UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
DEPARTAMENTO DE GENÉTICA
PROGRAMA DE PÓS-GRADUACÃO EM GENÉTICA

CARLOS ANDRÉ DOS SANTOS SILVA

PEPTÍDEOS ANTIMICROBIANOS DE ESPÉCIES DA FAMÍLIA EUPHORBIACEAE: IDENTIFICAÇÃO ESTRUTURAL E FUNCIONAL

Recife
2019

CARLOS ANDRÉ DOS SANTOS SILVA

PEPTÍDEOS ANTIMICROBIANOS DE ESPÉCIES DA FAMÍLIA EUPHORBIACEAE: IDENTIFICAÇÃO ESTRUTURAL E FUNCIONAL

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.
Área de concentração: Biotecnologia.

Orientadora: Ana Maria Benko-Iseppon

Coorientador: Sergio Crovella

Cláudia Sampaio de Andrade Lima

Recife

2019

Catalogação na fonte:
Bibliotecária Claudina Queiroz, CRB4/1752

Silva, Carlos André dos Santos
Peptídeos antimicrobianos de espécies da família Euphorbiaceae: identificação estrutural e funcional / Carlos André dos Santos Silva - 2019.
205 folhas: il., fig., tab.

Orientadora: Ana Maria Benko-Iseppon
Coorientadores: Sergio Crovella
Cláudia Sampaio de Andrade Lima
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Genética. Recife, 2019.

Inclui referências e anexos.

1. Defensina 2. *Manihot esculenta* 3. Bioinformática
I. Benko-Iseppon, Ana Maria (Orientadora) II. Crovella, Sergio (Coorientador) III. Lima, Cláudia Sampaio de Andrade (Coorientadora) IV. Título

581.35 CDD (22.ed.) UFPE/CB-2020-032

CARLOS ANDRÉ DOS SANTOS SILVA

PEPTÍDEOS ANTIMICROBIANOS DE ESPÉCIES DA FAMÍLIA EUPHORBIACEAE: IDENTIFICAÇÃO ESTRUTURAL E FUNCIONAL

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética.

Aprovado em 25/02/2019

BANCA EXAMINADORA:

_____

Drª. Ana Maria Benko-Iseppon
Universidade Federal de Pernambuco

_____

Dr. Antônio Mauro Rezende
Instituto Aggeu Magalhães

_____

Dr. Marcelo Zaldini Hernandes
Universidade Federal de Pernambuco

_____

Drª. Isabelle Freire Tabosa Viana
Instituto Aggeu Magalhães

_____

Dr. José Ribamar Costa Ferreira Neto
Universidade Federal de Pernambuco

Aos meus pais Carlos Pedro e Maria Cícera, que dignamente me apresentaram à importância da família e ao caminho da honestidade e persistência, além de indicar que a maior herança que poderiam me doar seria a oportunidade do conhecimento que eu pudesse adquirir em vida.

AGRADECIMENTOS

A Deus, que pela fé manteve minha sanidade mental nos momentos de dificuldade durante um período de amadurecimento e aprendizado.

A minha orientadora Ana Benko que aceitou o desafio da orientação mesmo sem me conhecer como pessoa e estudante. Além das inúmeras oportunidades concedidas durante esta jornada. Sempre terá minha graditão.

Aos meus coorientadores Sergio Crovella e Cláudia Sampaio por todo opoio e oportunidades concedidas.

A minha família que mesmo distante ajudou na estabilidade emocional principalmente no período que estive em outro país, em especial à minha esposa Lívia que esteve sempre comigo em cada passo deste projeto.

A realização de um projeto de pesquisa como este só foi possível com o apoio de vários colaboradores. Inicialmente ao nosso grupo de pesquisa que me treinou e preparou para este momento no conhecimento sobre bioinformática e genética.

Al professor Tossi che ci ha accolto nel suo laboratorio a Trieste. Ai miei amici Salvio, Sharade, Ottavia ed Erik che hanno contribuito direttamente o indirettamente ad un ambiente ospitale e informato, amicizie che conserveró per il resto della mia vita.

Por fim, porém não menos importante, à UFPE pela estrutura e oportunidade desde doutorado, à FACEPE pela bolsa de pesquisa financiada, ao programa Biologia Computacional da CAPES que através da Rede InterSys financiou o doutorado sanduíche para Itália, bem como ao Departamento de Genética e ao Programa de Pós-graduação em Genética pela excelência no conhecimento e na formação de profissionais.

“if it can’t be better, it has to be different”

Lord Jim O’Neill, 2018

RESUMO

A resistência antimicrobiana representa um problema de saúde pública que vem crescendo nas últimas décadas, obrigando a comunidade científica a procurar novas alternativas terapêuticas. Nesse cenário, os peptídeos antimicrobianos (AMPs, *antimicrobial peptides*) fornecem uma opção promissora contra uma ampla gama de microrganismos patogênicos. AMPs podem ser encontrados em uma vasta variedade de organismos com diversos mecanismos de ação o que os tornam potenciais candidatos para o desenvolvimento de antibióticos. O presente trabalho teve como objetivo identificar genes codificantes para AMPs e seus produtos a partir de plantas da família Euphorbiaceae, gerando um peptídeo sintético com potencial atividade contra diversos patógenos. Foram selecionadas sequências de defensinas em banco de dados, as quais foram utilizadas como sondas para busca de defensinas de Euphorbiaceae. No total, 12 sequências foram encontradas e caracterizadas. Após a amplificação e sequenciamento, os AMPs tiveram sua estrutura analisada e foram modificados, obedecendo a uma série de pré-requisitos e de análises de bioinformática, selecionando-se uma defensina (PDef-Me1) para síntese. Sua atividade antimicrobiana foi testada contra diversos patógenos, incluindo cepas resistentes a antibióticos, obtendo-se melhores resultados contra *Staphylococcus aureus* (16 $\mu g.ml^{-1}$), *Acinetobacter baumannii* (64 $\mu g.ml^{-1}$) e *Candida parapsilosis* (64 $\mu g.ml^{-1}$). Além disso, ensaios realizados apontaram para uma baixa toxicidade indicando o potencial terapêutico de PDef-Me1. Embora sejam necessários mais testes, PDef-Me1 mostrou potencial para testes *in vivo*, para seu uso como um futuro composto farmacêutico antimicrobiano de amplo espectro.

Palavras-chave: Defensina. *Manihot esculenta*. Bioinformática.

ABSTRACT

Antimicrobial resistance represents a public health problem that has been growing in recent decades, forcing the scientific community to search for new therapeutic alternatives. In this scenario, antimicrobial peptides (AMPs) provide a promising option against a wide range of pathogenic microorganisms. AMPs can be found in a wide variety of organisms with diverse mechanisms of action which make them potential candidates for the development of antibiotics. The present study aimed to identify genes coding for AMPs and their products from plants of the family Euphorbiaceae, generating a synthetic peptide with potential activity against several pathogens. Defensins sequences were selected from the database, which were used as seeds to search for Euphorbiaceae defensins. However, 12 sequences were found and characterized. After amplification and sequencing, the AMPs had their structure analyzed and were modified, following a series of prerequisites and bioinformatics analyzes, by selecting a defensin (PDef-Me1) for synthesis. Its antimicrobial activity was tested against several pathogens, including strains resistant to antibiotics, obtaining better results against *Staphylococcus aureus* (16 $\mu g.ml^{-1}$), *Acinetobacter baumannii* (64 $\mu g.ml^{-1}$) and *Candida parapsilosis* (64 $\mu g.ml^{-1}$). In addition, assays performed indicated low toxicity indicating the therapeutic potential of PDef-Me1. Although further testing is needed, PDef-Me1 has shown potential for *in vivo* testing for its use as a future broad spectrum antimicrobial pharmaceutical compound.

Key words: Defensins. *Manihot esculenta*. Bioinformatics.

LISTA DE FIGURAS

Figura 1 - Principais Mecanismos de Resistência a Antibióticos 20
Figura 2 - Principais euforbiáceas de importância econômica 24
Figura 3 - Mecanismo de defesa imunológica da planta 34
Figura 4 - Estruturas tridimensionais dos AMPs 38
Figura 5 - Estrutura tridimensional de defensinas de plantas 39
Figura 6 - Alinhamento da sequência de aminoácidos..... 40
Figura 7 - Duas classes de defensinas de planta..... 41
Figura 8 - Modelos de permeabilização da membrana 45
Figura 9 - O modelo do mecanismo de ação Shai-Matsuzaki-Huang 48
Figure 10 - Plant antimicrobial peptides features considering..... 63
Figure 11 - Three-dimensional structure of plant..... 65
Figure 12 - Alignment of the conserved domain region..... 134
Figure 13 - Alignment of defensins amplified from the genome. 136
Figure 14 - Three-dimensional models of Def-Me1, Def-Me2, and Def-Me3 . 138
Figure 15 - Relative expression of defensin genes in *M. esculenta* 139
Figure 16 - Alignment of all the defensins found in the *M. esculenta* 141
Figure 17 - Three-dimensional structure of PDf-Me1 showing the four ……. 159
Figure 18 - Alignment of the Def-Me1 sequence with PDef-Me1. 159
Figure 19 - Three-dimensional structures *ab initio* of PDf-Me1 160
Figure 20 - Graphs associated to the molecular dynamics. 161
Figure 21 - On the right side, the three-dimensional structure. 162
Figure 22 - CD spectra of PDef-Me1. 163
Figure 23 - Cell viability plot of rat macrophages in contact. 165
Figure 24 - Hemolytic activity plot of PDef-Me1. 166

LISTA DE TABELAS

Tabela 1 - Defensinas e seu amplo espectro de atividades biológicas. 43
Table 2 - Plant defensins used for transformation of various plant. 92
Table 3 - Plant Snakins, LTPs, α-Thionins, Heveins and Thaumatins. 94
Table 4 - Forward and Reverse Primers of *M. esculenta*. 129
Table 5 - Antimicrobial screening of the *M. esculenta* defensin. 135
Table 6 - Antimicrobial screening of the amplified sequences. 136
Table 7 - Parameters obtained in standard curve analyzes. 140
Table 8 - Models for PDef-Me1 with different possibilities for the formation…155
Table 9 - *In silico* evaluation of antimicrobial activity using the four. 159
Table 10 - Energies regarding the simulations of the three models. 162
Table 11 - Antimicrobial activity of PDef-Me1 against different. 164

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AChE	Acetilcolinesterase
AMP	Peptídeo antimicrobiano (do inglês *Antimicrobial Peptide*)
AMR	Resistência bacteriana (do inglês *Antimicrobial Resistance*)
APG	Grupo de filogenia de angiospermas (do inglês *Angiosperm Phylogeny Group*)
CS-αβ	Cisteína estabilizada por α e β (do inglês *Cysteine Stabilized by α and β*)
Cys	Cisteína (do inglês *Cysteine*)
E.c	*Escherichia coli*
EDL	Extrato de folhas (do inglês *Extract from the leaves*)
ET	Etileno
ETI	Imunidade desencadeada por efetor (do inglês *Effector triggered Immunity*)
ETR	Progen Tyrus ou Imperador (do inglês *Progen Tyrus or Emperor*)
hBD	Defensina-beta humana (do inglês *Human beta-defensin*)
HNP	Peptídeo de neutrófilos humanos (do inglês *Human neutrophil peptide*)
HR	Reação de hipersensibilidade (do inglês *Hypersensitivity reaction*)
JA	Ácido jasmônico (do inglês *Jasmonic acid*)
MAPK	Proteína quinase ativadas por mitógenos (do inglês *Mitogen-activated protein kinase*)
ME	*Manihot esculenta*
MIC	Concentração mínima inibitória (do inglês *minimal inhibitory concentration*)
MTT	Determinação da viabilidade de célula (do inglês 3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina)
ORFs	Quadro de leitura aberta (do inglês *Open Reading Frame*)
PAMPs	Padrões moleculares associados a patógenos
PhD1 / 2	Defensina de *Petunia hybrida* 1 / 2 (do inglês *Petunia hybrida defensin* 1 / 2)
PRRs	Receptores de reconhecimento de padrões (do inglês *Pattern Recognition Receivers*)

PTI	Imunidade desencadeada por PAMP (do inglês PAMP *triggered Immunity*)
RC	*Ricunus communes*
RENISUS	Relação Nacional de Plantas Medicinais de Interesse ao Sistema Único de Saúde
RIPs	Proteínas inativadoras de ribossoma
ROS	Espécies reativas de oxigênio (do inglês *Reactive oxygen species*)
RT-qPCR	Reação em cadeia da polimerase em tempo real (do inglês *Real-time polymerase chain reaction*)
S.a	*Staphylococcus aureus*
SA	Ácido salicílico
SAR	Resistência adquirida sistêmica (do inglês *Systemic acquired resistance*)
SPFS	Síntese de peptídeo em fase sólida (do inglês *Solid-phase peptide synthesis*)
HMM	Modelo oculto de Markov (do inglês, *Hide Markov Model*)
Regex	Expressão regular (do inglês, *Expressão Regular*)

SUMÁRIO

1 INTRODUÇÃO 15

1.1 OBJETIVOS 17

1.1.1 Objetivo geral 17

1.1.2 Objetivos específicos 17

2 REVISÃO DA LITERATURA 19

2.1 RESISTÊNCIA BACTERIANA 19

2.2 FAMÍLIA EUPHORBIACEAE COMO PRODUTORA EM POTENCIAL DE COMPOSTOS QUÍMICOS 22

2.2.1 Aspectos botânicos gerais e importância da família Euphorbiaceae ... 22

2.2.2 Euphorbiaceae rica em moléculas bioativas com potencial uso na medicina terapêutica 25

2.3 MECANISMOS DE DEFESA DA PLANTA 33

2.4 PEPTÍDEOS ANTIMICROBIANOS (AMPs) 35

2.4.1 Origem e Classificação 36

2.4.2 Defensinas 37

2.4.3 Mecanismo de ação dos AMPs 44

2.4.3.1 Permeabilidade da membrana 45

2.4.3.2 Modelo barril 46

2.4.3.3 Modelo toroidal 46

2.4.3.4 Modelo tapete 47

2.4.3.5 Modelo detergente 47

2.4.3.6 Modelo Shai-Matsuzaki-Huang 47

2.4.3.7 Comprometimento das funções intracelulares 48

2.4.4 AMPs como uma nova classe de agentes terapêuticos 50

2.4.5 AMPs sintéticos 51

2.5 BIOINFORMÁTICA: UMA FERRAMENTA IMPORTANTE NA BUSCA DE NOVOS FÁRMACOS 54

3 PLANT ANTIMICROBIAL PEPTIDES: STATE OF ART AND PERSPECTIVES IN THE OMICS ERA 59

4 *MANIHOT ESCULENTA* DEFENSINS: PROSPECTION, STRUCTURAL ANALYSIS AND EXPRESSION 125

5 NOVEL SYNTHETIC ANTIMICROBIAL PEPTIDE PDEF-ME1 MODIFIED FROM A *MANIHOT ESCULENTA* DEFENSIN 151

6 DISCUSSÃO GERAL 172

7 CONCLUSÕES 174

REFERÊNCIAS 176

ANEXO A – CURRÍCULO LATTES 197

ANEXO B – SUPLEMENTARES 203

1 INTRODUÇÃO

A resistência de patógenos aos medicamentos tradicionais compreende um grave problema de saúde pública, o qual vem crescendo nas últimas décadas, inexistindo até o momento uma solução capaz de reverter este cenário. A resistência microbiana é geralmente resultante de um fenômeno natural de adaptação dos patógenos aos compostos antimicrobianos. Tal situação é agravada pelo fato de que, uma vez que um patógeno se torna resistente a um antibiótico, essa característica pode ser transmitida para sua progênie ou mesmo para outros microrganismos do mesmo ambiente.

Atualmente os tratamentos disponíveis não têm sido eficazes contra a resistência a antibióticos desenvolvida por algumas cepas bacterianas. Contudo, pesquisas vêm sendo desenvolvidas na busca por novas moléculas com ação antimicrobianas que tenham um amplo espectro de atividade. Neste cenário, compostos antimicrobianos derivados de plantas têm sido prospectados, para combater doenças provocadas por bactérias, fungos, protozoários e vírus, frequentemente com poucos efeitos colaterais.

O Brasil abriga uma extensa diversidade vegetal, com a mais diversa flora do planeta, apresentando um enorme potencial fitoterapêutico e biotecnológico. Apesar desta potencialidade, ainda são poucos os estudos envolvendo a prospecção de moléculas bioativas da flora nativa, sendo insipiente a utilização comercial de recursos genéticos, quando comparada ao seu notório potencial. A exploração farmacológica da biodiversidade brasileira é basilar e existe um vastíssimo campo a ser explorado.

Durante milhões de anos a interação planta-patógeno resultou na coevolução de moléculas de defesa, como metabólitos secundários presentes nas plantas, com função antimicrobiana na defesa contra doenças. Esses compostos variam desde substâncias inseticidas a moléculas bioativas como terpenos, esteroides, alcaloides, saponinas, taninos, ligninas, flavonoides e peptídeos. De fato, compostos com atividade anticâncer, anti-inflamatória, anticoagulante, antibacteriana, moluscicida, antidiarreica e antiviral têm sido prospectados em vegetais, incluindo plantas do Nordeste brasileiro.

Entre as moléculas associadas à defesa vegetal, outras classes de compostos que possuem importância biológica, com destaque para componentes proteicos e peptídicos, incluindo peptídeos antimicrobianos (AMPs). Alguns AMPs vegetais

apresentam uma notável atividade antimicrobiana contra fungos fitopatogênicos, bactérias e vírus. Devido à sua atividade antimicrobiana rápida e potente contra agentes patogênicos resistentes às drogas tradicionais, os AMPs vêm sendo considerados como novas promessas terapêuticas contra agentes infecciosos.

O presente trabalho pretende identificar e caracterizar peptídeos antimicrobianos a partir de plantas da família Euphorbiaceae, com ênfase para defensinas, selecionando um peptídeo para testes *in vivo* e *in vitro*. Tendo em vista o grande potencial antimicrobiano e anti-inflamatório das plantas dessa família, considera-se que o peptídeo candidato selecionado (em sua forma nativa ou modificada) tem potencial para testes *in vivo* com vistas ao desenvolvimento de um novo agente terapêutico.

1.1 OBJETIVOS

1.1.1 Objetivo geral

- ✓ Avaliar peptídeos antimicrobianos em, no mínimo, duas espécies selecionadas da família Euphorbiaceae, inferindo sobre sua estrutura, função, expressão diferencial e potencial uso medicinal.

1.1.2 Objetivos específicos

1. Selecionar duas espécies da família Euphorbiaceae com base na bibliografia etnobotânica especializada, bem como na facilidade de obtenção e cultivo das espécies candidatas.

2. Avaliar perfis cromatográficos e fitoquímicos das espécies selecionadas, avaliando a abundância e diversidade das moléculas observadas.

3. Selecionar sequências dos principais peptídeos-alvo usando-as como sonda (*seed sequences*) para a identificação de ortólogos nos bancos de dados públicos.

4. Identificar e descrever os domínios, assinaturas e regiões conservadas das sequências identificadas, comparando-as com os depositados nos bancos de dados, bem como reconhecer e analisar quadros de leitura aberta (ORF) destas sequências, inferindo seus padrões estruturais.

5. Desenhar *primers* para amplificação e isolamento de genes envolvidos na defesa das espécies de interesse, para amplificação, clonagem e sequenciamento dos produtos obtidos, permitindo inferências sobre estrutura genômica, análise de ORF, predição funcional e modelagem molecular.

6. Analisar o padrão de expressão dos genes identificados como diferencialmente expressos *in vitro* através da técnica de qPCR, utilizando bibliotecas de cDNA

disponíveis a partir de tecidos das referidas espécies submetidas à inoculação por patógeno.

7. Realizar síntese de um peptídeo previamente selecionado e testá-lo *in vitro* contra patógenos humanos selecionados.

2 REVISÃO DA LITERATURA

2.1 RESISTÊNCIA BACTERIANA

Desde a descoberta da penicilina por Alexander Fleming, em 1928 até os primeiros antibióticos sintéticos (caso da sulfonamida; Gerhard Domagk, 1935), o uso excessivo e inadequado de agentes antimicrobianos acelerou o processo da resistência antimicrobiana (AMR), considerando-se que esta seja uma das maiores ameaças à saúde que a humanidade enfrentará nas próximas décadas (Tortora et al. 2010; O'Neill 2016).

