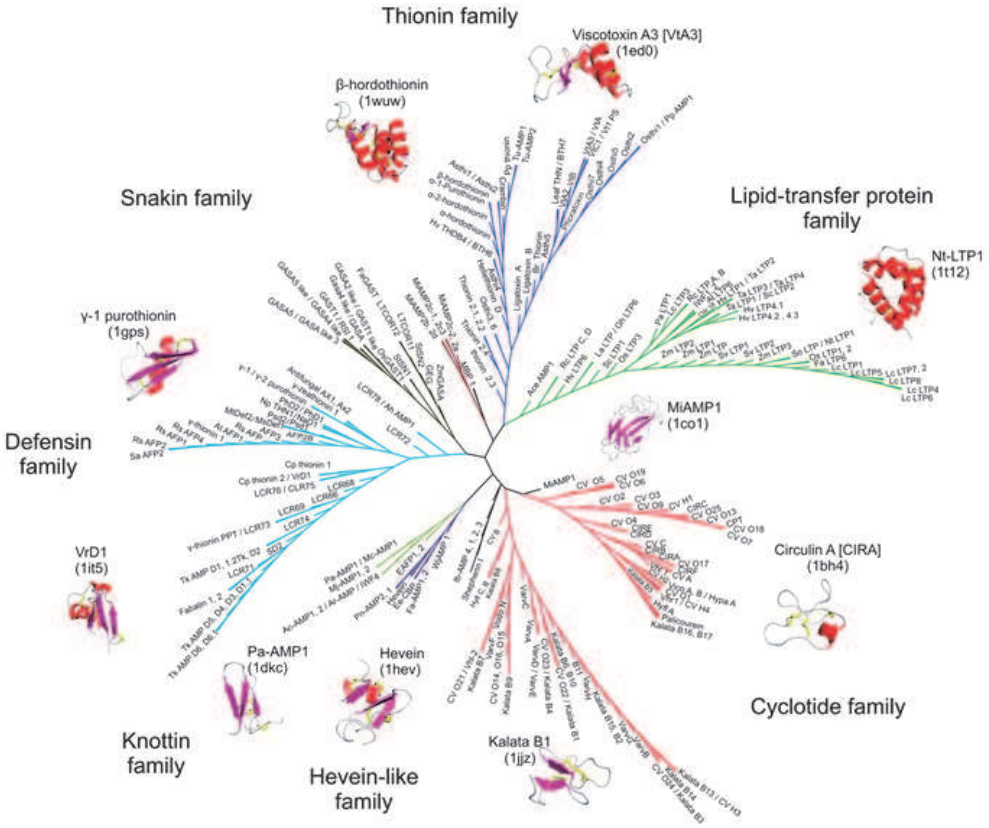

Fig. 2. Structural and phylogenetic representation of plant AMPs compiled in PhytAMP database. α-helices and β-sheets are respectively shown in red and purple (from original by Hammami et al., 2009)

Aiming to achieve more effective peptides, natural/original sequences may be re-arranged (Boman et al., 1989). Several protein databases and analyses tools (e.g. Swiss-Prot and links) are available, as softwares designed specifically to deal with predicting and modifying peptide structure and physicochemical improvement as ArgusLab, Anthewin and Peptool (detailed usage described by Wang, 2007; Hao et al., 2008). Simulations within *in silico* environments have been applied to test AMPs topology concerning biological effects and to unravel their probable mode of action, usually by interactions that destabilize lipid bilayers

(e.g. maculatin, that changes the lysis mechanism depending on lipid structure of target membrane; Bond et al., 2008).

Sometimes, naturally occurring AMP structure is not the most effective. The influence of 2 disulfide bonds in a small β-sheet AMP (Ib-AMP1) from *Impatiens balsamina* seeds was verified as not essential for antimicrobial activity, because the synthetic linear analogs displayed by 4.8-fold higher inhibitory specificity than the wild-type peptide (Wang et al., 2009). Also the seed antimicrobial peptide Cy-AMP1 from *Cycas revoluta* was analyzed on its chitin-binding ability, a well-conserved feature in other AFP as knottin and hevein (Yokoyama et al., 2009): antifungal activity of the peptide was strongly reduced after variations in chitin-binding motifs.