A resistência desenvolvida pelos microrganismos é um resultado inevitável do frágil equilíbrio e da coevolução entre as bactérias e os antibióticos. Porém, devido ao seu curto tempo de geração e à facilidade de acumular mutações, as bactérias têm infinitamente mais chances em adquirir resistência a antibióticos, que o ser humano tem de criar agentes antimicrobianos (Huttner et al. 2013). Essa resistência surge como consequência da pressão seletiva pelo uso excessivo de antibióticos, situação que oferece uma vantagem competitiva para as linhagens bacterianas mutantes (Ferri et al. 2017). No ambiente hospitalar ou ambulatorial, a disseminação de um clone resistente pode ser rápida, com graves consequências para os pacientes e a equipe envolvida. Esta propagação é facilitada pela transmissão horizontal de genes interespécies, falta de saneamento e controle hospitalar, bem como devido ao aumento da frequência global de viagens e comércio, facilitando a dispersão da doença (Ferri et al. 2017).

Nestes bilhões de anos de evolução dos seres vivos, o mundo microbiano tem acumulado uma enorme diversidade de mecanismos metabólicos e de proteção que podem ser utilizados como resposta a uma agressão externa, incluindo antibióticos. Esta resposta evolutiva para a pressão seletiva de antimicrobianos é a base do rápido desenvolvimento de resistência em bactérias e vírus que, para sobreviver, se multiplicam e produzem uma descendência resistente que progressivamente vai substituindo as cepas não resistentes (Aminov 2010).

O conhecimento dos mecanismos bioquímicos e genéticos envolvidos na AMR é fundamental para estabelecer medidas de controle da resistência bacteriana. Embora alguns patógenos possam ser mais aptos do que outros em adquirir e ou transmitir resistência pelo arsenal genético, tal capacidade em geral é causada por alguns fatores ilustrados na **Figura 1**, tais como: (1) inativação do antibiótico

diretamente na molécula bioativa por alterações químicas, geralmente promovidas por enzimas bacterianas (Wright 2005), (2) modificação do alvo, levando à perda de sensibilidade ao antibiótico (Lambert 2005), (3) permeabilidade ao antibiótico e (4) mudança na bomba de efluxo (Li et al. 2015).

As bombas de efluxo também fornecem resistência aos antibióticos estruturalmente diferentes, incluindo as quinolonas, uma recente família de antibióticos sintéticos (Nikaido e Pagès 2012). Isso indica que, pelo menos em alguns casos, a resistência aos antibióticos seria uma função emergente que foi recentemente selecionada devido ao uso de antibióticos para o tratamento de infecções. As bombas de efluxo também se enquadram nesta categoria, pois exibem múltiplas funções relevantes para a fisiologia bacteriana, além da resistência antibiótica mediadora, embora a resistência aos antibióticos seja provavelmente apenas uma função secundária (mais recente) dessas bombas (Martinez 2009a; Martinez 2009b).

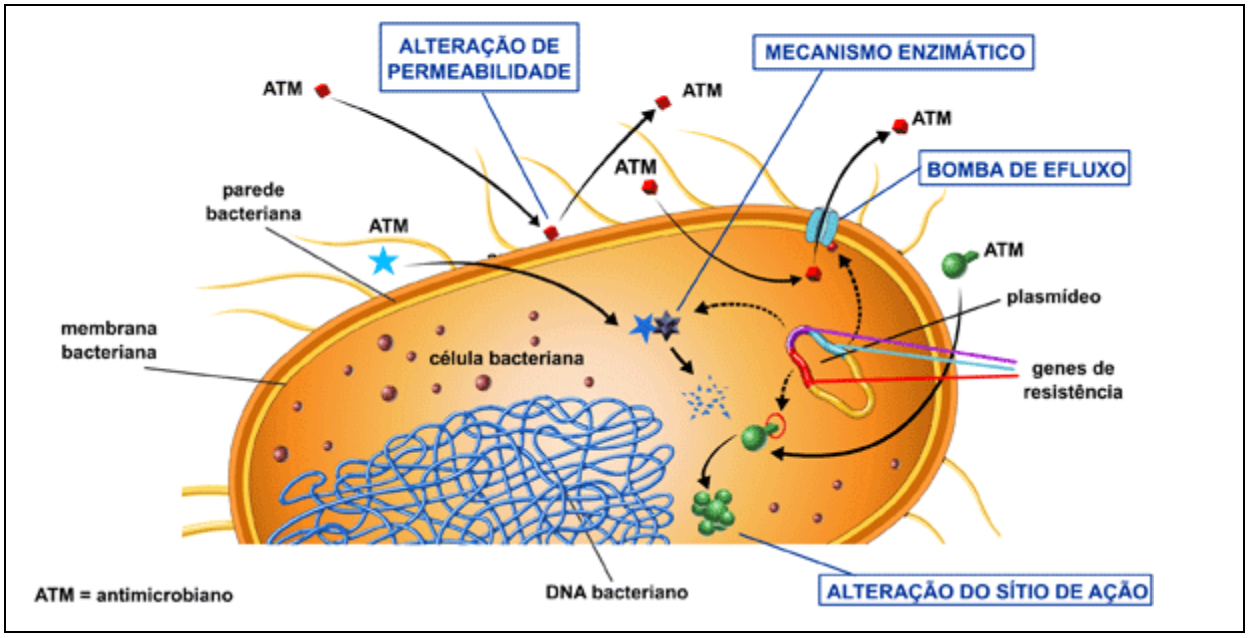

Figura 1 - Mecanismos de resistência a antibióticos. Nos quadros em azul, os principais mecanimos de resistência utilizados por bactérias. (Fonte: http://www.brasilescola.com/biologia/resistencia-das-bacterias-aos-antibioticos.htm. Acesso em 7 de dezembro de 2016).

De fato, algumas bombas de efluxo estão envolvidas na virulência bacteriana, no tráfego das moléculas e, sobretudo, na desintoxicação não apenas de metabólitos intermediários ou de compostos tóxicos como metais pesados e solventes, mas

também de antibióticos naturalmente produzidos por outros microrganismos (Alvarez-Ortega et al. 2013). Estas características primordiais das bombas de efluxo provavelmente potencializaram a diversidade funcional e estrutural como consequência de uma pressão seletiva. Para entender o surgimento dos fenótipos de resistência, é importante entender os papéis e a diversidade destas bombas em um contexto não apenas clínico, mas evolutivo, especialmente para patógenos oportunistas, que apresentam predisposições particulares para desenvolver rapidamente novas resistências aos antibióticos (Youenou et al. 2015).

Além disso, os elementos de resistência podem ser adquiridos através de transferência horizontal de elementos genéticos tais como a transferência de infecções via bacteriófagos, integrons, transposons e incorporação de plasmídeos ou fragmentos de DNA portadores de genes de resistência a antibióticos (Avison et al. 2000; Liaw et al. 2010; Hu et al. 2011; McArthur et al. 2013). No entanto, na análise genômica comparativa dos testes experimentais de dois isolados de *Stenotrophomonas maltophilia* – sendo uma cepa de isolado clínico e outra endofítica (K279a e R551-3) – ficou evidente que a maioria dos genes de resistência aos antibióticos, e especialmente os genes codificadores da bomba de efluxo, não estão associados a elementos genéticos móveis (Ryan et al. 2009).

Nos últimos tempos novos mecanismos de resistência resultaram no desenvolvimento simultâneo de resistência a várias classes de antibióticos criando cepas bacterianas resistentes a múltiplas drogas, também conhecidas vulgarmente como superbactérias (World Health Organization 2015).

A descoberta de novos fármacos pode ser potencializada por uma melhor compreensão dos mecanismos de resistência, bem como das interações sinérgicas entre eles, o que contribuiria também no auxílio à terapia clínica, otimizando a administração de antibióticos e reduzindo o seu uso excessivo. No entanto, devido ao limitado entendimento da complexidade de todos os eventos de resistência antimicrobiana, ainda se enfrenta muitos desafios que possam contornar os efeitos da interação evolutiva patógeno-hospedeiro que acabam levando à resistência (Li et al. 2015).

Além do estudo dos mecanismos de resistência e dos esforços no desenvolvimento de novos fármacos, outras formas de combate à AMR são necessárias, como por exemplo, conscientizar a população de forma geral para o uso

adequado dos antibióticos. No estudo de Bhattacharya et al. (2016), foi avaliado o impacto de uma campanha informativa entre agosto de 2014 e janeiro de 2015 na Inglaterra, através principalmente de mídias sociais e sites informativos, além de esclarecimento de dúvidas da população. De forma geral, dos 47.158 visitantes ao site, 26,5% se compromteram em se tornar Guardiões de Antibióticos, termo utilizado no trabalho para aqueles que combateriam a AMR, seja de forma pessoal ou informando outras pessoas. Destes, 69% eram profissionais de saúde. Após os resultados, que superaram as expectativas iniciais do trabalho, os autores concluíram que são necessárias outras ações semelhantes para melhorar o envolvimento com o público-alvo e determinar se estas campanhas têm um impacto no consumo de antibióticos e no comportamento de prescrição entre o público e os profissionais de saúde.

Embora esses resultados tragam esperanças, existe ainda uma demanda social progressiva por incentivos à pesquisa para o desenvolvimento de novos antibióticos, além da necessidade de melhorias na política de regulamentação após a disponibilização no mercado, que não vise apenas o lucro, mas sim os benefícios à sociedade. Uma vez que as políticas atuais não preconizam a correção do modelo econômico atual de uso e produção de antibióticos, poucas empresas investem em pesquisas inovadoras que atendam as necessidades de saúde mais urgentes da AMR (Outterson et al. 2016).

2.2 FAMÍLIA EUPHORBIACEAE COMO PRODUTORA EM POTENCIAL DE COMPOSTOS QUÍMICOS

2.2.1 Aspectos botânicos gerais e importância da família Euphorbiaceae

Os primeiros registros sobre Euphorbiaceae acorreram no século XIX, quando Jussieu (1824), Baillon (1858) e Mueller (1873) publicaram importantes descobertas em relação à classificação da família. Somente 57 anos mais tarde Bentham e Hooker (1880) revisaram o trabalho de Baillon e Muller, publicando no *Journal of the Linnean Society*, fazendo extensas notas sobre o seu próprio sistema de classificação. Pax (Pax 1910; 1924) parcialmente assistido por Kathe Hoffmann, descreveu a maioria dos gêneros de Euphorbiaceae. Apenas em 1931 (Pax e Hoffmann 1931) foi disponibilizada uma sinopse revisada das Euphorbiaceae, a qual tem sido utilizada como a classificação básica da família.

Posteriormente Webster (1967) e Webster e Carpenter (2002) passaram longos anos estudando e classificando novas espécies da família, reunindo informações morfológicas e taxonômicas importantes. A família passou por vários tratamentos taxonômicos, fundamentados principalmente em estudos filogenéticos com dados moleculares (APGII 2003; Wurdack et al. 2004; APGIII 2009), apresentando marcantes diferenças em relação à classificação tradicional revisada e proposta por Webster (Webster 1994; Secco et al. 2012).

Existem dificuldades na classificação desta família devido à diversidade de espécies, de modo que não é surpreendente que apenas alguns botânicos tenham um conhecimento geral de toda a família, como destacado por Punt (1962), cujo trabalho ainda reflete a realidade atual.

A família Euphorbiaceae tem distribuição cosmopolita sendo considerada uma das maiores e mais diversas famílias de Angiospermas. Abrange atualmente cerca de 8.000 espécies distribuídas em aproximadamente 299 gêneros, sete subfamílias (Phyllanthaceae, Oldfieldioideae, Peroideae, Cheilosoideae, Acalyphoideae, Crotonoides e Euphorbiaceae) e 47 tribos em todo o mundo (Webster 1975; Webster 2014), ocorrendo preferencialmente em ambientes tropicais e subtropicais (Pax e Hoffmann 1931; Watson e Dallwitz 1992; Webster 1994; Alves 1998; Tomlinson 2016). No Brasil, há cerca de 63 gêneros e estimadas 940 espécies distribuídas em todo o território (Cordeiro et al. 2015).

Podem se apresentar como árvores, arbustos, subarbustos e, menos frequentemente, ervas ou trepadeiras, monoicas ou dioicas. As folhas apresentam filotaxia alterna, sendo às vezes opostas ou verticiladas, simples, pecioladas, inteiras a profundamente lobadas com margem inteira ou de outro tipo. Flores unissexuais, geralmente actinomorfa dispostas na região proximal. Flores estaminadas, na região distal do eixo. Frutos esquizocarpos ou capsulídios, em geral globosos, 3-lobados; columela usualmente persistente, frequentemente delgada. Sementes na maioria elipsoides, planas na face interna, convexas na externa, endospermadas, com testa lisa a rugosa, carunculadas ou não; embrião reto com cotilédones planos, membranáceos, arredondados, mais largos do que a radícula (Webster e Carpenter 2002; Lima e Pirani 2008; Webster 2014). No entanto, segundo Webster (2014), as informações sobre membros da família Euphorbiaceae ainda são confusas, pela amplitude das tribos e sua complexidade.

As Euphorbiaceae têm se destacado por sua importância econômica na alimentação humana (Gleadow et al. 2016), na produção de látex (Friolani et al. 2017) e de óleos (Román-Figueroa et al. 2016), além de amplo uso na medicina popular (Ruangnoo et al. 2017). Em um levantamento das espécies ocorrentes no Brasil com potencial para o desenvolvimento de drogas antimicrobianas, Benko-Iseppon e Crovella (2010) indicaram 29 espécies de oito gêneros de Euphorbiaceae, compreendendo a segunda família mais representada no estudo (superada apenas pelas Fabaceae).

Certas euforbiáceas nativas são utilizadas na alimentação humana como a *Manihot esculenta* (Crantz) (**Figura 2**A), também conhecida como mandioca ou macaxeira, da qual se extrai a farinha de mandioca e o polvilho (Allem et al. 1975; Aloys e Hui Ming 2006). Consumida em larga escala em todo o país, trata-se de uma importante fonte de amido para boa parte da população brasileira (Sánchez et al. 2009). Os tubérculos de uma variedade dessa espécie são amplamente consumidos e delas pode ser produzida uma bebida alcoólica tradicional da Amazônia, a “tiquira” (Braga 1976).

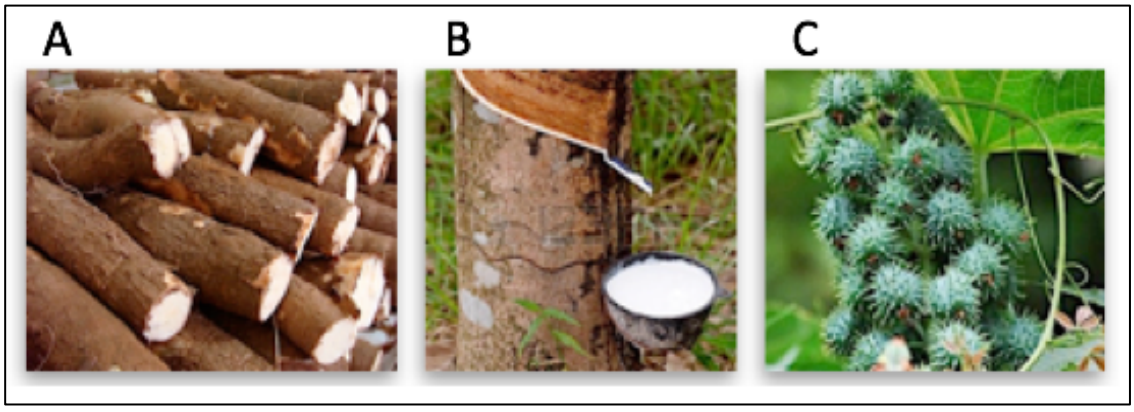

Figura 2 - Principais euforbiáceas de importância econômica. Em: **A** – *Manihot esculenta* **B** – *Hevea brasiliensis*; **C** – *Ricinus communis*. Fonte: Google imagens.

Outros representantes da família movimentaram grandes riquezas no Brasil, especialmente pela extração de látex para a produção de borracha natural, durante o ciclo da borracha no Brasil, como a espécie *Hevea brasiliensis (*Aublet), também conhecida como seringueira (**Figura 2**B), uma espécie nativa da floresta amazônica, que movimentou a economia do Acre e da Amazônia até ser cultivada com maior produtividade e menores custos no Sri Lanka e nas florestas do arquipélago Malaio (Batista 1976; Batista 2007).

Outra importante espécie é a mamoneira *Ricinus communis* (Linnaeus) (**Figura 2**C), nativa da África, cujos frutos são utilizados para a produção de óleos, em especial o óleo de rícino, fibras vegetais e compostos químicos usados na medicina e na indústria (Braga 1976). Além disso, os óleos extraídos de espécies como *Euphorbia tirucalli* (Linnaeus) (Van Damme 2001), *Jatropha curcas* (Linnaeus) (Achten et al. 2010), *M. esculenta* (Crantz) (Adeniyi et al. 2007), *R. communis* (Benavides et al. 2007), entre outros, podem ser empregados na produção de biodiesel.

A família também apresenta também espécies de importância ornamental, como a Poinsétia [*Euphorbia pulcherrima* (Willd)]. Outros usos incluem a produção de madeira, uso no artesanato e utilização em programas de reflorestamento, entre outros (Mwine e Van Damme 2011). Além dos usos mencionados, a família Euphorbiaceae vem sendo considerada como uma importante fonte de ervas medicinais (com aplicações no tratamento de afecções em humanos ou para uso veterinário), bem como agrícola (Mwine e Van Damme 2011; Afolayan et al. 2014).

2.2.2 Euphorbiaceae rica em moléculas bioativas com potencial uso na medicina terapêutica

Ao longo de sua existência, o homem tem extraído recursos da natureza para atender às suas necessidades básicas, também buscando por medicamentos para o tratamento de um amplo espectro de doenças. As plantas formaram a base dos sofisticados sistemas de medicina tradicional, com os registros mais antigos, datando de cerca de 2600 a.C., documentando os usos de aproximadamente 1000 substâncias derivadas de plantas na Mesopotâmia. Estes incluem óleos de cedro *Cedrus atlantica* (Endl.), cipreste *Cupressus sempevirens* (Linnaeus), alcaçuz *Glycyrrhiza glabra* (Linnaeus), mirra C*ommiphora myrrha* (Nees) e o sumo de papoula *Papaver somniferum* (Linnaeus), todos ainda hoje utilizados no tratamento de doenças variando de tosse e resfriados a infecções parasitárias e inflamação (Borchardt 2002).

Muitas plantas da família Euphorbiaceae estão incluídas na Lista Nacional de Plantas Medicinais de Interesse do Sistema Único de Saúde (RENISUS), um relatório do Ministério da Saúde brasileiro que inclui 71 espécies de plantas medicinais que visa orientar estudos e pesquisas que possam subsidiar a elaboração, o desenvolvimento e a inovação na busca por potenciais fitoterápicos para gerar

produtos farmacêuticos de Interesse no sistema de saúde pública brasileiro (Brasil 2009; Félix-Silva et al. 2014a).

Em relação à biodiversidade do reino vegetal, as plantas parecem ser fonte inegável de remédios para combater diversas enfermidades, incluindo bactérias multirresistentes (Subramani et al. 2017). As propriedades medicinais de várias plantas têm sido investigadas nos últimos anos em todo o mundo devido ao seu potencial farmacológico, baixa incidência de efeitos colaterais e baixo custo (Gadamsetty et al. 2013; Voukeng et al. 2017). Dentre estas as plantas da família Euphorbiaceae têm sido amplamente utilizadas na medicina popular para vários fins, incluindo usos antiofídicos, anti-inflamatórios, anti-hemorrágicos, hemostáticos, cicatrizantes, sinusite, inchaços, furúnculos, gonorreia, disenteria, bronquite, doenças do aparelho digestivo, febre, dor nos rins, reumatismo e hipertensão (Irvine 1961; Di Stasi et al. 2002; Burkill 2004; de Albuquerque et al. 2007; Benko-Iseppon e Crovella, 2010; Félix-Silva et al. 2014a), entre outros usos. Euphorbiaceae podem possuir comprovadamente atividade anti-inflamatória, antioxidante e analgésica (Wandji et al. 2000; Nkeh-Chungag et al. 2001) atuando também como depressores do Sistema Nervoso Central (Sudharshan et al. 2009). Estudos fitoquímicos anteriores em algumas espécies desta família apontam a presença de flavonoides, glicosídeos, saponinas, terpenos e/ou esteroides, fenol, alcaloides, etc. (Wandji et al. 2000; Wandji et al. 2003; Nenkep et al. 2008; Awanchiri et al. 2009; Fannang et al. 2011; Félix-Silva et al. 2014b).