Both MsDef1 and MtDef4 defensins, potent growth inhibitors of several filamentous fungi including *Fusarium graminearum*, induced plasma membrane permeabilization; however, MtDef4 is more efficient based on its unique γ-core motif, indicating that it defines specific antifungal properties of each defensin, and so may help de novo design of more effective AMP (Sagaram et al., 2011). The barley derived α-Hordothionin (αHTH), another membrane-permeabilizing peptide with broad-range antimicrobial activity, is supposed to act as small water-selective channel, through the αHTH double α-helix core when the peptide interacts with anions (Oard, 2011); conserved cysteine and tyrosine residues lined pore walls, resembling aquaporins that delivers water molecules to the lipid bilayer center, what may lead to localized membrane disruption.

The well known chitin-binding lectin hevein has served as template to synthetic mutant peptides that interact with chitin oligosaccharides (main components of fungi cell walls), but mutant AMP versions may present decrease in the association kinetics to target chito-oligosaccharides (Chávez et al., 2010). Such results, provided by nuclear magnetic resonance followed by image modelling analysis tools, pointed that mutant and parent Hev32 peptides three-dimensional structures were quite similar, including orientations of the three key Trp aromatic residues; hence, it was supposed that the mutant lower affinity relied on distinct topology orientation of key side chains and protein-sugar intermolecular essential hydrogen bonds.

Structural and functional design of AMPs from plants manipulated to be active in plants must attend to the agricultural demand for novel antimicrobial compounds, more specifically in plant disease control, with lower toxicity to consumers and environment (reviewed by Montesinos & Bardaji, 2008). In medicine and therapeutics for infectious diseases, AMPs effects on human cells can be widely verified in different gene expression levels, allowing to accurately confirming expected antimicrobial action without undesirable side effects (Ulrich-Merzenich et al., 2007). Also, synthetic peptides engineering have contributed with novel insights for antimicrobial drugs and treatments, by testing in vitro and in vivo several amino acid sequences putatively harbouring antagonistic effects to microorganisms (Choi & Moon, 2009).

Although every class or family of AMP has specific features in structure, activity and potential technological applications, few are so remarkably intriguing as the generally named cyclotides, very stable plant cyclic peptides which harbour many potential technological usages in pharmaceutical and agricultural strategies, according to their various bioactivities and fitness as protein-engineering templates (Henriques & Craik, 2010). Cyclotides compise the largest known family of head-to-tail cyclic peptides, have approximately 30 amino acid residues with a complex structure containing a circular peptide backbone and a cystine knot. They are mainly found in plants of Violaceae and

Rubiaceae families, and supposed to act mostly in plant protection. In addition to insecticidal properties, cyclotides may have cytotoxic, anti-HIV and antimicrobial effects, among other activities as to inhibit neurotensin binding ability (Gerlach et al., 2010). A cyclotide in alkaloid fraction from root bark of *Discaria americana* (Rhamnaceae) was isolated and structurally determined, but did not inhibit the growth of challenged bacteria significantly (Giacomelli et al., 2004). However, other cycletides extracted from *Scutia buxifolia*, also a Rhamnaceae family member, were much more efficient to inhibit bacterial cells than *Discaria*-derived cycletides, although no antifungal effect was observed (Morel et al., 2005).

Regarding prospection of structure-function clues, cyclic peptides isolated from Euphorbiaceae species have been intensively studied due to their rigid three-dimensional conformation, considered to be essential for bioactivity over lipid membranes (Barbosa et al., 2011). As example, cyclotide labaditin and derived synthetic open chain analogs were chemically and virtually analysed in comparison over their interaction with lipid bilayers, and results suggested the native labaditin had greater membrane insertion. A possible mechanism for this is based on initial hydrophobic interaction with the lipid membrane followed by conformational change, peptide adsorption and internalization; indeed, native labaditin reduced viability in Gram-positive bacteria (Barbosa et al., 2011).