Devido à grande capacidade de produzir diversas substâncias e moléculas bioativas, a família Euphorbiaceae vem sendo estudada ao longo dos anos e prospectada sua potencialidade na medicina terapêutica. Um exemplo envolve o trabalho de Méité et al. (2010) que investigaram a atividade laxativa do extrato foliar aquoso de *Mareya micrantha* (Benth.) em ratos em diferentes doses (100, 200 e 400 mg/kg de peso corporal), sendo a atividade laxativa determinada com base no peso da matéria das fezes. Os efeitos do extrato aquoso de folhas de *M. micrantha* e óleo de rícino também foram avaliados no trânsito intestinal, acumulação de líquido intestinal e secreção de íons. A prospecção fitoquímica do extrato revelou a presença de flavonoides, alcaloides, taninos, polifenóis, esteróis e politerpenos. O extrato aquoso nas diferentes doses produziu atividade laxante significativa e reduziu a constipação induzida pela loperamida de um modo dose-dependente. O efeito do

extrato a 200 e 400 mg/kg foi semelhante ao do fármaco de referência picossulfato de sódio (5 mg/kg). Os autores concluíram que os resultados do extrato aquoso de *M. micrantha* possuía uma atividade laxante significativa, dando suporte ao uso tradicional em fitoterapia (Méité et al. 2010).

Appiah-Opong et al. (2011) avaliaram a atividade antiplasmodial com diferentes extratos de *Tridax procumbens* (Compositae) e *Phyllanthus amarus* (Schum) (Euphorbiaceae), ambas usadas na medicina tradicional para tratar a malária. Estudos anteriores demonstraram os efeitos anti-tripanossoma, antibacteriano e anti-HIV. A atividade antiplasmodial dos extratos foi avaliada com o ensaio de hipoxantina 3H utilizando parasitas resistentes à cloroquina (Dd2) *Plasmodium falciparum*. O ensaio colorimétrico com base em tetrazólio modificado foi também utilizado para avaliar as atividades protetoras/antiplasmodiais dos glóbulos vermelhos e a citotoxicidade dos extratos. Os resultados mostraram que as plantas possuíam atividades antiplasmodiais. Os extratos aquosos e etanólicos de *P. amarus* foram os mais ativos, produzindo valores de IC_{50} de 34,9 µg/ml e 31,2 µg/ml, respectivamente, no ensaio à base de tetrazólio. No ensaio de hipoxantina as plantas produziram valores de IC50 de 24,8 µg/ml (*Tridax procumbens*) e 11,7 µg/ml (*P. amarus*). Nenhum dos extratos, dentro da faixa de concentração (1.9-500 µg/ml) estudada produziu qualquer toxicidade evidente para as células vermelhas de humano. Os resultados indicam que as duas plantas têm atividade contra parasitas resistentes à cloroquina, como *P. falciparum* (Dd2). Os principais tipos de extratos com atividade antiplasmodiais foram de aguaso e etanólico (Appiah-Opong et al. 2011).

No estudo de Elya et al. (2012), determinaram a atividade inibitória da α-glucosidase de extratos de etanol a 80% de folhas e galhos de algumas plantas das Apocynaceae, Clusiaceae, Euphorbiaceae e Rubiaceae. O teste de atividade inibitória da α-glucosidase foi realizado *in vitro* utilizando métodos espectrofotométricos. Em comparação com o controle da acarbose (IC_{50} 117,20 µg/mL), 37 de 45 amostras mostraram ser mais potentes inibidores da α-glucosidase com valores de IC_{50} na gama de 2,33-112,02 µg/mL. Por fim, os autores destacam a importância de purificar estes extratos e investigar seus mecanismos de ação, bem como realizarem testes *in vivo* (Elya et al. 2012).

Na extensa revisão de Alves et al. (2012) sobre o gênero *Croton* (Euphorbiaceae) no Nordeste do Brazil, destacam-se várias espécies com diferentes

propriedades farmacológicas (Fontenelle et al., 2008). Devido à grande diversidade e importância medicinal deste gênero, os autores sugerem que estudos de taxonomia, ecologia, genética, fitoquímica e etnobotânica ainda são necessários, bem como a promoção do desenvolvimento de produtos e estratégias de conservação *in situ* e *ex situ*. Visando este potencial, Blank et al. (2017) relataram o desenvolvimento de uma formulação à base de óleo essencial de *Croton tetradenius* com menor atividade fungistática contra *Fusarium solani* testada de 0,5 $mL.L^{-1}$ e atividade fungicida de 5 $mL.L^{-1}$, dando origem a uma patente.

Gadamsetty et al. (2013) investigaram as atividades antioxidantes, anti-inflamatórias e citotóxicas *in vitro* de extratos com diferentes tipos de solventes obtidos de folhas da euphorbiácea *Drypetes sepiaria* (Wight Arn.). O extrato de metanol apresentou melhores resultados em comparação com os outros para atividade antioxidante e foi capaz de inibir a inflamação *in vitro* por cerca de 85-90% e *in vivo* de 40-45%. No ensaio de citotoxicidade o extrato metanólico apresentou IC50 de 10 ug/ml, mostrando um grande potencial medicinal, além de baixa citotoxicidade (Gadamsetty et al. 2013).

Na extensa revisão de Sabandar et al. (2013), sobre o gênero *Jatropha*, além de citar a importância do estudo etnobotânico em vários continentes, dizendo que espécies deste gênero têm sido prospectadas para dor de estômago, inchaço, inflamação, hanseníase, disenteria, vertigem, anemia, diabete, bem como para tratar HIV e tumores, oftalmia, micose, úlceras, malária, doenças de pele, bronquite, asma e o uso como afrodisíaco. Também citaram que espécies de *Jatropha* podem ser de uso ornamental e como seu uso como biocombustíveis. Em relação às substâncias encontradas, citam desde alcaloides, peptídeos cíclicos, diterpenos a diversos compostos já relatados por outros autores. Nos extratos e compostos purificados de plantas deste gênero foram descritas atividades citotóxica, antitumoral, antimicrobiana, antiprotozoários, anticoagulantes, imunomoduladoras, anti-inflamatória, antioxidantes, protoscolicidas, inseticidas, moluscicidas, inibidores de acetilcolinesterase (AChE). Desta maneira, concluíram que espécies do gênero *Jatropha* possuem um forte potencial, como fonte de novas drogas devido às suas várias atividades promissoras, indicando a necessidade de estudos mais aprofundados sobre o desenvolvimento de fármacos com os extratos e constituintes desde gênero (Sabandar et al. 2013).

Gangwar et al. (2014) fizeram uma extensa revisão sobre uma Euphorbiaceae (*Mallotus philippinensis* Muell. Arg.) com foco na etnofarmacologia e fitoquímica. Segundo os autores as espécies de *M. philippinensis* são conhecidas por conterem compostos naturais diferentes, principalmente fenóis, diterpenoides, esteroides, flavonoides, cardenólideos, triterpenoides, cumarinas e alguns fenóis específicos como bergenina, mallotophilippinens, rottlerin e isorottlerin. Estes últimos já foram isolados, identificados e apresentaram atividades biológicas como antimicrobiana, antioxidante, antiviral, citotóxica, antioxidante, anti-inflamatória, inibição da proteína de atividade imunorreguladora contra células cancerígenas. Por fim os autores concluem que as euforbiáceas são uma valiosa fonte de moléculas naturais medicinalmente importantes e fornece suporte convincente para seu uso futuro na medicina moderna. No entanto, o conhecimento existente é muito limitado, sendo necessária a geração de dados mais detalhados relativos à toxicidade de forma geral dos extratos e seus compostos purificados (Gangwar et al. 2014).

Félix-Silva et al. (2014b) avaliaram as propriedades antiofídicas do pinhão-manso (*Jatropha gossypiifolia* L.) a partir do extrato aquoso de suas folhas. Sua análise fitoquímica revelou a presença de açúcares, alcaloides, flavonoides, taninos, terpenos e/ou esteroides e proteínas. O extrato foi capaz de inibir as atividades enzimáticas e biológicas induzidas pelo veneno de serpente jararaca *in vitro* e *in vivo*. Em conclusão, os resultados demonstraram que o extrato dessa espécie possui uma atividade antiofídica, incluindo ação sobre os efeitos locais, sugerindo seu uso como nova fonte de moléculas bioativas contra o veneno de serpente (Félix-Silva et al. 2014b).

Apesar do uso na medicina popular de *J. gossypiifolia*, mais estudos toxicológicos são necessários para seu uso de forma confiável. Almeida et al. (2015; 2016) avaliaram os efeitos tóxicos, citotóxicos e genotóxicos do látex e de extratos etanólicos e aquosos (folhas) de *J. gossypiifolia*, utilizando *Allium cepa* como sistema teste. Em todas as concentrações testadas (0,001 a 10 $mL.L^{-1}$) as três amostras apresentaram queda significativa no valor médio de crescimento radicular e índice mitótico, com exceção de 1,25 $mL.L^{-1}$, quando comparado ao controle negativo. As concentrações de 1,25, 2,5 e 5 $mL.L^{-1}$ induziram aderências cromossômicas, metáfases C e/ou pontes cromossômicas, como efeitos genotóxicos. A frequência significativa de pontes cromossômicas também indicou potencial mutagênico para os

cromossomos de *J. gossypiifolia* como discutido no artigo. A presença de micronúcleos na administração dos extratos também indicou ação mutagênica no nível cromossômico. No extrato etanólico, a aneugenicidade parece ser a principal atividade, provavelmente como resultado da ação de terpenos e/ou flavonoides, enquanto no extrato aquoso a ação clastogênica parece ser a principal atividade, presumivelmente como consequência do efeito de flavonoides e/ou saponinas. Assim, os autores sugerem que os extratos desta espécie devem ser usados com muita cautela para fins fitomedicinais.

Por se tratar de uma espécie utilizada na medicina popular, Wu et al. (2019) realizaram uma extensa revisão sobre a farmacologia específica e atributos toxicológicos de *Jatropha gossypiifolia* e seus metabólitos bioativos. Os autores enumeraram as principais propriedades farmacológicas da espécie, incluindo atividades antiinflamatória, antineoplásica, antimicrobiana, antioxidante e anticolinesterásica e anti-hipertensiva. Contudo, espécies de *Jatropha* são também conhecidas pelo seu potencial tóxico, primariamente relacionado ao conteúdo de látex e a compostos presentes nas sementes. Além disso, os mecanismos potenciais das atividades farmacológicas reportadas ainda não foram totalmente elucidados, demandando pesquisas complementares.

Villanueva et al. (2015) estudaram a atividade citotóxica de três isolectinas purificadas a partir do látex *Euphorbia trigona* (Miller) baseados na hipótese que as lectinas seriam proteínas inativadoras de ribossomos (RIPs) e, portanto, potentes inibidores da síntese de proteínas em células e em sistemas livres de células. Foram isoladas três isolectinas (ETR1, ETR2 e ETR3) por cromatografia de troca aniônica. Tanto ETR1 como ETR3 produziram uma única banda em SDS-PAGE sob condições redutoras, correspondendo a um peso molecular de 32 $g.mol^{-1}$, enquanto que ETR2 produziu duas bandas correspondentes a 31 e 33 $g.mol^{-1}$. As lectinas estudadas foram termoestáveis até 60 °C e a sua atividade observada foi mantida através do intervalo de pH 5-12. Estas lectinas revelaram-se como potentes inibidores da síntese de proteínas eucarióticas num sistema de células livres. A análise de citometria de fluxo revelou a atividade antiproliferativa destes para as linhas celulares A549, HeLa, H116, HL-60. Como conclusão Villanueva et al. (2015) afirmaram que as isolectinas de *E. trigona* são RIPs com atividade citotóxica em relação a linhas celulares de câncer humano testadas (Villanueva et al. 2015).

Araújo et al. (2015) investigaram os efeitos citotóxicos e genotóxicos do extrato etanólico de *Euphorbia hyssopifolia* em cultura de células HepG2. As principais classes encontradas na triagem fitoquímica foram terpenos, esteroides e flavonoides. As concentrações de extrato usadas no ensaio de MTT não mostraram atividade citotóxica. Por outro lado, a atividade genotóxica foi verificada em 0,1 e 1,0 $mg.mL^{-1}$ no teste do cometa alcalino. Além disso, a concentração de 1,0 $mg. mL^{-1}$ induziu danos celulares graves, levando à morte no ensaio de micronúcleos em bloco de citocinese, indicando um efeito citotóxico. Por fim os autores alertam que o uso do extrato de *E. hyssopifolia* para fins medicinais deve ser evitado, pois concentrações acima de 0,01 $mg.mL^{-1}$ podem representar risco à saúde humana devido aos efeitos citotóxicos e/ou genotóxicos.

Conforme informado na revisão de Haque et al. (2015), a espécie *Mallotus philipiensis* (Lam.) (Euphorbiaceae) vem sendo utilizada há muito tempo na medicina popular na Índia. Além de ser utilizada como corante comum, a citada espécie compreende uma fonte rica de substâncias e compostos biológicos, sendo também utilizada no tratamento de doenças cutâneas, bronquite, diarreia, de problemas de infecção urogenital, para combater o câncer, diabetes, icterícia e a malária, entre outros. Existem muitos constituintes químicos documentados como fenóis, esteroides diterpenoides, triterpenoides, flavonoides, cumarinas, isocumarinas, cardenólidos e particularmente fenóis. Compostos como bergenina, mallotophilippinens, rottlerina e isorottlerina foram isolados e identificados, relatando-se diferentes atividades biológicas, tais como antimicrobiana, citotóxica, antifúngica, antiviral, antioxidante e anti-inflamatória. Alguns compostos já foram implementados há algum tempo em formulações para o tratamento de sarna e comichão, de forma segura. Os autores concluem em sua revisão que *M. philippinensis* compreende uma fonte preciosa de medicamento natural e fornece suporte persuasivo para seu uso potencial em medicamentos modernos (Haque et al. 2015).

No estudo de revisão de Ernst et al. (2015), os autores destacaram a importância da pesquisa em usos medicinais das espécies de *Euphorbia* e sua importância como fonte de produtos naturais. Além disso, os autores salientam que o subgênero medicinal *Chamaesyce* (atualmente posicionado no gênero *Euphorbia*) necessita de estudos adicionais investigando a diversidade química associada ao elevado valor medicinal (Ernst et al. 2015). Neste cenário, Kumari (2018), avaliou

diferentes extratos de *Euphorbia hirta* L., sendo o extrato metanólico das folhas o mais ativo contra as bactérias testadas, especialmente contra *E. coli* que apresentou um halo de inibição por volta de 25 mm, considerado um resultado promissor.

Sendo a picada de serpente um dos problemas de saúde no Brasil, Gomes et al. (2016) investigaram a capacidade do extrato aquoso das folhas de uma euforbiácea (*Jatropha mollissima* Pohl) frente aos efeitos locais induzidos por veneno de serpente do gênero *Bothrops*. Após alguns perfis cromatográficos com análise de detecção de matriz de diodo e análise de espectrometria de massas do extrato aquoso de folha, foi confirmada a presença de flavonoides, isoschaftoside, schaftoside, isoorientina, orientina, vitexina e isovitexina. Doses entre 50 e 200 mg/kg do extrato administradas por via intraperitoneal, apontam para um potencial inibitório significativo contra os efeitos locais (hemorragia cutânea local, edema local, migração leucocitária e miotoxicidade) induzidos pelo veneno de serpentes, como *Bothrops erythromelas* e *B. jararaca*. Os autores concluíram que esses resultados demonstram que o extrato de *J. mollissima* apresenta potencial inibitório, especialmente contra o veneno de botrópicos, sugerindo seu potencial como adjuvante no tratamento de picadas de serpentes (Gomes et al. 2016).

Voukeng et al. (2017) obtiveram o extrato metanólico das folhas da euforbiácea *Elaeophorbia drupifera* (Thonn.), aplicando cromatografia para isolar compostos e avaliar sua atividade antimicrobiana contra um painel de bactérias Gram-negativas e positivas, incluindo fenótipos resistentes a múltiplas drogas. Os extratos apresentaram uma variedade de metabólitos com diferentes valores de concentração mínima inibitória contra as bactérias testadas. A subfração EDLc3 foi mais ativa do que todos os compostos isolados e os autores sugerem que a mesma merece investigações adicionais, apresentando potencial para o desenvolvimento de um futuro fármaco. Os autores concluíram que as euforbiáceas compreendem uma fonte preciosa de compostos químicos fitoterápicos, inclusive para combater bactérias multirresistentes (Voukeng et al. 2017).

Além de todas as moléculas bioativas citadas anteriormente, as Euphorbiaceae produzem uma gama diversificada de terpenos, muitos dos quais têm atividades farmacológicas. Apesar do interesse nos terpenos, atualmente sua biossíntese é difícil e pouca compreendida (King et al. 2014).

Os diterpenóides são compostos de 20 átomos de carbono derivados do precursor comum pirofosfato de geranilgeranilo. A biossíntese de diterpenoídeos é um processo essencial em plantas, sendo necessário para a produção de clorofilas, tocotrienol e giberelinas. Além desses metabólitos primários, algumas famílias de plantas desenvolveram a capacidade de produzir uma gama diversificada de metabólitos secundários como diterpenóides (Zerbe et al. 2013). A família Euphorbiaceae é bastante conhecida na medicina etnobotânica pela capacidade de produção de metabólitos (Mwine e Van Damme 2011) e análises dos extratos de várias espécies da família revelaram que em muitos casos a atividade biológica pode ser atribuída a diterpenóides particulares (Beutler et al. 1989).

Por fim, vale ressaltar que a grande disponibilidade de informações genéticas (especialmente genomas e transcriptomas) levou ao uso de técnicas genéticas que permitiram a implementação da tecnologia biossintética combinatória e mineração de dados ômicos. O conhecimento adquirido permitiu identificar moléculas desconhecidas, incluindo novas estruturas bioativas que podem ser otimizadas usando a química combinatória, gerando novos candidatos a drogas para muitas doenças. Cragg e Newman (2013) destacam que a natureza teve três bilhões de anos de adaptação e evolução, na geração de diversos compostos bioquímicos, sendo que estamos agora apenas arranhando a superfície para explorar a sua diversidade molecular.

2.3 MECANISMOS DE DEFESA DA PLANTA

A proteção contra os desafios que ameaçam a vida é um determinante crítico de auto-sobrevivência, incluindo a defesa contra estresses bióticos, como por exemplo predação por insetos e ataque por fitopatógenos (como fungos, bactérias, vírus e nematoides). Assim, os mecanismos de defesa evoluem à medida que combatem outros organismos em ambientes em mudança. Além de outros modos de proteção, as plantas desenvolveram uma resposta de defesa hipersensível (HR) contra estresses bióticos. Na HR, as plantas reconhecem a presença de patógenos através de assinaturas estruturais chamadas padrões moleculares associados a patógenos (PAMPs) através de receptores transmembranar (Dodds e Rathjen 2010).

O reconhecimento inicial de patógenos pelas plantas é análogo ao sistema animal na medida em que os receptores de reconhecimento de padrões (PRRs) na

superfície da célula hospedeira detectam a presença de PAMPs, representada por pequenos motivos de moléculas maiores que são essenciais para sobrevivência (Janeway e Medzhitov 2002). A imunidade desencadeada por PAMPs (PTI) ativa uma miríade de processos, incluindo cascatas de proteínas quinase ativadas por mitógenos (MAPK), produção de espécies reativas de oxigênio (ROS), sinalização hormonal e expressão gênica (**Figura 3**). A imunidade conferida através deste mecanismo é referida como imunidade desencadeada por PAMP (Schwessinger e Zipfel 2008).

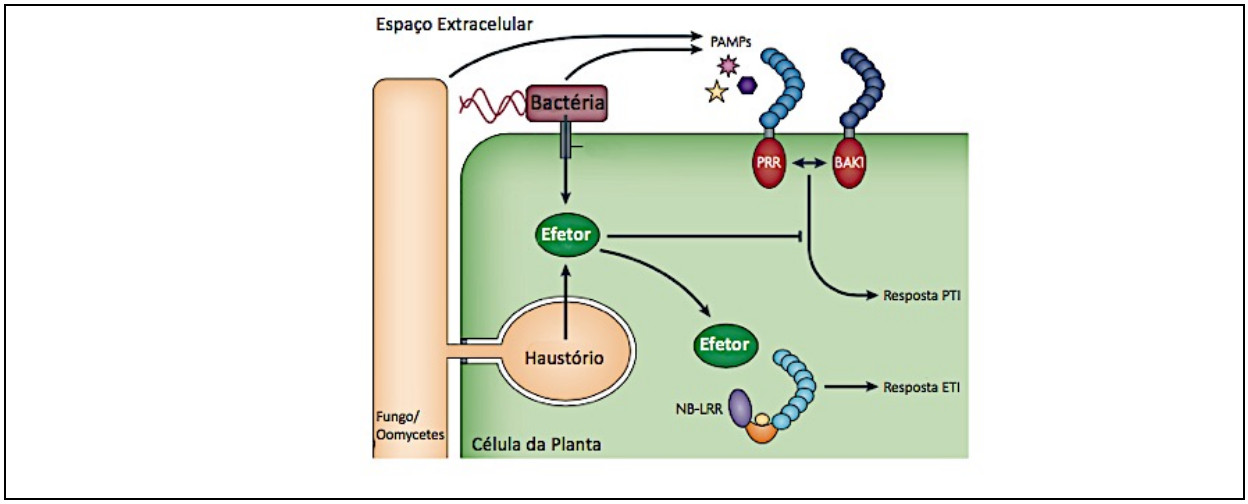

Figura 3 - Mecanismo de defesa imunológica da planta. Fonte: Baseado em Dodds e Rathjen (2010).

Os agentes patogênicos bem-sucedidos suprimem a PTI através do carreamento de proteínas efetoras de virulência ao hospedeiro. Por sua vez, o segundo ramo da imunidade das plantas, é definido por outro conjunto de receptores de plantas amplamente intracelulares, cujos produtos dos genes de resistência (R) são ativados. Esses receptores reconhecem proteínas efetoras específicas de forma direta ou indireta, ativando a “imunidade desencadeada por efetor” (ETI), uma resposta de defesa da planta mais forte que pode culminar na morte celular programada (Jones e Dangl 2006).

O PTI e o ETI estimulam diferentes caminhos de biossíntese e sinalização hormonal. O ETI desencadeia biossíntese e sinalização de ácido salicílico (SA), levando à resistência adquirida local e sistêmica (SAR) contra patógenos biotróficos (Metraux et al. 1990; Delaney et al. 1994). A SAR é a indução de resistência à doença de amplo espectro em tecidos distais não infectados, ativados por infecção patogênica

local, que resulta na necrose tecidual (HR) (Ward et al. 1991). Por outro lado, o PTI estimula a biossíntese de etileno (ET) (Felix et al. 1999). O ET e o ácido jasmônico (JA) ativam sinergicamente as defesas das plantas contra patógenos necrotróficos (Thomma et al. 1998). Além disso, existe uma interação antagonista entre a sinalização JA / ET e SA (Niki et al. 1998). Ambos os caminhos induzem a expressão de peptídeos antimicrobianos (AMP) de diferentes maneiras.

Desta maneira, ao longo da história evolutiva da relação planta-patógeno foram selecionadas nas plantas estratégias para combater os mecanismos de evasão desenvolvidos pelos patógenos (Chisholm et al. 2006). As mudanças abrangentes associadas à defesa de HR para patógenos desviam os recursos que de outra forma seriam usados para o crescimento e o desenvolvimento da planta de modo que, os estresses bióticos levam à redução da produtividade da planta por baixa-regulação de genes fotossintéticos e consequentemente redução da atividade fotossintética (Brown 2002; Bolton 2009).

Em termos de seleção natural, adaptações que geram menos custos energéticos às plantas tendem a ter sua frequência aumentada, como os AMPs que possuem a característica de terem um baixo custo energético para o hospedeiro com um amplo e potente espectro de ação. Por isso, não é surpreendente que, além das fontes de animais e plantas, os AMPs também foram identificados em microrganismos, incluindo bactérias e fungos (Benko-Iseppon et al. 2010; Paiva e Breukink 2013).

2.4 PEPTÍDEOS ANTIMICROBIANOS (AMPS)

Além da resposta de defesa hipersensível e proteínas de resistência (R), as plantas também empregam barreiras através da parede celular e sintetizam peptídeos antimicrobianos (AMPs). Os peptídeos antimicrobianos são moléculas de defesa de baixa massa molecular com uma vasta e potente atividade inibitória contra uma ampla gama de organismos, incluindo fungos (Han et al. 2016), bactérias (Liu et al. 2016), alguns vírus (Mathieu et al. 2017), como também predação por insetos (Fürstenberg-Hägg et al. 2013). Pertencem a um diverso e abundante grupo de moléculas que são produzidas tanto em plantas quanto em animais, agindo como componentes importantes da imunidade inata (Broekaert et al. 1997; Franco 2011).

Em animais, esses peptídeos antimicrobianos constituem parte do sistema imune inato (Lehrer et al. 1993; Benko-Iseppon et al. 2010). Em plantas, proteínas e peptídeos antimicrobianos formam um sistema de defesa comparável à imunidade inata em animais, protegendo-as assim do ataque de certos patógenos e pragas (Shewry e Lucas 1997; Gallo et al. 2002). Esses componentes vêm sendo isolados de diferentes espécies de plantas e a partir de diferentes órgãos como flores, folhas, frutos, tubérculos, raízes, e especialmente sementes (Broekaert et al. 1997; Benko-Iseppon et al. 2010; Nawrot et al. 2014; Goyal e Mattoo 2016).

2.4.1 Origem e Classificação

De forma geral os peptídeos antimicrobianos são derivados de proteínas precursoras não funcionais (o termo não funcional é utilizado para aqueles que não têm atividade biológica conhecida). Contudo, novas evidências indicam que esses peptídeos seriam derivados de proteínas funcionais, diretamente traduzidos a partir de pequenos quadros de leitura aberta (*Open Reading Frames*, ORFs), sem o envolvimento de um precursor ou sendo, ainda, codificados por transcritos primários de micro RNAs. Estes novos tipos de peptídeos aumentam a complexidade da peptidômica. Alguns AMPs são derivados de proteínas precursoras que necessitam de processamento para produzir um peptídeo funcional (Tavormina et al. 2015; Goyal e Mattoo 2016).

Os peptídeos são classificados de acordo com sua origem, estrutura primária e secundária, presença de resíduos de cisteínas (que se conectam em pares, formando pontes dissulfeto conferindo alta estabilidade a esses peptídeos) ou carga líquida (Astafieva et al. 2013). Alguns possuem α-hélice, outros β-folha, ou ambas as estruturas secundárias, α e β. Na sua maioria são ricos em aminoácidos básicos, fornecendo-lhes uma carga positiva (catiônicos) e pH fisiológico. Em relação à quantidade de aminoácidos, variam de >20 até cerca de 100 aminoácidos (Stotz et al. 2013; van der Weerden e Anderson 2013; Nawrot et al. 2014; Goyal e Mattoo 2016). Os peptídeos também foram classificados com base na sua origem como proteínas precursoras e/ou geradas pelo processamento pós-traducional em peptídeos maduros (Tavormina et al. 2015). As estruturas tridimensionais dos AMPs estão representadas categoricamente de acordo com o sistema prevalente na **Figura 4**.

2.4.2 Defensinas

Desde o início de 1990, foram realizados muitos estudos com AMPs, catiônicos de plantas ricos em cisteínas, hoje conhecidos como defensinas (Broekaert et al. 1995; Broekaert et al. 1997; Segura et al. 1998). As defensinas vegetais são um dos tipos de AMPs mais estudados, foram primeiramente descritas nas sementes de trigo (*Triticum turgidum*) e cevada (*Hordeum vulgare*) (Colilla et al. 1990; Mendez et al. 1990). A caracterização inicial dessas proteínas foi na família proteica das tioninas (Carrasco et al. 1981), devido às similaridades na massa molecular, na sequência de aminoácidos e no número de cisteínas. No entanto, estudos posteriores revelaram a existência de diferenças no padrão das pontes dissulfeto, demonstrando que estas duas famílias de peptídeos não estão relacionadas (Bruix et al. 1993). Depois de comparar sua semelhança estrutural e funcional com os AMPs previamente caracterizados encontrados em insetos e mamíferos, esses peptídeos foram renomeados como defensinas vegetais (Terras 1995; Broekaert et al. 1995). Desde então o número de defensinas de plantas descritas tem aumentado rapidamente (Bruix et al. 1993; Fant et al. 1998; Bloch et al. 1998; Almeida et al. 2002; Antcheva et al. 2006; Lacerda et al. 2014; Goyal e Mattoo 2016).

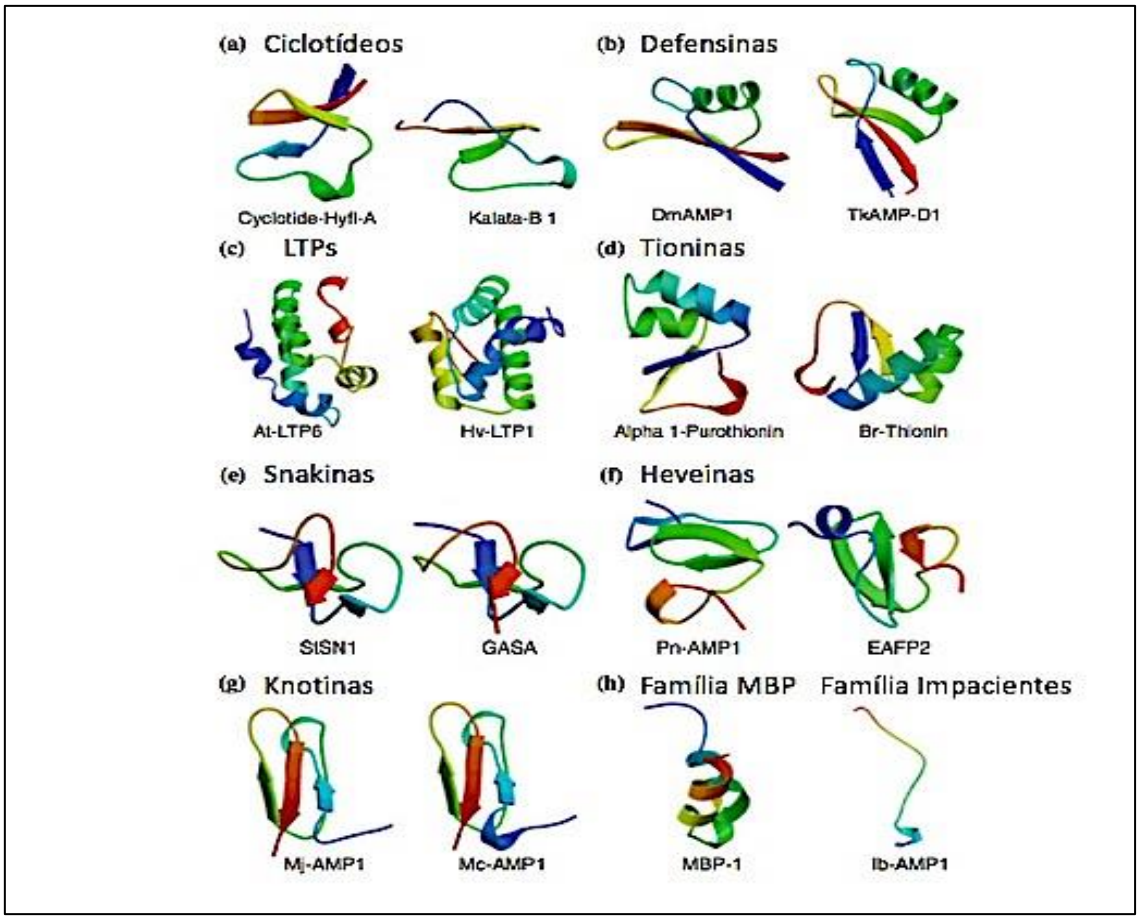

Figura 4 - Estrutura tridimensional das principais categorias e peptídeos antimicrobianos. Fonte: Adaptado de Goyal e Mattoo (2016).

As defensinas são pequenos peptídeos com tamanho variando entre 45 e 54 aminoácidos, sendo geralmente catiônicas, com uma assinatura conservada de cisteínas, que podem formar três a quatro pontes dissulfeto na sequência de Cys1-Cys8, Cys2-Cys5, Cys3-Cys6 e Cys4-Cys7 (**Figura 5**) (Lay e Anderson 2005). No entanto, algumas defensinas possuem estruturas diferenciadas. Por exemplo, defensinas isoladas de flores de petúnia (*Petunia hybrida,* PhD1 e PhD2) apresentam em sua estrutura 10 resíduos de cisteínas formando cinco pontes dissulfeto. A ponte dissulfeto adicional, localizada após a α-hélice e a primeira folha β não afeta a estrutura tridimensional típica da defensina. A quinta ponte dissulfeto parece reforçar uma ligação de hidrogênio conservada e confere maior estabilidade termodinâmica à defensina (Lay 2003; Janssen et al. 2003).

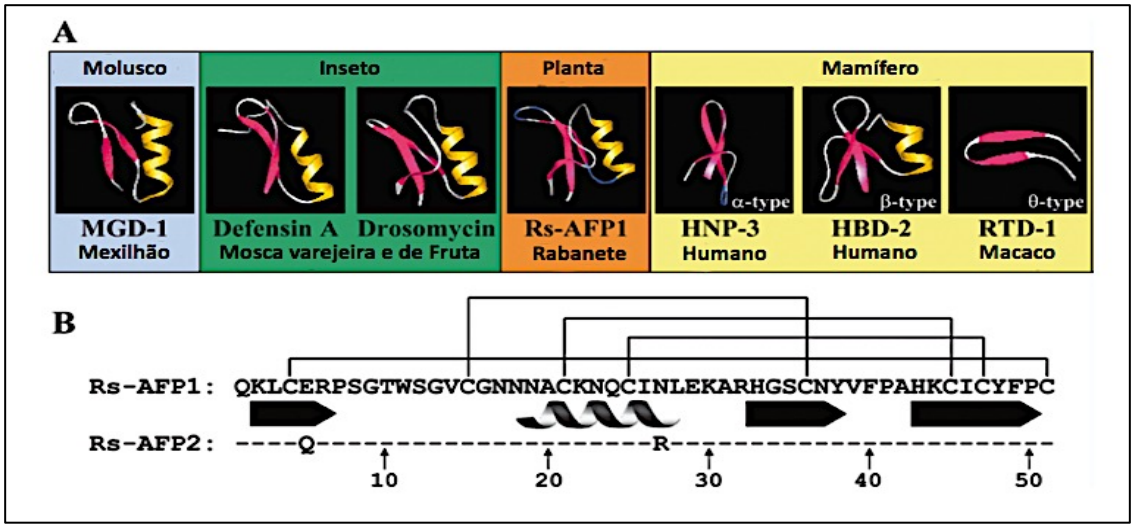

Figura 5 - Estrutura tridimensional de defensinas de plantas, invertebrados (insetos e moluscos) e vertebrados (mamíferos). B – Sequência de aminoácidos de rabanete (*Raphanus sativus*), Rs-AFP1 e 2. Os traços indicam resíduos de aminoácidos idênticos. As linhas de conexão entre os resíduos de cisteína representam ligações dissulfeto e a espiral e as setas indicam a localização da alfa-hélice e das folhas beta, respectivamente. Fonte: Adaptado de Thomma et al. (2002).

Existe uma relação entre a estrutura tridimensional global de defensinas vegetais, defensinas de insetos e beta defensinas de mamíferos. Tais características sugerem que as defensinas são peptídeos conservados nos eucariotos, originados antes da divergência evolutiva de plantas e animais (Thomma et al. 2002).

A quantidade de resíduos de cisteínas conservadas formando pontes dissulfeto, principalmente próximo dos terminais N e C (Cys1-Cys8), conferem uma extraordinária estabilidade, pela conformação de cadeia tripla de folhas β antiparalelas e a estrutura de α-hélice CS-αβ (**Figura 6**) (Goyal e Mattoo 2016). A integridade das pontes dissulfeto e da conformação estrutural é essencial para a atividade antimicrobiana, embora a estabilidade da estrutura não esteja ligada diretamente com a atividade (Sagaram et al. 2011; Lacerda et al. 2014). Com base em uma análise mutacional, foi demonstrado que a defensina Rs-AFP2 possuía dois locais importantes para a atividade antifúngica contra o fungo *Fusarium culmorum*. O primeiro envolvia as cadeias β2 e β3, formando uma ligação altamente hidrofóbica. A segunda foi composta pela ligação da cadeia β1 e α-hélice (De Samblanx et al. 1997). Estas duas regiões são importantes para a atividade antifúngica e podem constituir dois locais que contatam um único receptor predito. Alternativamente, a presença de dois locais poderia ser indicativa de duas características distintas, necessárias para a atividade antifúngica de Rs-AFP2, tal como a ligação de Rs-AFP2 ao seu receptor por

um lado e a subsequente permeabilização da membrana por outro lado (De Samblanx et al. 1997; Schaaper et al. 2001).

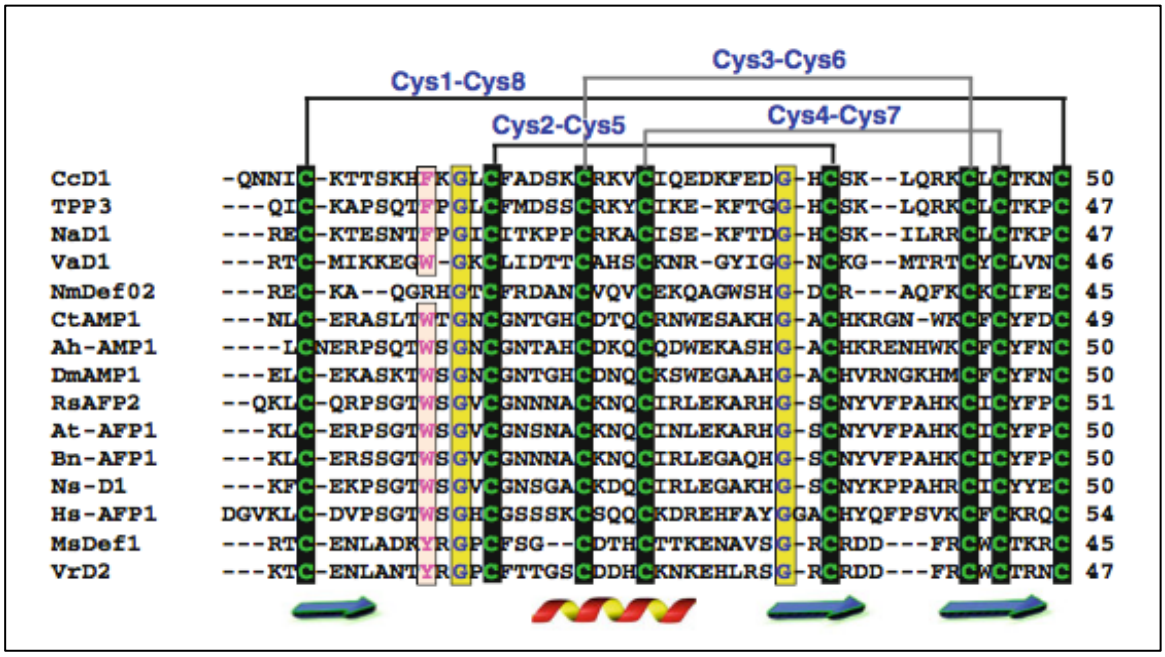

Figura 6 - Alinhamento da sequência de aminoácidos de diferentes defensinas de plantas. No alinhamento destacado na cor verde mostram as cisteínas altamente conservadas, já as linhas sólidas na parte superior, mostram as ligações entre as cisteínas formando pontes dissulfeto. Os resíduos de glicina em amarelo se mostram igualmente conservada com a preferência de um aminoácido aromático em rosa que precede o primeiro resíduo de glicina. As setas representam folhas betas, já a espiral representa a alfa-hélice. Fonte: Adaptado de Goyal e Mattoo (2016).

Em relação à sua origem e estrutura, as defensinas são classificadas em dois grupos a partir dos seus tipos de precursores. No primeiro e maior, a proteína precursora é composta por uma sequência sinal de retículo endoplasmático que direciona as proteínas para o espaço extracelular (**Figura 7A**). Este fragmento muitas vezes é ácido e pode também desempenhar um papel na atividade supressora até a maturação, seguido de um domínio de defensina madura (Lay e Anderson 2005). Na segunda, inclui defensinas produzidas como precursores mais longos, com pró-domínio C-terminal (**Figura 7B**), mais comuns em solanáceas (Gu et al. 1992; Milligan e Gasser 1995; Brandstädter et al. 1996; Lay 2003) e em tecidos de frutos (Aluru et al. 1999). Esse pró-domínio pode contribuir para o amadurecimento da defensina, agindo como uma chaperona intramolecular e/ou impedindo as interações deletérias entre as defensinas e outras proteínas celulares ou membranas lipídicas, neutralizando a atividade tóxica durante a translocação através da via secretora (Michaelson et al. 1992; Bohlmann e Broekaert 1994; Florack e Stiekema 1994; Lay e Anderson 2005; Aerts et al. 2008; Carvalho e Gomes 2011; Santana 2012).