Some plant AMPs have so many unique structural features which impair its insertion into any previous, well-characterized AMP family. This is the case of antimicrobial peptide Ib-AMP1, formed by a 20-residue disulfide-linked beta-sheet and usually found in the seeds of *Impatiens balsamina*. Using it as template molecule, synthetic analogs were obtained in order to check the relevance of 2 disulfide bonds on the antimicrobial activity and specificity (Wang et al., 2009).

Beyond conventionally detected cationic antimicrobial peptides, there are also anionic antimicrobial peptides/proteins (AAMPs), reported since 1980s and accepted as important components of innate immune systems of plants and animals (Harris et al., 2009). AAMPs present activity against bacteria, fungi, viruses and insects, but noteworthy their antimicrobial activity is believed to be secondary. Structures vary from alpha-helical peptides in amphibians to cyclic cystine knots in some plant peptides, and certain AAMPs are suggested to link metal ions forming cationic salt bridges with negatively charged lipids of microbial membranes. In bioinformatics context, softwares and analysis parameters must be adjusted and present enough flexibility to cope in a suitable manner with such huge structural and charge inversion, if compared to data obtained from "conventional" antimicrobial peptides dynamics and biological function.

3.3 Heterologous expression

Several AMPs, mostly defensins, have been studied through gene cloning and expression in heterologous systems (Kovaleva et al., 2011). In this context, perhaps one of the main contributions of bioinformatics is the codon-usage optimization for heterologous expression of AMPs, depending on host organism.

Heterologous expression of AMPs also refers to increasing interest for production of antimicrobial compounds such as the defensins, whose applications include health, agriculture and industry as targets. Specifically to protect food or bio-fuel crops, several strategies have been proposed and tested in order to isolate and produce defensins. Experimental viability is still mostly constrained in academic research, where defensins have been heterogously expressed in bacteria, yeasts, fungi and plants (Padovan et al.,

2010b); on the other hand, (bio)-chemical synthesis is not usual yet for commercial production purposes, and here the most striking challenge is to keep correct protein/peptide folding *in vitro*.

A novel defensin-like peptide, isolated from *Nicotiana megalosiphon*, NmDef02 was heterologously expressed in the yeast *Pichia pastoris*, and the purified recombinant protein was found to display antimicrobial activity in vitro against important plant pathogens. Constitutive expression of NmDef02 gene in transgenic tobacco and potato plants enhanced resistance against various plant microbial pathogens, including the oomycete *Phytophthora infestans*, causal agent of the economically important potato late blight disease, under greenhouse and field conditions (Portieles et al., 2010).

Other type of AMP, a defensin from cowpea seeds was heterologously assessed on its putative alpha-amylase inhibitory action probably involved in protection against pests (Dos Santos et al., 2010). Its cDNA was cloned into plasmidial expression vector and transformed into *Escherichia coli* cells; the recombinant peptide was then purified via affinity chromatography, identified by sequencing and submitted to alpha-amylase inhibition assay together with seeds-isolated defensin. Both peptides inhibited alpha-amylases from weevil (*Callosobruchus maculatus*) but were not able to inhibit mammalian alpha-amylases.

Heterologous expression, supported by comprehensive *in silico* prediction and peptide design tools, will probably keep being one of the most helpful tools for biological research over plant AMP, based on success in literature. However, large scale production of AMP establishment will depend on results of future studies where the main tasks shall be the engineering/re-design of more stable and self-folding peptides, and definition of optimized biotechnological procedure to cost-effectively produce the peptide, as molecular farming transgenic plants.

4. AMP-coding genes promoter analysis

The search on regulatory genomic regions, mainly promoter elements, has presented increasing usefulness to start unravelling the control mechanisms of activation of AMP-coding genes. These are known to be expressed in defense signalling against microbial pathogens, involving several transduction components that depend on the action of hormones as jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA). An *in silico* approach with potential applicability on tracking regulatory pathways of AMP coding genes is their promoter sequence analysis.

It is known that JA and ET signaling pathways are synergistic for activation of AMPs, especially the defensin PDF1.2. The coding pdf1.2 is targeted and expressed after activation by ORA59 transcription factor, a APETALA2/Ethylene response Factor (AP2/ERF)-domain protein, which is dependent of JA and ET signaling pathways. The pdf1.2 promoter contains two GCC boxes that were confirmed to be the ligation site for ORA59 transcription factor, enabling PDF1.2 coding gene to respond simultaneously to both hormones (Zarei et al., 2011).

The characterization of tissue-specific and pathogen-inducible promoters is essential for localized expression of defense-related genes as AMP. Wheat and rice defensin genes expressed in early developing grain and during grain germination were compared regarding their promoters activity, through stable transformation with promoter-GUS reporter fusion constructs (Kovalchuk et al., 2010). Activity was detected mainly in ovary before and at anthesis in both transgenic cereal species, but differences concerning one or other species were

observed in the expression of transgenic constructs in reproductive tissues. Even so, wheat and rice promoters were strongly induced by wounding in leaf, stem and grain.
Specifically for plant lipid transfer proteins (LTPs), which are very unkown on antimicrobial function although being well-known in other cellular activities, the study of gene promoter sequence may reveal defense-related aspects. From a *Vitis vinifera* genomic library 2,100-bp fragment, the coding region and the promoter of a lipid transfer protein 1 (VvLTP1) was screened, revealing several defense-related *cis*-regulatory elements, like MYB-boxes (Laquitaine et al., 2006). The expression of VvLTP1 promoter-GUS fusion construct in *Arabidopsis thaliana* indicated the antifungal response of VvLTP1 from grape.

5. AMP from plant-related microorganisms

The growing number of distinct species whose genomes, transcripts, proteins and other molecular data are being deposited in public databases makes more reasonable to consider antimicrobial peptides produced by other organisms, specially in the cases where these species, although not plants, have strict ecological relationship with host or neighboring plants. Several examples have been described in literature normally including well-known or potential endophytes, which produce AMPs in a predictable symbiotic context. Obviously, the applications derived from this type of research are supposed to be useful within sustainable biological control of pathogens and pests.
The mycelium-forming actinomycetes of the genus Frankia (well known producers of bioactive compounds) are commonly found as symbionts in actinorhizal plants, performing facultative nitrogen-fixing. Bioinformatic analysis of the strains ACN14a, CcI3, and EAN1pec by genomes prediction and by intact cells MALDI-TOF allowed the identification of putative coding regions and molecules associated to cyclic peptides, siderophores, pigments, signaling molecules and specialized lipids, from which some cyclotides and lipid-transfer proteins are considered to be essential for host-endophyte recognizing and to inhibit other competitor microorganisms, as pathogens (Udwary et al., 2011).
Pyoverdines (PVDs), high affinity siderophores well studied in *Pseudomonas aeruginosa*, were searched *in silico* in *P. fluorescens* SBW25 (a plant growth-promoter endophyte) complete but not annotated genome, where 31 genes putatively involved in PVD biosynthesis, transport or regulation, could be identified (Moon et al., 2008). Since pyoverdine-mediated iron uptake is essential for this endophyte, structural analysis of its PVDs was achieved and defined it as a partly cyclic seven residue peptide backbone, which makes the bacteria able to utilize a wide variety of exogenous PVDs.
Another interesting example can be traced for alamethicin, a membrane-active AMP produced by root symbiont fungus *Trichoderma viride* that permeabilises plasma membrane, mitochondria and plastids of *in vitro* cultured plant cells by creating voltage-dependent pores (Aidemark et al., 2010). Cultured plant cells pre-treated with pathogen elicitors did not get resistant to alamethicin, while those treated with cellulase did; this suggested that different membrane lipid composition induced by cellulase may render the cells resistant to alamethicin, in a mechanism where possible cellulase-secreting pathogens (as several phytopathogenic fungi) would suffer alamethicin action in their membranes. Other fungus-derived AMPs have been described as candidates for production and biotechnological uses in plant protection, as the cystein-rich antifungal peptide AcAFP, secreted by *Aspergillus clavatus* (Skouri-Gargouri et al., 2010).

Development of applications derived from plant-related microorganisms AMP in biological control of pathogens and pests will depend on ecological modeling studies, even if it starts from a single AMP being produced by an endophyte but biologically active in the host plant.