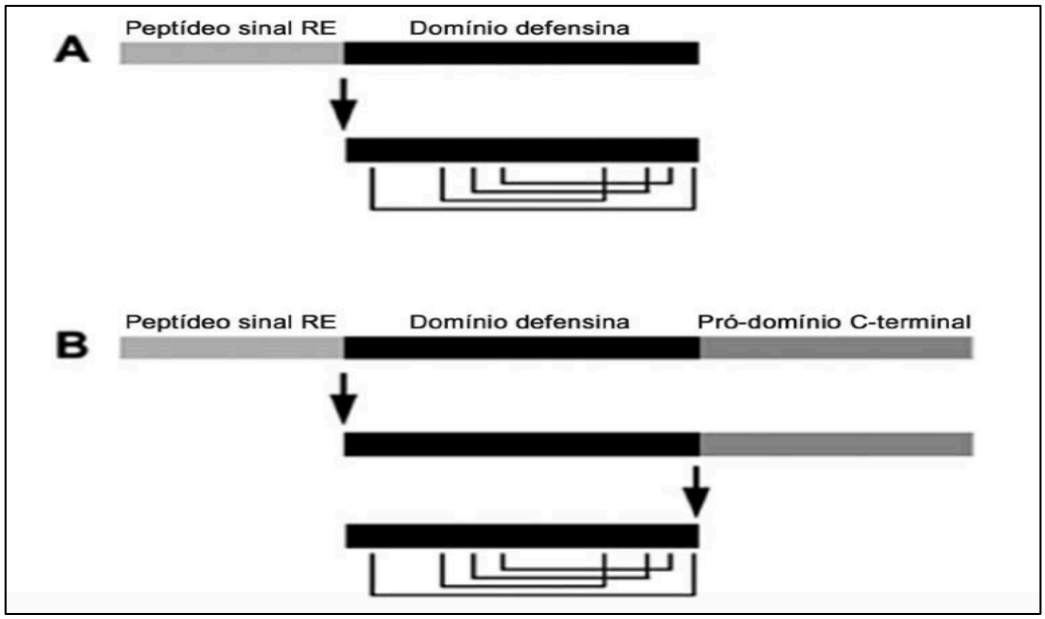

Figura 7 - Duas classes de defensinas de plantas. (A) A maioria das defensinas vegetais possuem uma sequência de peptídeo sinal (RE) além do domínio maduro. (B) Em algumas solanáceas, isolaram-se clones de cDNA que codificam defensinas vegetais com um pró-domínio C-terminal adicional após o domínio maduro. Fonte: Adaptado de Lay e Anderson (2005).

Inicialmente pensou-se que as defensinas só estariam presentes nas sementes de angiospermas, onde a maioria delas foi isolada (Carvalho e Gomes 2011; Lacerda et al. 2014), muitas delas caracterizadas em nível molecular, bioquímico e estrutural (Broekaert et al. 1995; De Samblanx et al. 1997; Thomma et al. 2002). Porém descobriu-se que esses peptídeos estão aparentemente distribuídos em quase todos os órgãos das plantas, incluindo folhas (Terras 1995; Kragh et al. 1995; Komori et al. 1997; Segura et al. 1998; Lay 2003), vagens (Chiang e Hadwiger 1991), tubérculos (Moreno et al. 1994), frutos (Meyer et al. 1996; Aluru et al. 1999; Wisniewski et al. 2003), raízes (Sharma e Lönneborg 1996), casca do tronco (Moreno et al. 1994; Wisniewski et al. 2003) e tecidos florais (Gu et al. 1992; Moreno et al. 1994; Karunanandaa et al. 1994; Milligan e Gasser 1995; Li e Gray 1999; Park et al. 2002; Lay 2003). Além disso, as defensinas vegetais também estão localizadas nas células do xilema, do estômato, do parênquima e outras áreas periféricas (Kragh et al. 1995; Segura et al. 1998; Chen et al. 2002).

A presença de defensinas nos diferentes tecidos é consistente com o papel defensivo desses peptídeos, uma vez que cada um desses tecidos pode compreender o local do primeiro contato com um patógeno potencial (Carvalho e Gomes 2011).

Além da sua localização, as defensinas podem ser classificadas de acordo com a sua atividade biológica, seja contra bactérias, vírus e fungos (**Tabela 1**). Podem

ainda apresentar ação imunomoduladora, auxiliar na cicatrização de feridas, além de apresentar outras funções coadjuvantes. As defensinas podem auxiliar no controle de infecções e inflamações, podendo apresentar sinais quimiotácticos para células imunes, pela indução de secreção de citoquinas e quimiocinas, bem como neutralizar exotoxinas e endotoxinas (van der Weerden e Anderson 2013; Pachón-Ibáñez et al. 2017).

Tabela 1 - Defensinas e seu amplo espectro de atividades biológicas.

Atividade Biológica	Nome	Referência
Antibacteriana	MBD-3	Manko et al. 2017
	N1 e N3	Yang et al. 2017
	Chrysophsin-1	Tripathi et al. 2017
Anti-inflamatória	hBD2	Li et al. 2017
	hBD3	Bedran et al. 2014
Antifúngica	JBTX	Becker-Ritt et al. 2017
	Ts1	Santussi et al. 2017
	PAF112 e PAF118	Garrigues et al. 2017
Viral	APOBEC3G	Lafferty et al. 2017
	HPIV3	Mathieu et al. 2017

Embora esses peptídeos possam apresentar uma capacidade inibitória do crescimento microbiano, apenas algumas defensinas vegetais têm apresentado atividade contra bactérias (Lacerda et al. 2014). A maioria das defensinas reportadas na literatura apresenta atividade antifúngica, sendo efetivas no combate de diversos tipos de fungos patogênicos e fitopatogênicos e leveduras (Garrigues et al. 2017; Santussi et al. 2017; Becker-Ritt et al. 2017). Esses peptídeos de defesa são classificados como proteínas promíscuas, pois mostram inúmeras atividades biológicas. Como exemplo, a família de defensinas isoladas de *Vigna unguiculata*, nas quais diferentes formas homólogas podem atuar como inibidores antifúngicos, antibacterianos e enzimáticos (Franco, 2011).

Apesar da ocorrência de muitos relatos na literatura sobre a atividade de defensinas de plantas, poucas delas tiveram o seu mecanismo de ação elucidado (Huang et al. 2012). Devido ao seu caráter catiônico, sabe-se que essas moléculas interagem especificamente com compostos de membrana de carga negativa do patógeno, como receptores lipídicos de bactérias, fosfolipídios e esfingolipídios de membranas fúngicas (Wilmes et al. 2011; Vriens et al. 2014).

2.4.3 Mecanismo de ação dos AMPs

Os AMPs, sejam de plantas ou de mamíferos, podem distinguir o hospedeiro dos seus alvos microbianos. Estudos indicam que a disparidade estrutural das membranas procarióticas e eucarióticas contribui para a seletividade do AMP (Zasloff 2002; Yeaman 2003; Yount e Yeaman 2013).

A parede celular protetora de um microrganismo é provavelmente o primeiro ponto de contato com AMPs. A parede celular fúngica é complexa, montada em muitas camadas, sendo de até 80% de heteropolissacarídeos, apresentando ainda a camada mais interna composta de bicamadas lipídicas com proteínas de quitina e β-glucano. Uma parede de células bacterianas Gram negativas contém membrana de bicamada lipídica interna envolvida por uma fina camada de peptidoglicano, adicionalmente, existe uma membrana externa composta por fosfolipídios e lipopolissacarídeos, que fornecem uma carga negativa à superfície da membrana. Em contrapartida, as bactérias Gram-positivas contêm apenas uma camada muito espessa de peptidoglicano adjacente à membrana interna. O enriquecimento da camada de peptidoglicano com polissacáridos ácidos – tais como os ácidos teicoico e lipoteicoico – confere uma carga negativa à membrana. Por outro lado, a parede celular de plantas possui características distintas de celulose, hemicelulose, ligninas, havendo ausência de peptidoglicano, quitina e β-glucano. Em fosfolipídios de membrana de mamíferos, a fosfatidilcolina e a fosfatidiletanolamina são neutras (Goyal e Mattoo 2016).

Apesar das diferenças estruturais significativas em organismos procarióticos, os AMPs são conhecidos por estabelecer interação na membrana desses patógenos. A estrutura dos AMPs com regiões hidrofóbicas de carga positiva interage com as cabeças polares carregadas negativamente e o núcleo hidrofóbico das membranas microbianas. Uma carga positivamente elevada nos AMPs catiônicos faz com que haja uma interação eletrostática, facilitando assim a sua ligação inicial às membranas com carga negativa dos patógenos. Existem vários parâmetros estruturais que contribuem para a toxicidade e especificidade do alvo, como estrutura, carga, hidrofobicidade e anfipaticidade (Yeaman 2003).

A ligação de um AMP a um patógeno não é necessariamente realizada apenas por interações eletrostáticas, mas também pelo reconhecimento de componentes específicos da parede celular do microrganismo. Contudo, Dutta et al. (2018) propuseram que os AMPs contêm pelo menos uma região amiloidogênica que poderia

auxiliar na autoagregação dos peptídeos, apresentando uma possível estrutura oligomérica em forma de anel, desta forma potencializando seu mecanismo de ação. Uma vez que o contato tenha sido feito através de uma interação inicial e o AMP tenha se ligado ao alvo, o efeito tóxico sobre o patógeno pode então ser exercido de duas formas gerais, por permeabilização da membrana ou por comprometimento das funções intracelulares (Thevissen et al. 2003; Goyal e Mattoo 2016).

2.4.3.1 Permeabilidade da membrana

A permeabilização da membrana ocorre após a interação do AMP com o sítio alvo do patógeno, resultando na dissipação do gradiente eletroquímico através da membrana, fragmentação da membrana, vazamento de íons e outros conteúdos celulares e, em última análise, morte celular (Shai 2002). A concentração limiar de um AMP é necessária para induzir a permeabilização e o fenômeno é dependente do tempo (Wimley 2010). Outras deformações estruturais da membrana ocorrem ao longo do tempo. A permeabilidade da membrana pode ocorrer de diferentes maneiras dependendo da interação pela estrutura de um AMP. Modelos foram propostos para explicar o efeito dos AMPs nas membranas (**Figura 8**).

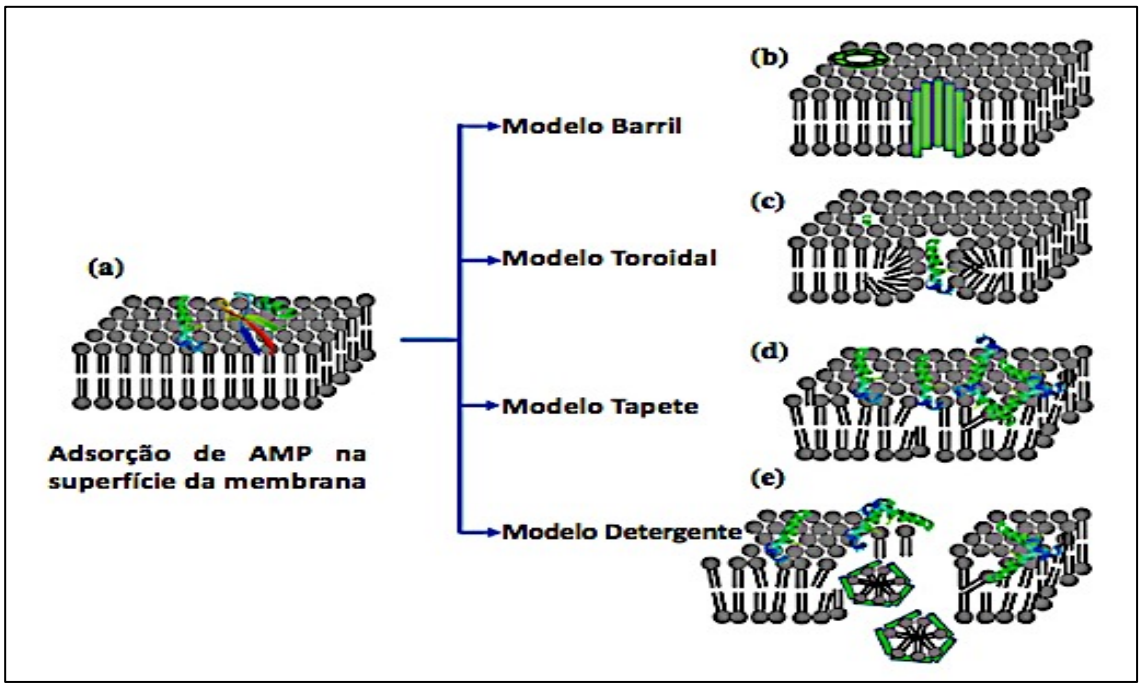

Figura 8 - Modelos de permeabilização da membrana por peptídeos antimicrobianos. (a) Ligação inicial do AMP na superfície da membrana. (b, c) Formação de poros, podendo desestruturar a membrana. (d) Modelo tapete. (e) Modelo detergente. Fonte: Adaptado de Goyal e Mattoo (2016).

2.4.3.2 Modelo barril

Neste modelo, as moléculas de AMP ligam-se à membrana alvo com um acúmulo de monômeros de peptídeos realizando interação com a membrana e diminuindo sua espessura. Quando a concentração crítica de peptídeos é atingida os aglomerados de peptídeos formam um poro transmembranar do tipo barril (**Figura 8**b). As regiões hidrofóbicas da α-hélice ou das folhas β de um AMP se alinham paralelamente a parte da cadeia acila dos fosfolipídeos da membrana e a face hidrofílica forma a superfície interna do poro para a passagem de íons, água, além de componentes intracelulares (Bechinger 2004; Kozma et al. 2012; Batista 2015). O tamanho dos poros pode variar dependendo do peptídeo e do grau de agregação. Um estudo de caso com alameticina, um peptídeo de 20 resíduos produzido pelo fungo *Trichoderma viride*, evidencia o mecanismo de ação do tipo barril (Aidemark et al. 2010). Esse modelo é, entretanto, considerado de formação rara durante a ação de peptídeos, sendo o modelo toroidal (**Figura 8**c) o mais aceito (Yang et al. 2001; Batista 2015).

2.4.3.3 Modelo toroidal

O modelo toroidal é um mecanismo conjugado de formação de poros e de ruptura da membrana. Sua principal diferença para o modelo barril é a intercalação de AMP na bicamada lipídica induz curvatura positiva das cabeças polares fosfolipídicas perpendiculares ao plano da membrana. O peptídeo proporciona uma alternativa mais forte de interações hidrofóbicas e hidrofílicas do que interações intramoleculares de moléculas lipídicas (Shai 2002; Jenssen et al. 2006; Bocchinfuso et al. 2009). A presença do peptídeo rompe as interações hidrofóbicas de moléculas lipídicas e favorecendo seu realinhamento para criar poros toroidais (Figura 8c). Em contraste com o modelo barril, os grupos lipídicos nos poros toroidais são expostos ao lúmen de um poro. Em estudos com peptídeos Magainina 2 e PGLa, observou-se que esses AMPs se ligam preferencialmente a membranas carregadas negativamente e aparentemente causam sua ruptura pela formação de poros transmembranares toroidais. Os ângulos de inclinação calculados para Magainina 2 e PGLa estavam em excelente concordância com experiências recentes de RMN de estado sólido (Pino-Angeles et al. 2016).

2.4.3.4 Modelo tapete

O modelo tapete é um mecanismo de ação que não forma poros. Nele os peptídeos se inserem no núcleo hidrofóbico da membrana, porém se orientam em paralelo à superfície da membrana, recobrindo-a como um tapete. Uma forte interação eletrostática entre os grupos fosfolipídicos de cabeça polar carregados negativamente e o peptídeo catiônico, realizam uma distorção na estrutura da membrana, permitindo a chegada de peptídeos adicionais (Figura 8d). À medida que o peptídeo atinge o seu limiar, a desintegração da membrana ou a lise celular é induzida (Shai 1999; He e Lazaridis 2013). Nguyen et al. (2011) sugerem que esse mecanismo também possa ser a primeira etapa do processo de peptídeos que atuam formando poros. Este modelo foi proposto pela primeira vez para explicar o efeito tóxico de cecropin P1 da hemolinfa de uma traça, o qual se alinha paralelamente à superfície da membrana. Alguns exemplos de peptídeos que realizam esse tipo de mecanismo são a dermaseptina S (Strahilevitz et al. 1994; Shai 2002; Raja et al. 2013) e outros análogos, como cecropinas (Gazit et al. 1995) caerina (Wong et al. 1997) e mastoparan X (Whiles et al. 2001; Guo et al. 2011).

2.4.3.5 Modelo detergente

Esta é uma versão estendida do modelo tapete na qual o peptídeo interage através de um mecanismo semelhante ao modelo do tapete, levando ao colapso catastrófico da membrana. As moléculas peptídicas formam micelas com a membrana fragmentada de forma semelhante à ação de um detergente (Figura 8e). A repartição abrangente da membrana permite que o citoplasma extravase, resultando na morte celular (Bechinger e Lohner 2006; Goyal e Mattoo 2016).

2.4.3.6 Modelo Shai-Matsuzaki-Huang

Este modelo propõe que a interação dos peptídeos que aderem à face extracelular da membrana plasmática tenha a capacidade de tracionar a bicamada, reduzindo a sua espessura, e permitindo a formação de poros transitórios (**Figura 9**). Neste modelo, algumas moléculas de peptídeos podem atravessar os poros, alcançando a face interna da membrana e outros elementos intracelulares do microrganismo (Zasloff 2002).

Em geral, os peptídeos que usam este mecanismo matam patógenos em baixas concentrações, como é o caso do peptídeo nisina, uma molécula anfipática de

14 aminoácidos produzida por *Lactococos lactis*, que opera em concentrações nanomolares. Além disso, algumas defensinas de plantas usam uma estratégia similar, a exemplo de *Dahlia merckii* 1 (DmAMP1), uma defensina isolada da semente de dália (*D. merckii*) com atividade antifúngica (Brötz et al. 1998; Thevissen et al. 2000).

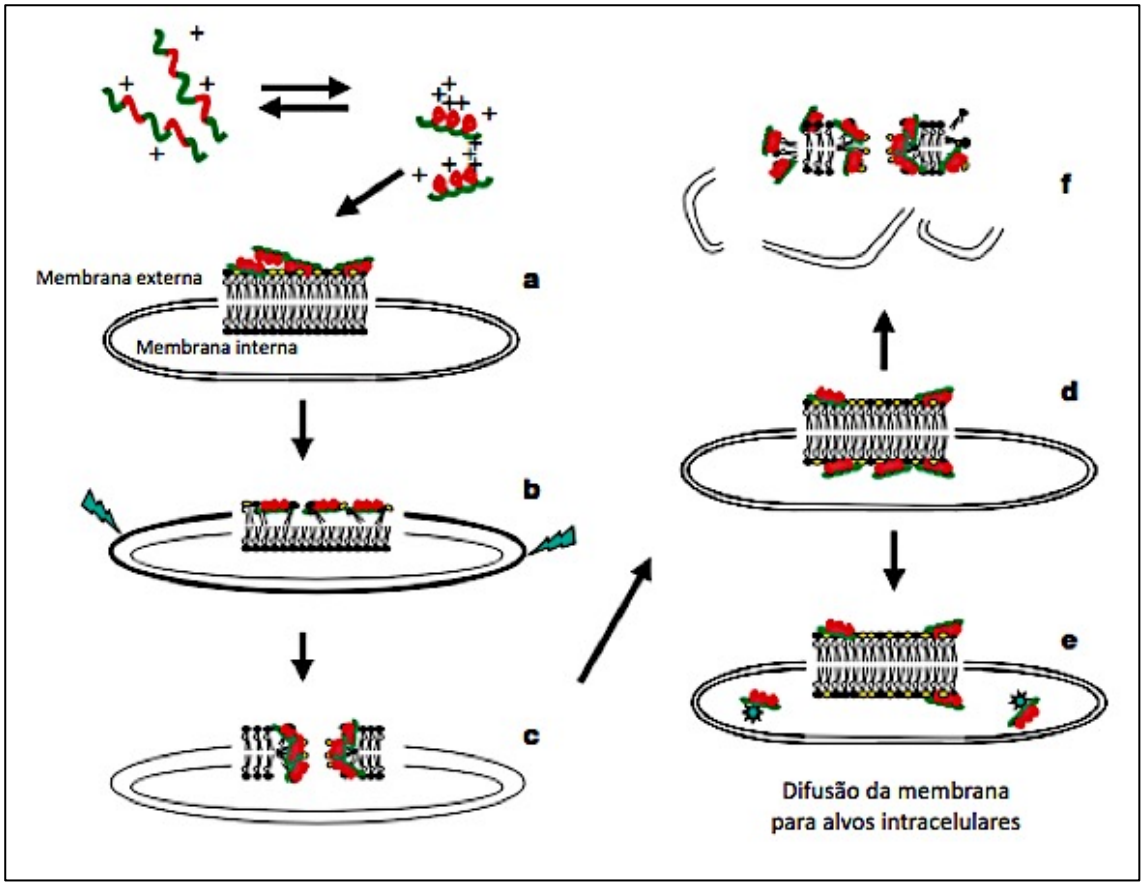

Figura 9 - O modelo do mecanismo de ação Shai-Matsuzaki-Huang de um peptídeo antimicrobiano. Um peptídeo alfa-hélice é representado. (A) Os peptídeos se aderem pelo Modelo Tapete. (B) A área de superfície da superfície externa se expande em relação a interna, resultando em tensão dentro da bicamada (setas irregulares). (C) Transição de fase e formação de poros. (D) Transporte de lipídios e peptídeos para o interior do corpo. (E) Difusão de peptídeos em alvos intracelulares (em alguns casos). (F) Colapso da membrana em fragmentos e ruptura física da membrana da célula-alvo. Fonte: Adaptado de Zasloff (2002).

2.4.3.7 Comprometimento das funções intracelulares

A permeabilização da membrana é considerada um importante atributo da atividade antimicrobiana. No entanto, evidências recentes sugerem que existam outros mecanismos de ação dos AMPs, além da interrupção das funções da membrana (Brogden 2005; Muñoz et al. 2013). Em alguns casos o grau de permeabilização de alguns peptídeos não aparentou relacionado com a sua atividade,

uma vez que em alguns testes o microrganismo sobreviveu durante um período de tempo prolongado após a interrupção da membrana. Num outro estudo, os fragmentos ativos de um peptídeo bovino (catelicidinas) Bac7 não permeabilizaram a membrana de *Escherichia coli*, mas proporcionou uma redução de 2-5 log na contagem de células viáveis (Gennaro e Zanetti 2000). Estudos *in vitro* demonstraram a capacidade que um AMP tem de se associar a alvos intracelulares, tais como ácidos nucléicos, proteínas ou enzimas, o que sugere um mecanismo de ação que não envolve apenas as membranas.

Tem sido um consenso que o mecanismo de ação dos AMPs seja resultante da combinação da permeabilização da membrana e da inibição das funções intracelulares. Para facilitar a internalização do peptídeo no citoplasma, pode haver uma alteração transitória ou permanente da estrutura da membrana, o que pode aumentar o efeito letal de um AMP. Outras possibilidades como o transporte passivo de peptídeo (Henriques et al. 2006) e/ou um processo ativo dependente de energia (Kim et al. 2001) são significativos. Muitas defensinas antifúngicas de plantas interagem com esfingolipídios ou fosfolipídios de membrana fúngica, posteriormente, algumas delas são internalizadas e induzem morte celular, como por exemplo, MtDef4, NaD1 e Psd1 (Kaewklom et al. 2016).

Os detalhes do mecanismo da ação para algumas defensinas foram revistos exaustivamente por Vriens et al. (2014) com foco nas defensinas RsAFP2, MsDef1, MtDef4, NaD1 e Psd1. As observações apontam uma variedade de mecanismos pela qual essas defensinas de plantas com atividade antifúngica afetam a viabilidade das células microbianas. Uma vez dentro da célula, um AMP pode atacar as organelas celulares e afetar as suas funções. Diferentes AMPs podem interagir com diversos alvos intracelulares interferindo em sua funcionalidade, assim como diferentes AMPs podem também interagir no mesmo alvo (Goyal e Mattoo 2014).

Graças a essa diversidade, os AMP possuem atividade antimicrobiana de amplo espectro, de modo que a atividade antimicrobiana não se restringe a sua própria população patogênica, mas também muito além do filo ou reino. Esta característica é evidente a partir do seu mecanismo de ação, onde os AMPs atacam as paredes das células microbianas, além de se ligar a domínios específicos. A atividade através de uma gama de patógenos de diversos reinos foi observada em AMPs isolados de plantas que mostraram atividade antimicrobiana contra patógenos de mamíferos,

incluindo humanos. Por outro lado, AMPs isoladas de insetos, artrópodes, anfíbios, humanos, entre outros, apresentam toxicidade contra uma variedade de fitopatógenos (Hammami et al. 2009). Além do mais, alguns AMPs podem ser modificados visando a uma diminuição de sua citotoxicidade para as células hospedeiras mantendo sua atividade antimicrobiana. Esta propriedade dos AMPs aumentou o alcance de sua aplicação no manejo de doenças de seres humanos ou de outros mamíferos de interesse comercial, bem como na defesa vegetal. Curiosamente, AMPs vegetais mostraram resultados promissores no combate a células cancerosas humanas (Marques et al. 2017).

2.4.4 AMPs como uma nova classe de agentes terapêuticos

O interesse recente em AMPs para aplicações terapêuticas desenvolveu-se em parte devido à necessidade de novos antibióticos, uma vez que as bactérias passaram a apresentar resistência às moléculas antibióticas tradicionais (Ahmad et al. 2013; Yang et al. 2017). Presumivelmente, as bactérias foram expostas aos AMPs por milhões de anos e, com exceção de poucas espécies (como, por exemplo, espécies de *Burkholderia*), não foram relatadas resistências a todos os tipos de AMPs (Loutet e Valvano 2011). Notavelmente, o desenvolvimento de resistência contra AMPs ocorreu em um grau muito menor, pois não poderia haver um mecanismo geral de resistência aos AMPs, sendo necessários múltiplos caminhos como, por exemplo, ataque a múltiplos alvos hidrofóbicos e/ou polianiônicos (Friedrich et al. 2001; Peschel e Sahl 2006; Fjell et al. 2011). Desta forma, os AMPs surgiram como potenciais candidatos na busca de novos agentes terapêuticos contra a resistência bacteriana (Hancock e Sahl 2006; Vaara 2009; Ahmad e Laughlin 2010; Laughlin e Ahmad 2010; Yeung et al. 2011; Liu et al. 2016; Lafferty et al. 2017).

Uma série de peptídeos com atividade antimicrobiana segue em vários estágios de análise edesenvolvimento, com possíveis aplicações clínicas incluindo atividade anticancerígena, imunomoduladora, cicatrização de feridas, como drogas carreadoras de substâncias, adjuvantes de vacinas, reguladores da defesa inata e agentes pró e anti-inflamatórios (Yeung et al. 2011). As aplicações gerais em desenvolvimento incluem antibióticos tópicos e antissépticos, atividade anti-inflamatória, infecções hospitalares e respiratórias (Fjell et al. 2011).

Os esforços atuais para desenvolver AMPs para aplicações terapêuticas específicas incluem terapias para doenças da mucosa bucal (Okamura et al. 2011; Li et al. 2017b), infecções por biofilmes (de la Fuente-Núñez et al. 2016), septicemia bacteriana (Brandenburg et al. 2011; Silva et al. 2015), terapia adjuvante antimalárica (Achtman et al. 2012) e contra *Staphylococcus aureus* resistente à meticilina (Fitzgerald-Hughes et al. 2012; Liu et al. 2016).

Desde o ano 2000 cerca de 22 novos antibióticos foram desenvolvidos e 49 compostos estavam em desenvolvimento clínico. Pelo menos 15 peptídeos ou peptídeos miméticos para aplicações terapêuticas estão em desenvolvimento ativo, havendo uma predominância de compostos derivados de produtos naturais presentes nos últimos estágios de testes sugerindo a possibilidade de que produtos naturais como AMPs possam ter uma maior probabilidade de sucesso (Butler e Cooper 2011; Fjell et al. 2011; Butler et al. 2013; Butler et al. 2017). Os possíveis pontos fracos do uso de AMPs como candidatos a novos antibióticos incluem uma baixa atividade devido à susceptibilidade das proteólises, citotoxicidade celular inespecífica, altos custos de produção, perda de atividade, potencial interferência na imunidade inata do hospedeiro e interferência com a flora normal. Porém essas barreiras iniciais vêm sendo superadas com o desenvolvimento de peptídeos sintéticos estáveis, mais econômicos e com potente e amplo espectro de ação (Tan et al. 2017). Assim, há esperança de que os AMPs gerem uma nova geração de fármacos antimicrobianos com uma ampla gama de aplicações tópicas e sistêmicas no tratamento de diversos tipos de infecção (Vaara 2009; da Rocha Pitta et al. 2010; Sun et al. 2016).

2.4.5 AMPs sintéticos

A síntese de peptídeos percorreu um longo caminho, começando com (Fischer e Fourneau 1901) e o desenvolvimento de grupos protetores de carbamato por Bergmann e Zervas (1932), seguido da síntese de oxitocina pelo vencedor do Prêmio Nobel (du Vigneaud et al. 1954) e pelo método de síntese de peptídeo em fase sólida (SPFS) revolucionário desenvolvido por (Merrifield 1963). Os avanços modernos no campo dos peptídeos sintéticos, como a automação total da montagem da síntese, a otimização contínua das metodologias e a introdução de técnicas de ligação para expandir o escopo da síntese química total de proteínas, estão contribuindo para uma maior implementação e aceitação da síntese em fase sólida para muitos laboratórios

de pesquisa em todo o mundo. Este desenvolvimento levou a um campo próspero com vários peptídeos no mercado e centenas deles atualmente passando por testes clínicos (Vlieghe et al. 2010; Kaspar e Reichert 2013). Em particular, os avanços metodológicos nas últimas quatro décadas foram cruciais para o crescimento e o sucesso da pesquisa de peptídeos (Muttenthaler et al. 2015).

O método de SPFS baseia-se no crescimento, resíduo por resíduo, da cadeia peptídica presa covalentemente pelo seu aminoácido carboxiterminal a sítios reativos existentes em um suporte sólido (resina). A característica insolúvel da resina possibilita que todas as etapas da síntese sejam realizadas em um único frasco de reação contendo uma placa porosa filtrante. Essa placa retém a resina desde o início até o final da síntese, o que evita a troca do frasco de reação, simplificando o manuseio e impedindo perdas de produto. O uso da resina insolúvel permite que a eliminação de todos os reagentes, solventes e subprodutos das diversas etapas do ciclo sintético seja feita por simples filtração em placa porosa. Esse procedimento faz com que o método se torne simples e rápido, além de permitir o uso de grandes quantidades de reagentes. Esse procedimento também garante reações mais eficientes e quantitativas em todas as etapas do ciclo sintético. Somado a isto, como as etapas sintéticas atualmente são padronizadas e repetitivas, é possível a automação desse método de síntese peptídica (Muttenthaler et al. 2015; Bergmann et al. 2017).

Devido a estes avanços metodológicos, pesquisas recentes com AMPs têm se concentrando em métodos de SPFS, bem como em métodos de síntese “de novo” ou por modificação de modelos naturais que possam ser otimizados visando à redução do tamanho das sequências peptídicas, seja de forma empírica ou computacional. Estes peptídeos sintênticos para serem considerados potencial candidatos precisam apresentar uma ampla atividade antimicrobiana, com também uma baixa hemólise e citotoxicidade (Xu et al. 2014; de la Fuente-Núñez et al. 2016).

De um modo geral são usadas três abordagens para a produção de AMPs sintéticos. A primeira delas é o estudo fundamentado em modelos, onde sequências de AMPs com atividade conhecida ou toxicidade reduzida são modificadas a partir das sequências de aminoácidos, muitas vezes modificando um único aminoácido no peptídeo para identificar aminoácidos e posições que são importantes para a atividade

antimicrobiana, sendo que neste tipo de método o uso dos recursos computacionais geralmente é limitado (Jiang et al. 2011; Fjell et al. 2011).

A segunda abordagem compreende estudos biofísicos, em contraste com o método anterior baseado em modelo, em que os peptídeos são tratados como um "texto" formado por "letras" de aminoácidos que possuem propriedades individuais como valores de carga e hidrofobicidade (Mátyus et al. 2007). Os estudos biofísicos são motivados por modelagem e visam entender a atividade do AMP e as suas variantes modificadas, examinando a estrutura dos peptídeos em ambientes hidrofóbicos ou modelando peptídeos em nível atômico. Exemplos desses tipos de estudos incluem modelagem molecular fundamentada em perturbações de energia livre, simulação de dinâmica molecular, bem como cálculos termodinâmicos das interações de peptídeos com membranas (Mátyus et al. 2007; Bolintineanu e Kaznessis 2011; Fjell et al. 2011; Liang e Kelemen 2017).

A terceira abordagem é chamada de “estudo de rastreio virtual”, podendo ser usado quando a síntese é demasiadamente cara e as técnicas biológicas, como a ‘*phage display’*, não podem ser aplicadas (Pande et al. 2010; Li et al. 2017a). Essa abordagen tem a vantagem de ter apenas alguns pressupostos a priori, pois procura imputar estruturas peptídicas com base em sequências primárias. Em contraste com os estudos de simulação computacional, estudos de triagem virtual não tentam necessariamente criar modelos com resultados imediatamente e facilmente interpretáveis. Em vez disso, os métodos numéricos são utilizados para determinar as propriedades quantificáveis dos peptídeos (descritores), como a carga e a hidrofobicidade a partir da estrutura primária e características físico-químicas dos peptídeos, os quais são usados para relacionar essas propriedades com atividades biológicas (Engel 2006; Fjell et al. 2011).

Desta forma, a crescente disponibilidade e uso de estratégias inovadoras de desenho de peptídeo assistido por computador tem um potencial considerável para aumentar a descoberta de peptídeos terapêuticos de nova geração e de peptídeos miméticos como antimicrobianos não apenas para as bactérias alvo que se tornaram resistentes aos antibióticos existentes, mas também contra protozoários causadores de doenças, helmintos, insetos e fungos de importância médica e agropecuária (Sakata e Winzeler 2007; Fjell et al. 2011).

2.5 BIOINFORMÁTICA: UMA FERRAMENTA IMPORTANTE NA BUSCA DE NOVOS FÁRMACOS

O termo "bioinformática" foi usado pela primeira vez em meados da década de 1980, a fim de descrever a aplicação da ciência da informação e da tecnologia nas ciências da vida. A definição era naquele tempo muito geral, abrangendo tudo, desde a robótica à inteligência artificial. Posteriormente, a bioinformática passou a ser de certa forma prosaicamente definida como o uso de computadores para recuperar, processar, analisar e simular informações biológicas. Segundo Ramsden (2015), essas definições não conseguiam captar a centralidade da informação biológica, definindo a bioinformática como a ciência e a forma pela qual a informação é gerada, transmitida, recebida, armazenada, processada e interpretada em sistemas biológicos ou, mais sucintamente, a aplicação da ciência da informação à biologia.

Devido ao caráter interdisciplinar, a bioinformática consiste em uma mistura de habilidades e conhecimentos tais como matemática aplicada, estatística, informática, química e bioquímica, para resolver problemas biológicos. Fornece algoritmos, bancos de dados, interfaces de usuário e ferramentas estatísticas. Além disso, a bioinformática torna possível a realização de análise de dados complexos, incluindo genômica, proteômica, metabolômica e todas as outras "ômicas", compreendendo geralmente um esforço duradouro e desafiador (Fatumo et al. 2014; Emmert-Streib et al. 2014).

Na era pós-genoma humano, a habilidade de gerar dados em escala genômica de muitos componentes moleculares e celulares, permite obter *insights* sobre a patogênese e mecanismos moleculares causais, pelo menos a princípio. As abordagens computacionais fornecem métodos automáticos, consistentes e robustos para extrair informações de dados de alta dimensionalidade. Infelizmente, a transição prática de dados para informação e conhecimento revelou-se, em geral, um grande obstáculo e, atualmente, há esforços para encontrar abordagens e métodos padrões para este problema (Emmert-Streib et al. 2015).

Com o advento da bioinformática, ferramentas como técnicas de mineração fornecem um meio para resolver este problema, seja no auxílio nos processos de recuperação e de extração de informações. Neste contexto, a mineração de textos é muito importante, sendo atualmente utilizada para triar artigos para termos biológicos, incluindo nomes de moléculas, interações biológicas e moleculares. Ao adicionar

significado ao texto, essas técnicas produzem uma análise mais estruturada do conhecimento textual do que simples buscas de palavras, podendo fornecer ferramentas poderosas para a produção e análise de modelos de biologia de sistemas (Ananiadou et al. 2006).

O desenvolvimento de terminologias e ontologias consistentes permite a padronização de informações na mineração de textos, que pode ser usado para modelar processos biológicos mais amplos e inspirar novos alvos e estratégias para o desenvolvimento de novas drogas. A padronização conceitual também facilita a organização consistente de dados experimentais e permite a fusão de diversos recursos, aumentando o volume total de dados para análise e o número de padrões descobertos dentro de uma amostra experimental. Essas terminologias, quando organizadas, possibilitam a extração mais consistente de relações ou redes de interação (como a ativação de um gene por outro) em uma passagem posterior através de textos. Com este tipo de representação de conhecimento estruturado, os computadores podem simular o processo de raciocínio de cientistas humanos em grande escala (Garten e Altman 2009; Yao et al. 2010).

Desta forma, a biologia está se tornando uma ciência de dados, e os avanços futuros dependerão de fortes colaborações entre biólogos experimentais e computacionais. Aspectos da biologia computacional estão se integrando em todos os níveis da medicina e dos cuidados direcionados à saúde humana. Tanto os profissionais médicos como o público precisam estar bem informados e instruídos sobre essas mudanças, a fim de realizar o pleno potencial dessa nova fronteira na medicina, sem medo dos avanços tecnológicos (Fogg e Kovats 2015).

Este novo cenário de técnicas computacionais e experimentais favorece pesquisas como a descoberta de novos agentes antimicrobianos, desde a extração de dados textuais, experimentais e clínicos, até a construção de modelos de redes integradas de processos moleculares, bem como direcionada à análise experimental destas moléculas. A modelagem molecular ou biologia estrutural envolve o domínio mais estabelecido da biologia computacional, que pretende predizer e modelar propriedades de alvos biológicos integrando a química, a biofísica, a cristalografia experimental e a informática. Tais abordagens, em conjunto com ferramentas estabelecidas de química computacional, são capazes de modelar as propriedades e derivações de fármacos e sua interação com alvos, propiciando a geração de modelos

moleculares que constituem a peça central da descoberta e do desenvolvimento computacional de novos fármacos (Yao et al. 2010).

A bioinformática tornou o uso de banco de dados uma ferramenta acessível e intuitiva para consultas que ajudam a impulsionar a descoberta de novos fármacos. Na mesma linha, uma análise abrangente das bases de dados disponíveis, contendo compostos regulares, deve ajudar a racionalizar o motivo pelo qual alguns compostos são capazes de interagir ou não com determinadas proteínas (Labbé et al. 2016).

A identificação de agentes químicos na descoberta direcionada a novos fármacos envolve a coordenação de sistemas químicos, biológicos e sociais altamente complexos e requer um alto investimento de capital (Oberholzer-Gee e Inamdar 2004). O ciclo típico de descoberta e desenvolvimento de fármacos, do conceito ao mercado, leva aproximadamente 14 anos (Myers e Baker 2001), e o custo varia de 0,8 a 1 bilhão de dólares (Moses et al. 2005). Desta maneira, desenvolvimentos rápidos em química combinatória e tecnologias de rastreio de alto rendimento têm proporcionado um ambiente para acelerar o processo de descoberta de fármacos, permitindo que grandes bibliotecas de compostos sejam rastreadas e sintetizadas em curto espaço de tempo (Lobanov 2004; Shekhar 2008).

Embora o investimento no desenvolvimento de novos medicamentos tenha crescido significativamente nas últimas décadas, a produção não é positivamente proporcional ao investimento, devido à baixa eficiência e alta taxa de falha na descoberta de fármacos, além do tempo necessário para colocar um novo fármaco no mercado, passando por todas as etapas de testes (Apic et al. 2005).

A concepção de fármacos assistidos por ferramentas computacionais é um dos métodos mais eficazes para atingir estes objetivos (Ou-Yang et al. 2012). As abordagens computacionais, se aplicadas com sabedoria, têm o potencial de reduzir substancialmente o custo do desenvolvimento de fármacos em até 50% através da ampliação do conjunto de alvos viáveis e da identificação de novas estratégias terapêuticas e abordagens institucionais para a descoberta de fármacos (Tan et al. 2010; Yao et al. 2010).

Desenvolver modelos computacionais dinâmicos em consonância com a maquinaria celular humana sempre foi um sonho da biologia computacional. Tais modelos poderiam explicar os resultados clínicos dos fármacos existentes e prever o efeito de novas derivações. No entanto, os recentes avanços na integração de dados

permitiram a construção de complexas redes baseadas em mineração de texto e experimentos de alto rendimento (Liu et al. 2009). A maioria destas redes é estática, mas à medida que elas se tornam mais precisas e incorporam a temporalidade dos processos biomoleculares, elas provavelmente possibilitarão a identificação e o rastreamento sistemático de novas drogas alvos. Até mesmo as redes estáticas atuais sugerem princípios de designer e métricas que poderiam acelerar a descoberta de drogas bem-sucedidas (Marrer e Dieterle 2007; Marrer e Dieterle 2008).