6. Novel AMP functions in plants

Relatively recent studies have added new insights in plant-derived AMP functions, other than classical antimicrobial activity. In fact, the ubiquitous presence of AMPs in distantly related taxa allows the concept that novel functions for derived AMP genes and products may have arisen during each species evolutionary history. Literature is rich in well-established as well as still unkown biological functions for AMP in plants. In this context, even no-peptidic biomolecules could be considered in a wider view.

Perillic acid is a terpenoid plant extract with antiinfective and anticancer properties, and is a small cyclic molecule structurally similar to salicylic acid. It is known to cause large-scale membrane thinning, a clearly possible antimicrobial activity through a membrane-lytic mechanism very close to that of AMPs (Khandelia et al., 2010). Indeed, also subproteins or subpeptides may be relevant to antimicrobial activity *in silico* prospection. Plant-specific insert domain (PSI) is a region of about 100 amino acid residues, contained in several plant aspartic protease (AP) precursors. The PSI from potato aspartic protease 1 was purified after heterologous expression, and was able to kill pathogenic spores in a dose-dependent manner, without deleterious effect on host plant, and through lytic interaction with microbial cell wall/or membrane (Muñoz et al., 2010).

In roots of host legume plants, a complex and evolutionary successful symbiosis takes place with nitrogen-fixing *Rhizobium* bacteria. Surprisingly, the bacteria irreversible differentiation to bacterioid form is also dependent of plant factors, nodule-specific cysteine-rich (NCR) peptides, that are driven to bacterial membrane and cytosol (van de Velde et al., 2010). Since NCRs are similar to AMPs, it is very likely that the host plant adopted effectors from innate immune system for symbiosis, resulting in a control mechanism to the endosymbiotic bacteria cell fate.

Other proteins initially thought to be not related to AMPs have been well linked to regulation of defense mechanisms that include such peptides. Examples have been described where specific phosphatases are key responsive proteins to pathogen infection and induce plant defensins (Widjaja et al., 2010). In addition, some AMPs may be effectors as well as regulators of other molecules. Plant lipid transfer proteins (LTPs) are ubiquitous lipid-binding proteins involved in diverse stress responses; for example, 14 LTPs from *Tamarix hispida* Willd. were screened over possible functions in response to various abiotic stresses (Wang et al., 2009). Results showed that all 14 LTPs were expressed in roots, leaves and stems, in different levels according to the organ; also, some of them were induced by NaCl, PEG, $NaHCO_3$, $CdCl_2$ and ABA, suggesting novel roles beyond defense, and in abiotic stress tolerance. In flower buds of *Brassica campestris* L. ssp. *chinensis*, a putative LTP of 103 amino acids was characterized as being a membrane protein with a signal peptide at the N-terminus, and strongly related to pollen viability and male sterility (Tian et al., 2009).

In the endosperm cells undergoing programmed cell death, LTPs have been described as participating in recycling of endosperm lipids, or acting as protease inhibitors to protect growing cotyledons from released proteases (Eklund & Edqvist, 2003).

The mode of action of AMPs on external pathogen or pest cells seems to hide still unknow mechanisms: certain cyclotides are able to increase permeability across nematodes cuticle

layers, suggesting that one action of the such AMPs involves the interaction with the lipid-rich epicuticle layer at the pathogen worm surface (Colgrave et al., 2010).

7. Towards biotechnological applications

Identification of plant defense genes against pathogens and environmental stresses provides novelties to plant breeding, also genetic transformation (Vidal et al., 2003), mainly due cross-activity of distinct organisms AMPs that has significant potential in phytopatology and plant resistance improvement. As already confirmed, insect-derived AMP gene is able to be expressed in plant genome and its product can be correctly sorted in plant cell or tissue. The metchnikowin, a 26-amino acid residue proline-rich AMP from *Drosophila melanogaster*, was used for resistance in barley against pathogenic fungi (Rahnamaeian et al., 2009). In an interfamily transfer, transgenic tobacco (Solanaceae) and peanut (Fabaceae) plants expressed a defensin from mustard (Brassicaceae), effective against to phytopathogenic fungi (Anuradha et al., 2008).