Devido ao aumento na disponibilidade de informações sobre macromoléculas e pequenas moléculas biológicas, a aplicabilidade da descoberta computacional de fármacos foi expandida em quase todas as fases do processo de descoberta e desenvolvimento de fármacos, incluindo identificação, validação, descoberta e otimização de testes pré-clínicos. Ao longo das últimas décadas, os métodos computacionais aplicados à descoberta de fármacos foram melhorados, incluindo docking molecular, modelagem e mapeamento de farmacóforos, desenho de drogas *de novo*, cálculo de similaridade molecular e busca virtual baseada em sequências (Ou-Yang et al. 2012).

Abordagens computacionais utilizadas na descoberta de novos fármacos podem ser categorizadas com base na estrutura, ligante e na sequência ou tipo de fármaco. Os métodos baseados na estrutura, como a docking molecular e o desenho de drogas *de novo*, dependem do conhecimento da estrutura da macromolécula alvo. Tal conhecimento é obtido principalmente a partir de estruturas cristalinas, dados de ressonância magnética nuclear e modelos de homologia. Na ausência de estruturas tridimensionais de potenciais alvos, as ferramentas baseadas no ligante (incluindo a relação quantitativa estrutura-atividade, modelagem farmacófora, análise molecular de campo e avaliação de similaridade bidimensional ou tridimensional), podem fornecer evidências cruciais sobre a natureza das interações entre alvos de fármacos e seus ligantes, o que permite o desenvolvimento de modelos preditivos que são adequados para descoberta e otimização de potenciais fármacos (Acharya et al. 2011).

Nos últimos anos, para lidar com situações em que a estrutura-alvo e a informação do ligante estão indisponíveis, foram desenvolvidas abordagens baseadas em sequências, as quais utilizam ferramentas de bioinformática para analisar e

comparar múltiplas sequências para identificar alvos potenciais a partir do zero e conduzir a descoberta de novos candidatos (Tang et al. 2006).

A análise de sequências tem como objetivo comparar eficientemente as sequências de nucleotídeos e aminoácidos, permitindo aos pesquisadores imputar a função de um gene, considerando evidências de genes homólogos, muitas vezes de diferentes sistemas biológicos. A análise de sequências tornou-se agora uma parte indispensável da identificação do alvo na descoberta de fármacos numa fase inicial. Atualmente, todos os métodos individuais são incapazes de satisfazer as necessidades práticas na descoberta e desenvolvimento de fármacos. Portanto, estratégias combinatórias que empregam múltiplas abordagens computacionais têm sido frequentemente utilizadas com sucesso (Yao et al. 2010).

Embora seja evidente que os métodos computacionais tenham grande potencial na descoberta de novos fármacos, sempre será necessário acoplar as técnicas computacionais com ensaios experimentais, a fim de obter melhores resultados validando experimentalmente as predições realizadas (Kubinyi 2003; Kumar et al. 2006; Zhang 2011; Ou-Yang et al. 2012).

3. Capítulo I

PLANT ANTIMICROBIAL PEPTIDES: STATE OF ART AND PERSPECTIVES IN THE OMICS ERA

Current Protein & Peptide Science

Submitted: 13th December 2018 and accepted 24th Januery 2019

Authors:

Carlos André dos Santos Silva[1]; Marx Oliveira de Lima[1]; Lívia Maria Batista Vilela[1]; João Pacifico Bezerra-Neto[1]; José Ribamar Ferreira-Neto[1,2]; José Diogo Cavalcanti Ferreira[1]; Roberta Lane de Oliveira-Silva[1]; Carolline de Jesús Pires[1]; Flávia Figueira Aburjaile[1]; Marianne Firmino de Oliveira[1]; Ederson Akio Kido[2] and Ana Maria Benko-Iseppon[1]

Authors Affiliation

[1]Universidade Federal de Pernambuco, Departamento de Genética, Laboratório de Genética e Biotecnologia Vegetal, Recife, Pernambuco, Brazil.

[2]Universidade Federal de Pernambuco, Departamento de Genética, Laboratório de Genética Molecular, Recife, Pernambuco, Brazil.

Abstract

Even before the perception or the contact with pathogens, plants rely on guardian molecules that may be expressed constitutively, in a tissue/stage specific manner or, still, induced after pathogen expression. They are antimicrobial peptides (AMPs), small molecules, generally cysteine-rich, capable of preventing the establishment of invading pathogens. These peptides are usually expressed in gene families as defensins, snakins, LTPs, thionins, heveins, and cyclotides (among others) with a vast tertiary structure variation. Plants present a much higher number of AMP groups and isoforms than other groups of organisms, possibly due to gene duplications or genomic redundancy, and their sessile habit. Therefore, plants represent a vast resource of new AMP molecules. In order to organize and to facilitate access to information about these peptides many specific and general databases were built, also providing tools for peptide identification and characterization. The present review compiles information on AMP structural features at genomic, transcriptomic and protein level. Since AMPs are often difficult to recognize, based on simple BLAST alignments, a description of available database resources and bioinformatic tools for their identification is provided. Finally, we highlight the still almost unexplored biotechnological potential of plant AMPs in the generation of both transgenic plants resistant to pathogens, and new drugs or bioactive compounds for the treatment of human and animal diseases.

Keywords: Defensin, Lipid Transfer Protein, Hevein, Cyclotide, Snakin, Knotin, Macadamia β-barrelins, Impatiens-Like, Puroindoline, Thaumatin.

Introduction

Proteins and peptides are formed by small subunits composed of amino acids (aa), forming a chain that can range from a few tens to thousands of aa residues. Peptides are conventionally understood as having less than 50 aa [1]. Proteins, on the other hand, would be any molecule presenting higher amino acid content, being widely studied in plants. It is well known that the biochemical machinery necessary for the synthesis and metabolism of peptides is present in every living organism. From this machinery, a wide diversity of peptides is generated, justifying the growing interest in their study. As in animals, plant peptides are prevalent in intercellular communication, performing as hormones and growth factors [2].

Plant peptides can be multifunctional, and have been classified into two main categories (**Supplementary Figure S1**):

- Peptides with no bioactivity, primarily resulting from the degradation of proteins by proteolytic enzymes, aiming at their recycling;
- Bioactive peptides (BP), which are encrypted in the structure of the parent proteins and are released mainly by enzymatic processes.

The first group is innocuous regarding signaling, regulatory functions, and bioactivity. So far, it has been known that some of them may play an important role in nitrogen mobilization across cellular membranes [3]. The second group (BP) has a substantial impact on the plant cell physiology, and some peptides can act in the plant growth regulation (through cell-to-cell signaling), endurance against pests and pathogens by acting as toxins or elicitors, or even detoxification of heavy metals by ion-sequestration.

Comprising BPs, additional subcategorizations have been proposed. Tavormina et al. (2015) [4], based on the type of precursor, divided BPs into three groups (**Supplementary Figure S1**):

- Derived from functional precursors: originated from a functional precursor protein;
- Derived from nonfunctional precursors: originated from a longer precursor that has no known biological function (as a preprotein, proprotein, or preproprotein);
- Not derived from a precursor protein: some sORFs (small Open Read Frames; usually <100 codons) are considered to represent a potential new source of functional peptides (known as ‘short peptides encoded by sORFs’);

A more intuitive classification of BPs was further proposed according to their intracellular role by Farrokhi et al. (2008) [5] (**Supplementary Figure S1**):

- Phytohormone peptides: the characteristic feature of these peptides is the regulation of major plant physiological processes. They can be classified into those with signaling roles in non-defense functions or those with signaling roles in plant defense. Concerning the first group (**Supplementary Figure S1**), the peptide CLE25 (CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED 25) is one of the representatives. This peptide transmits water-deficiency signals through vascular tissues in *Arabidopsis thaliana*, affecting abscisic acid biosynthesis and stomatal control of transpiration in association with BAM (BARELY ANY MERISTEM) receptors in leaves [6]. Another example is the PLS (POLARIS) peptide that acts during early embryogenesis but later activates auxin synthesis, also affecting the cytokines synthesis and the ethylene response [7]. Regarding the second group, it includes peptides with signaling roles in plant defense, comprising at least four subgroups, including SYST (SYSTEMIN) (**Supplementary Figure S1**). SYST peptides were identified in Solanaceae members, like tomato and potato [8] (acting on the signaling response to herbivory. SYST leads to the production of a plant protease inhibitor that suppresses insect´s proteases [9]. Stratmann (2003) [10] suggested that in plants SYSTs act to stimulate the jasmonic acid signaling cascade within vascular tissues to induce a systemic wound response.
- Defense peptides or antimicrobials peptides (AMPs): to be fitted into this class, a plant peptide must fulfill some specific biochemical and genetic prerequisites. Regarding biochemical feature, an *in vitro* antimicrobial activity is required. Concerning the genetic condition, the gene encoding the peptide should be inducted in the presence of infectious agents [11]. In practice, this last requirement is not ever fulfilled since some AMPs are tissue-specific and are considered as part of the plant innate immunity, while other isoforms of the same class appear induced after pathogen inoculation [12].

Plant AMPs are the central focus of this review, comprising information on their structural features (at genomic, gene and protein levels), resources and bioinformatics tools available. Their biotechnological potential is also highlighted in the generation of both transgenic plants resistant to pathogens, and new drugs or bioactive compounds.

Overall Features of Plant AMPs

AMPs are ubiquitous host defense weapons against microbial pathogens. The overall plant AMP characterization regards the following variables (**Figure 1**): electrical charge, hydrophilicity, secondary and three-dimensional structures, and the abundance or spatial pattern of cysteine residues [12]. These features are primarily related to their defensive role(s) as membrane-active antifungal, antibacterial or antiviral peptide(s).

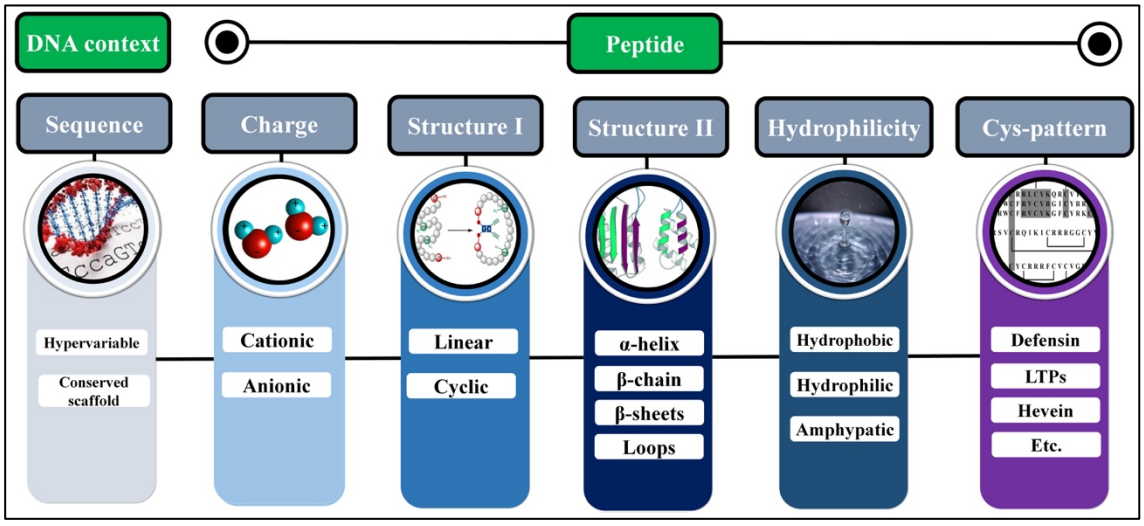

Figure 10 - Plant antimicrobial peptides features considering DNA sequence level, protein structure and physicochemical properties.

Regarding the nucleotide sequence, plant AMPs are hypervariable (**Figure 10**). This genetic variability provides diversity and the ability to recognize different targets. Regarding their charges, AMPs can be classified as cationic or anionic (**Figure 10**). Most plant AMPs have positive charges, which is a fundamental feature for the interaction with membrane lipid head groups of pathogens [13]. Concerning hydrophilicity, AMPs are generally amphipathic, i.e., they exhibit molecular conformation with both hydrophilic and hydrophobic domains [14]. With respect to its tridimensional structure, AMPs can be either linear or cyclic (**Figure 10**). Some linear AMPs adopt an amphipathic α-helical conformation whereas non-α-helical linear peptides generally show one or two predominant amino acids [15]. In turn, cyclic AMPs, including cysteine-containing peptides, can be divided into two subgroups, based on the presence of single or multiple disulfide bonds. A usual feature of these peptides is a cationic and amphipathic character, which accounts for their functioning as membrane- permeabilizing agents [15].

Considering the secondary structures, AMPs may exhibit α-helices, β-chains, β-pleated sheets and loops (**Figure 10**). Wang (2010) [16] classified plant AMPs into four families (α, β,

αβ, and non-αβ), based on the protein classification of Murzin et al. (1995) [17], with some modifications. AMPs of the 'α' family present α-helical structures [18], whereas AMPs from the 'β' family contains β-sheet structures usually stabilized by disulfide bonds [19,20]. Some plant AMPs showing an α-hairpin motif formed by antiparallel α-helices are stabilized by two disulfide bridges [19]. Such AMPs present a higher resistance to enzymatic, chemical or thermal degradation [21]. AMPs from the 'αβ' family having 'α' and 'β' structures are also stabilized by disulfide bridges. An example of AMP presenting 'αβ' structures are defensins, usually composed of a cysteine-stabilized αβ motif (CSαβ), an α-helix and a triple-stranded antiparallel β sheet stabilized mostly by four disulfide bonds [22]. Finally, AMPs that do not belong to the 'αβ' group exhibit no clearly defined 'α' or 'β' structures [18].

Plant AMPs are also classified into families, considering protein sequence similarity, cysteine motifs and distinctive patterns of disulfide bonds, which determine the folding of the tertiary structure [23]. Some of these AMP groups will be detailed in the next sections, with comments on their functions and scientific data availability.

Classification and structural features of plant AMPs at gene and protein levels

Defensin

The first plant defensins were isolated from wheat [24] and barley grains [25], initially called γ-hordothionins. Due to some similarities in cysteine content and molecular weight they were classified as γ-thionins. Later, the term 'γ-thionin' was replaced by 'defensin' based on the higher number of primary and tertiary structures of these proteins and also to their antifungal activities more related to insect and mammalian defensins than to plant thionins [26].

Plant defensins belong to a diverse protein superfamily called cis-defensin [27] and exhibit cationic charge, consisting of 45 to 54 aa with two to four disulfide bonds [26,28]. Plant defensins share similar tertiary structures and typically exhibit a triple-stranded antiparallel β sheet, enveloped by an α-helix and confined by intramolecular disulfide bonds [29] (**Figure 11.A**).

Defensins are known for their antimicrobial activity at low micromolar concentrations against Gram-positive and -negative bacteria [30], fungi [31], parasitic viruses and protozoa [32]. Additionally, they present inhibitory, insecticidal, and antiproliferative activity, acting as an ion-channel blocker, being also associated with protein synthesis [33].

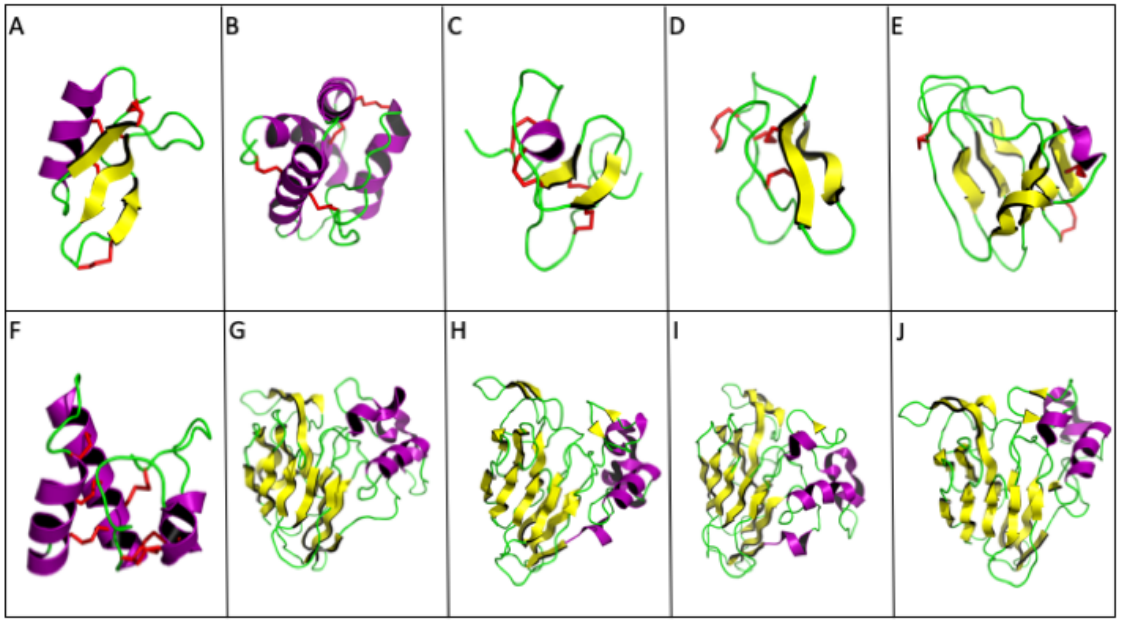

Figure 11 - Three-dimensional structure of plant antimicrobial peptide representatives. Yellow arrows correspond to β-sheets; α-helices are represented in purple and disulfide bonds in red. (**2.A**) Defensin NaD1 of *Nicotiana alata* (PDB ID: 1MR4). (**2.B**) Lipid Transfer Protein TaLTP1.1 isolated from wheat (PDB ID: 1GH1). (**2.C**) Hevein of *Hevea brasiliensis* (PDB ID: 1HEV). (**2.D**) Knottin Ep-AMP1 of *Echinopsis pachanoi* (PDB ID: 2MFS). (**2.E**) MiAMP1 peptide of *Macadamia integrifolia* (PDB ID: 1C01). (**2.F**) Snakin-1 of *Solanum tuberosum* (PDB ID: 5E5Q). (**2.G**) Thaumatin from *Thaumatococcus daniellii* (1RQW). (**2.H**) Zeamatin of *Zea mays* (1DU5, chain A). (**2.I**). Osmotin of *Nicotiana tabacum* (1PCV, chain A). (**2.J**) NP24-I Thaumatin-Like Protein (TLP) of *Solanum lycopersicum* (2I0W).

Lipid Transfer Protein (LTP)

Non-specific Lipid Transfer Proteins (nsLTPs) were first isolated from potato tubers [34] and are actually identified in diverse terrestrial plant species. They concern a large gene family, are abundantly expressed in most tissues, but absent in most basal plant groups as chlorophyte and charophyte green algae [35]. They generally include an N-terminal signal peptide that directs the protein to the apoplastic space [36]. Some LTPs have a C-terminal sequence that allows their post-translational modification with a glycosylphosphatidylinositol molecule, facilitating the integration of LTP on the extracellular side of the plasma membrane.

nsLTPs regard small proteins which were thus named because of their function of transferring lipids between the different membranes carrying lipids (non-specifically, the list includes phospholipids, fatty acids, their acylCoAs or sterols). They consist of approximately 100 aa and are relatively larger than other AMPs, such as defensins.

Depending on their sizes LTPs may be classified into two subfamilies: LTP1 and LTP2 with a relative molecular weight of 9 kDa and 7 kDa, respectively [37,38]. The limited sequence conservation turned this classification inadequate. Thus, a modified and expanded classification system was proposed, producing five main types (LTP1, LTP2, LTPc, LTPd, and LTPg) and

four additional types with a smaller number of members (LTPe, LTPf, LTPh, LTPj, and LTPk) [35]. The new classification system is not based on molecular size but rather on (i) the position of a conserved intron; (ii) the identity of the amino acid sequence and (iii) the spacing between the cysteine (Cys) residues (**Supplementary Figure S2)**. Although this latter classification system is the most recent, the conventional classification of types LTP1 and LTP2 has been maintained by most working groups.

LTPs nomenclature has been confusing and without consistent guidelines or standards. There are several examples where specific LTPs receive different names in separate articles. The lack of a robust terminology sometimes made it quite difficult, extremely time-consuming and sometimes frustrating to compare LTPs with different roles [36].

An additional nomenclature was also proposed by Salminen et al. (2016) [39], naming LTPs as follows: AtLTP1.3, OsLTP2.4, HvLTPc6, PpLTPd5 and TaLTPg7, with the first two letters indicating the species of plants (e.g. At = *Arabidopsis thaliana*, Pp = *Physcomitrella patens*), whereas LTP1, LTP2, LTPc indicate the type, while the last digit (here 3-7) regard the specific number given to each gene or protein within a given LTP type. For the sake of clarity, they recommend the inclusion of a point between the type specification and the gene number in LTP1 and LTP2. For LTPc, LTPd, LTPg and other types of LTP defined with a letter; the punctuation mark was not recommended. This latter classification system is currently recommended since it comprises several features of LTPs and is more robust than previous classification systems.