Many examples of structurally manipulated AMP followed by their transfer to crop plants resulted in increased resistance against phytopathogens: about 18 sequence-optimized AMPs have been transfected to plants with beneficial results for agricultural (Marcos et al., 2008; Jan et al., 2010; Ma et al., 2010; Eggenberger et al., 2011). Plant-derived AMPs are not just possible templates for bioactive molecule design, but candidates to nanotechnologies applied to crop protection as inner content of nanocapsules to be used in greenhouses or in the field to enhance plant resistance and or to control phytopathogens, pests or parasitic weeds (Perez-de-Luque & Rubiales, 2009; Pestana-Calsa et al., 2010; Imamura et al., 2010; Choi et al., 2009) or as integral domains fused to another host organ/tissue targeted protein to local delivery of AMPs in transgenics (Bryksa et al., 2010). Enginnered LTPs have the potential to be utilized as scaffolds to design hydrophobic ligand biosensors or to serve as drug carriers (Choi et al., 2007). Inclusion of plastid transformation technology to enhance yield of peptides accumulation in molecular farming approaches is also very expected in next few years (Oey et al., 2009), establishing another alternative to the production of next-generation antimicrobial peptides in plants, from plants or non-plant source organism.

Novel bioactive molecules produced by plant growth-stimulator endophytes have been comproved on suppressing the growth of bacterial and fungal plant pathogens, as the case of TOMM, a thiazole/oxazole-modified microcin produced by the soil bacterium *Bacillus amyloliquefaciens*. TOMM requires extensive posttranslational modification to become bioactive against other bacteria, involving host plant factors to achieve such activity (Kajula et al., 2010). How to solve this extensive need for proper peptide folding in other expression systems remains another good question for next years. Probably, answers will come with intensive *in silico* modeling to trial all theoretical three-dimensional intramolecular interactions.

By the way, AMP-target interaction has been a recurrent bioinformatic issue in human immunology and nutrition, concerning several allergenic AMPs. Immunodominance of certain T-cells epitopes have been screened by modeling, helping breeders to achieve the theoretical amino acid sequence of a given allergenic AMP that would have the lowest allergenic potential (Oseroff et al., 2010). AMPs have also been tested and used for medicine biotechnological applications, as cardiovascular functioning and diseases (Li, 2009).

A biotechnological focus on plant AMPs with potentially high impact in biofuels industry is the investigation of such peptides effects over bioethanol production, from alcoholic fermentation by yeasts as well as from promising cellulolytic filamentous fungi species. In both cases, it is likely that AMPs present in plant feedstock may still have inhibitory activity on these industrial microorganisms, and so, over industrial yield (Nierop et al., 2008). In the specific case of sugarcane and other potential crops for bioethanol production in Brazilian northeast, as sorghum, Opuntia and other 'caatinga'-adapted plant species, research efforts have been directed to quantify this inhibition (if significant) and to achieve alternative agronomical and/or breeding solutions to reduce it (Bioethanol Research Network of Pernambuco).

8. Conclusion

As presented, antimicrobial compounds have been relatively well studied, since a long period and usually from native traditional usage of plants. However, biological and chemical bioinformatics have focused phytochemicals prospection and effects, while plant antimicrobial peptides are left apart. Expansion of AMP prospection through *in silico* methodologies is in perfect adjustment to the huge amount of biological data still not screened. Several antimicrobial effects observed in some plant extracts may also be explained due to AMPs supposed to be in sample, but such valuable information still has to be generated in lab benches as well as in databases extensive computational analyses.

Plant biodiversity in natural and anthropical ecosystems provide almost infinite targets number to unravel novel AMP candidates. Achieving such results relies on the generation of molecular data from crop and wild plant species. "-Omics" based experiments will then be more profitable and reliable, making easier the application of scientific knowledge from molecular biology and bioinformatics to develop systems biology approaches in accordance to nowadays and future needs in medicine, agriculture and industry.

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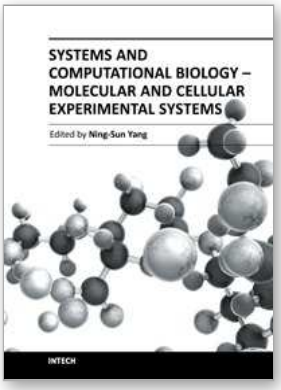

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