LTPs are small cysteine-rich proteins, having four to five helices in their tertiary structure (**Figure 11.B**), which is stabilized by several hydrogen bonds. Such a folding gives LTPs a hydrophobic cavity to bind the lipids through hydrophobic interactions. This structure is stabilized by four disulfide bridges formed by eight conserved cysteines, similar to defensins, although bound by cysteines in different positions. The disulfide bridges promote LTP folding into a very compact structure, which is extremely stable at different temperatures and denaturing agents [40–42]. This folding provides a different specificity of lipid binding at the LTP binding site, where the LTP2 structure is relatively more flexible and presents a lower lipid specificity when compared to LTP1 [43].

The first three-dimensional structure of an LTP was established for TaLTP1.1 based on 2D and 3D data of 1H-NMR, purified from wheat (*Triticum aestivum*) seeds in aqueous solution [44,45]. Currently, several three-dimensional structures of LTPs have been determined,

either by NMR or X-ray crystallography, either in their free, unbound form or in a complex with ligands.

Heveins

The heveins were first identified in 1960 in *H. brasiliensis*, but its sequence was determined later, whereas a similarity was detected to the chitin-binding domain of an agglutinin isolated from *Urtica dioica* (L.) [46] with eight cysteine residues forming a typical Cys motif [47].

The primary structure of the hevein consists of 29 to 45 aa, positively charged, with abundant glycine (6) and cysteine (8-10) residues [48] and aromatic residues [23,49]. The chitin-binding domain is a determinant component in the identification of hevein-like peptides whose binding site is represented by the amino acid sequence SXFGY / SXYGY, where X regards any amino acid [47,50]. Most heveins have a coil-β1-β2-coil-β3 structure that occurs by variations with the secondary structural motif in the presence of turns in two long coils in the β3 chain [23]. Antiparallel β chains form the central β sheet of the hevein motif with two long coils stabilized by disulfide bonds (**Figure 11.C**).

Although the presence of chitin has not been identified in plants, there are chitin-like structures present in proteins that exhibit strong affinity to this polysaccharide isolated from different plant sources [51]. The presence of three aromatic amino acids in the chitin-binding domain favors chitin binding by providing stability to the hydrophobic group C-H and the π electron system through van der Waals forces, as well as the hydrogen bonds between serine and N-acetylglucosamine (GlcNAc) present in the chitin structure [47,49]. This domain is commonly found in chitinases of classes I to V, in addition to other plant antimicrobial proteins, such as lectins and PR-4 (pathogenesis-related protein 4) members [52,53]. It may also occur in other proteins that bind to polysaccharide chitin [54], such as the antimicrobial AC-AMP1 and AC-AMP2 proteins of *Amaranthus caudatus* (Amaranthaceae) seeds which are homologous to hevein but lack the C-terminal glycosylated region [55]. Among the several classes of proteins mentioned, the proteins with a high degree of similarity to hevein are classes I and IV of chitinases [48].

Chitinases are known to play an essential role in plant defense against pathogens [56], also inhibiting fungal growth *in vitro* [57], especially when combined with β-1,3-glucanases [58] and interferes with the growth of hyphae, resulting in abnormal ramification, delay and swelling in their stretching [53]. However, it has been shown that heveins have a higher inhibitory

potential than chitinases and that their antifungal effect is not only related to the presence of chitinases [59]. Pn-AMP1 and Pn-AMP2 antimicrobial peptides with hevein domains have potent antifungal activities against a broad spectrum of fungi, including those without chitin in their cell walls [60], evidencing the statement raised by Van Parijs et al. (1991) [59]. The described modes of action of chitinases usually result in degradation and disruption of the fungal cell walls and plasma membranes due to the hydrolytic action of the enzyme, causing the extravasation of plasma particles [13,60]. Therefore, heveins have good antifungal activity, and only a few are active against bacteria, most of them with low activity [53,61].

Knottins (Cystine-knot peptides)

Knottins are part of the superfamily of cystine-rich peptides (CRPs), share the cystine-knot motif and therefore resemble other families as defensins, heveins, and cyclotides [62]. Their structure was initially identified by crystallography of a carboxypeptidase isolated from potato, showing the cystine-knot motif, 39 aa and six cysteine residues [63]. They are also called cystine-knot peptides, inhibitor cystine-knot peptides or even cystine-knot miniproteins because their mature peptide presents less than 50 aa, forming three interconnected disulfide bonds in the cystine-knot motif, characterizing a particular scaffold [64]. This conformation confers thermal stability at high temperatures. For example, the Cystine Stabilized β-sheet (CSB) motif derived from knottins presents stability at approximately 100ºC with only two disulfide bonds [65]. The knottins may have linear or cyclic conformation, however, both exhibit connectivity between the cysteines at positions 1-4C, 2-5C, 3-6C, forming a ring at the last bridge [64] (**Figure 11.D**).

In addition, knottins have different functions, such as signaling molecules [66], response against biotic and abiotic stresses [67], root growth [68], symbiotic interactions as well as antimicrobial activity against bacteria [69], fungi [70], virus [71], and insecticidal activity [72], among others. Knottins antimicrobial activity has been attributed to the action on the functional components of the plasma membrane, leading to alterations of lipids, ion flux, and exposed charge [73]. The accumulation of peptides on the surface of the membrane results in the weakening of the pathogen membrane [74] resulting in transient and toroidal perforations [73].

Macadamia (β-barrelins)

In the course of a large-scale survey to identify novel antimicrobial peptides from Australian plants [75,76], an antimicrobial peptide with no sequence homology was purified. Its cDNA was cloned from *Macadamia integrifolia* (Proteaceae) seeds, containing the complete peptide coding region. The peptide was named MiAMP1, being highly basic with an estimated PI of 10 and a mass of 8 kDa.

MiAMP1 is 102 aa long, including a 26 aa signal peptide in the N-terminal region, bound to a 76 aa mature region with six cysteine residues. Its three-dimensional structure was determined using NMR spectroscopy [76] revealing a unique conformation amongst plant AMPs, with eight beta-strands arranged in two Greek key motifs associated to form a Greek key beta-barrel (**Figure 11.E**). Due to its particularities, MiAMP1 was classified as a new

structural family of plant AMPs, and the name β-barrelins was proposed for this class [76]. This structural fold resembles a superfamily of proteins called *γ-crystallin-like* characterized by the precursors *βγ-crystallin* [77]. This family includes AMPs from other organisms, for example, WmKT a toxin produced by the wild yeast species *Williopsis mraki* [78].

MiAMP1 exhibited *in vitro* antimicrobial activity against various phytopathogenic fungi, oomycetes and gram-positive bacteria [75] with a concentration range of 0.2 to 2 μM generally required for a 50% growth inhibition (IC_{50}). In addition, the transient expression of MiAMP1 in canola (*Brassica napus*) provided resistance against blackleg disease caused by the fungus *Leptosphaeria maculans* [79], turning MiAMP1 potentially useful for genetic engineering aiming at disease resistance in crop plants.

There are few scientific publications with Macadamia-like peptides, maybe because they have been found only in primitive plant groups, being apparently absent or difficult to recognize in derived angiosperms, including most studied model and crop plants. In fact, peptides similar to MiAMP1 appear to play a role in the defense against pathogens in gymnosperms [80] including species of economic importance, thus deserving attention for their biotechnological potential.

Impatiens

Four closely related AMPs (Ib-AMP1, Ib-AMP2, Ib-AMP3, and Ib-AMP4) were isolated from seeds of *Impatiens balsamina* (Balsaminaceae) with antimicrobial activity to a variety of fungi and bacteria, with low toxicity to human cells in culture. They consist of only 20 aa in length and are the smallest antimicrobial peptides isolated from plants to date. Ib-AMPs are highly basic and contain four cysteine residues that form two disulfide bonds, with no significant homology with AMPs available in public databases. Sequencing of cDNAs isolated from *I. balsamina* revealed that all four peptides are encoded within a single transcript. Concerning the predicted Ib-AMP precursor protein, it consists of a pre-peptide followed by six mature peptide domains, each of them flanked by propeptide domains ranging from 16 to 35 aa in length (**Supplementary Figure S3**). This primary structure with repeated domains of alternating basic peptides and acid propeptide domains was reported before in plants [81].

Two-dimensional proton nuclear magnetic resonance (1H NMR) and circular dichroism (CD) were performed in IbAMP1 by Patel et al. (1998) [82] indicating that the peptide presents a turn but shows no evidence of helical or sheet structure over the analyzed temperature and pH range. NMR structural information was achieved in the form of proton-proton internuclear

distances deduced from NOEs (Nuclear Overhauser Enhancement) and dihedral angle restraints from spin-spin coupling constants that were used for distance geometry calculations.

Due to the difficulty in obtaining the correct disulfide bridges by chemical methods, three separate calculations were performed: without disulfide bridges and with two bridge formation alternatives. Calculations have shown that although the peptide is small, the cysteines restrict part of it to adopt a well-defined chain conformation. From residue 6 to 20, the main chain is well defined, whereas residues 1-5 in the N-terminal region present few restrictions and appear to be more flexible [82] .

Little is known about the mode of action of Impatiens-like AMPs. Lee et al. (1999) [83] investigated the antifungal mechanism of Ib-AMP1, noting that when oxidized (bound by disulfide bridges) there was a fourfold increase in antifungal activity against *Aspergillus flavus* and *Candida albicans* as compared to reduced Ib-AMP1 (without disulfide bridges). Confocal microscopy analyses have shown that Ib-AMP1 binds to the cell surface or penetrates cell membranes, indicating an antifungal activity by inhibiting a distinct cellular process, rather than ion channel or membrane pore formation. Modifications of these peptides have been carried out, indicating that the synthetic variant of Ib-AMP1 is totally active against yeasts and fungi. It has been proposed that the replacement of amino acid residues by arginine or tryptophan may improve by more than twice the antifungal activity [84]. Another study involving AMP modification generated a synthetic peptide without the disulfide bridges (i.e., a linear analog of Ib-AMP1) which showed an antimicrobial specificity 3.7-4.8 times higher than the wild-type Ib-AMP1 [85].

Puroindoline

Puroindolines (PINs) are small basic proteins that contain a single domain rich in tryptophan. These proteins were isolated from the wheat endosperm, have molecular masses around 13 kDa and a calculated isoelectric point higher than 10. At least two major isoforms (called PIN-a, and PIN-b) are known, which are encoded by *Pina-D1* and *Pinb-D1* genes, respectively. These genes share 70.2% identical coding regions but exhibit only 53% identity in the 3' untranslated region [86].

Both PIN-a and PIN-b contain a structure with ten conserved cysteine residues and a tertiary structure similar to LTPs, consisting of four α-helices separated by loops of varying lengths, with the tertiary structure joined by five disulfide bonds, four of which identical to ns-LTPs [86].

The conformation of the two PIN isoforms was studied by infrared and Raman spectroscopy. PIN-a and PIN-b have similar secondary structures comprising approximately 30% helices, 30% β-sheets and 40% non-ordered structures at pH 7. It has been proposed that the folding of both PINs is highly dependent on the pH of the medium. The reduction of the disulfide bridges results in a decrease of puroindolines solubility in water and to an increment of the ß-sheet content by about 15% at the expense of the α-helix content [87]. No high-resolution structure for any of the PIN isoforms is available, bringing challenges to understanding the function of their hydrophobic regions, with some evidence coming only from homolog peptides [86].

PINs are proposed to be functional components of wheat grain hardness loci, control core texture, besides antifungal activity [88–91]. Although the biological function of PINs is unknown, its involvement in lipid binding has been proposed. While LTPs bind to hydrophobic molecules in a large cavity, PINs interact only with lipid aggregates, i.e., micelles or liposomes, through a single stretch of tryptophan residues. This stretch of tryptophan residues is especially significant in the main form, PIN-a (WRWWKWWK), while it is truncated in the smaller form, PIN-b (WPTWWK) [92–94].

Puroindolines form protein aggregates in the presence of membrane lipids, controlled by their lipid structure. In the absence of lipids, these proteins may aggregate, but there is no accurate information on the relationship between aggregation and interaction with lipids. The antimicrobial activity of PINs is targeted to cell membranes. Charnet et al. (2003) [95] indicated that PIN is capable of forming ion channels in artificial and biological membranes that exhibit some selectivity over monovalent cations. The stress and Ca^{2+} ions modulate the formation and/or opening of channels. PINs may also be membranotoxins which may play a role in the plant defense mechanism against microbial pathogens.

Snakin

Snakins are cysteine-rich peptides first identified in potato (*Solanum tuberosum*) [96,97]. Due to their sequence similarity to GASA (Gibberellic Acid Stimulated in Arabidopsis) proteins, the snakins were classified as members of the snakin/GASA family [98]. The genes that encode these peptides have: (i) a signal sequence of approximately 28 aa (ii) a variable region, and (iii) a mature peptide of approximately 60 residues, with 12 highly conserved cysteine residues. These cysteine residues maintain the three-dimensional structure of the

peptide through the disulfide bonds, besides providing stability to the molecule when the plant is under stress [96,97,99,100] (**Figure 11.F; Supplementary Figure S4**).

Snakins may be expressed in different parts of the plant, like stem, leaves, flowers, seeds and roots [101–104], both constitutive or induced by biotic or abiotic stresses. *In vitro* activity was observed against a variety of fungi, bacteria and nematodes, acting as a destabilizer of the plasma membrane [96,105,106]. They were reported as important agents in biological processes such as cell division, elongation, cell growth, flowering, embryogenesis, and signaling pathways [107–110].

Cyclotide

The term cyclotide was created at the end of the past century to designate a family of plant peptides with approximately 30 aa in size and a structural motif called cyclic cystine knot (CCK) [111]. This motif is composed by a head-to-tail cyclization that is stabilized by a knotted arrangement of disulfide bridges, with six conserved cysteines, connected as follows: C1-2, C3-6, C4-5 [112]. Cyclotides are generally divided into two subfamilies, Möbius, and Bracelets, based on structural aspects. In addition to CCKs, two loops (between C1-2 and C4-5) have high similarity between different subfamilies, while other two loops (between C2-3 and C3-4) exhibit some conservation within the subfamilies [113,114] (**Supplementary Figure S5**).

To date, several cyclotides were identified in eudicot families such as Rubiaceae [115], Violaceae [116], Fabaceae [117] and Solanaceae [118], in addition to some monocots of Poaceae family [119]. In general, cyclotides may act in defense against a range of agents like insects, helminths or mollusks, in addition, they can also act as ecbolic [115], antibacterial [120], anti-HIV [71] and anticancer [121]. All these characteristics added to the stability conferred by the CCK motif turn these peptides into excellent candidates for drug development [122,123].

Thaumatin-like protein (TLP)

Thaumatins or Thaumatin-like proteins (TLPs) belong to the PR-5 (Pathogen-related protein) family and received this name due to its first isolation from the fruit of *Thaumatococcus daniellii* (Maranthaceae) from West Africa [124]. TLPs are abundant in the plant kingdom, being found in angiosperms, gymnosperms and bryophytes [124], being also identified in other organisms, including fungi [125,126], insects [127], and nematodes [128].

TLPs are known for their antifungal activity, either by permeating fungal membranes [129] or by binding and hydrolyzing β-1,3-glucans [130,131]. In addition, they may act to inhibit fungal enzymes, such as xylanases [132], α-amylases or trypsin [133]. Besides, the expression of

TLPs is regulated in response to some stress factors, such as drought [134], injuries [135], freezing [136], and infection by fungi [137,138], viruses and bacteria [139].

As to the TLP structure, this protein presents characteristic thaumatin signature (PS00316): G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)-[QG]-x(2,3)-C [140,141]. Most of the TLPs have molecular mass ranging from 21 to 26 kDa [124], possessing 16 conserved cysteine residues (**Supplementary Figure S6**) involved in the formation of eight disulfide bonds [142], which help in the stability of the molecule, allowing a correct folding even under extreme conditions of temperature and pH [143]. TLPs also contain a signal peptide at the N-terminal which is responsible for targeting the mature protein to a particular secretory pathway [124].

The tertiary structure presents three distinct domains, which are conserved and form the central cleft, responsible for the enzymatic activity of the protein, being located between domains I and II [144]. This central cleft may be of an acidic, neutral or basic nature depending on the binding of the different linkers / receptor. All TLPs from plants with antifungal activity have an acidic cleft known as motif REDDD due to five highly conserved amino acid residues (arginine, glutamic acid, and three aspartic acid; (**Supplementary Figure S6**), being very relevant for specific receptor binding of antifungal activity [129,145,146].

Crystallized structures were determined for some plant TLPs, such as thaumatin [147]; (**Figure 11.G)**, zeamatin [129] (**Figure 11.H)**, tobacco PR-5d [145] and osmotin [146] (**Figure 11.I)**, the cherry allergen PruAv2 [148] and banana allergen Ba-TLP [144] (**Figure 11.J)**, among other TLPs.

Some TLPs are known as sTLPs (small TLPs) due to the deletion of peptides in one of their domains, culminating in the absence of the typical central cleft. These sTLPs exhibit only ten conserved cysteine residues, forming five disulfide bonds, resulting in a molecular weight of approximately 16-17 kDa. They have been described in monocots, conifers, and fungi, so far [124,149,150]. Other TLPs exhibit an extracellular TLP domain and an intracellular kinase domain, being known as PR5K (PR5-like receptor kinases) [151] and are present in both monocots and dicots. For example, *Arabidopsis* contains three PR5K genes while rice has only one [124].

Bioinformatic approaches on plant AMPs

Databases

With the rapid growth in the number of available sequences, it is almost unfeasible to handle such amount of data manually. Thus, AMP sequences (as well as their biological

information) have been deposited in large general databases, such as UniProt and TrEMBL, which contain sequences of multiple origins [152,153]. In this sense, the construction of databases that deal directly with AMPs was an important step to organize the data.

During the past decade, several databases were built to support the deposition, consultation, and mining of AMPs. Thus, these databases can be classified into two groups: general and specific [154]. The specific databases can be divided into two subgroups: those containing only one specific group (defensins or cyclotides) and those containing data from a supergroup of peptides (plant, animal or cyclic peptides) (**Supplementary Table 1**). In general, both types of databases share some characteristics such as the way that the data are available or the access to tools to analyze AMPs.

The Collection of Antimicrobial Peptides ($CAMP_{R3}$) is a database that comprises experimentally validated peptides, sequences experimentally deduced and still those with patent data, besides putative data based on similarity [155–157]. The current version includes structures and signatures specific to families of prokaryotic and eukaryotic AMPs [157]. The platform also includes some tools for AMP prediction.

The antimicrobial peptide database (APD) [158,159] collects mature AMPs from natural sources, ranging from protozoa to bacteria, archaea, fungi, plants, and animals, including humans. AMPs encoded by genes that undergo post-translational modifications are also part of the scope, besides some peptides synthesized by multienzyme systems. APD provides interactive interfaces for peptide research, prediction, and design, statistical data for a specific group, or for all peptides available in the database.

The LAMP (Database Linking Antimicrobial Peptides) comprises natural and synthetic AMPs, which can be separated into three groups: experimentally validated, predicted, and patented. Their data were primarily collected from the scientific literature, from UniProt and from other databases related to AMPs [160].

DBAASP [161] contains information about AMPs from different origins (synthetic or non-synthetic) and complexity levels (monomers and dimers) that were retrieved from PubMed using keywords: antimicrobial, antibacterial, antifungal, antiviral, antitumor, anticancer and antiparasitic peptides. This database is manually curated and provides information about peptides that have specific targets validated experimentally. This database also includes information on chemical structure, post-translational modifications, modifications in the N/C terminal amino acids, antimicrobial activities, cell target and experimental conditions in which

a given activity was observed, besides information about the hemolytic and cytotoxic activities of the peptides [161].

Due to the diversity of AMPs and the need to accommodate the most representative subclasses, several databases were established focusing on specific types, sources or features. There are several ways to classify AMPs, and they can range from biological sources such as bacterial AMPs (bacteriocins), plants, animals, etc.; biological activity: antibacterial, antiviral, antifungal and insecticide, and based on molecular properties, such as the pattern of covalent bonds, 3D structure and molecular targets [162,163].

The 'Defensins Knowledgebase' is a database with manual curation and focused exclusively on defensins. This database contains information about sequence, structure, and activity, with a web-based interface providing access to information and enabling text-based search. In addition, the site presents information on patents, grants, laboratories, researchers, clinical studies and commercial entities [164,165].

The CyBase is a database dedicated to the study of sequences and three-dimensional structures of cyclized proteins and their synthetic variants, including tools for the analysis of mass spectral fingerprints of cyclic peptides, also assisting in the discovery of new circular proteins [166].

Finally, the PhytAMP is a database designed solely dedicated to plant AMPs based on information collected from the UniProt database and the scientific literature through PubMed [167].

Biological databanks (DB) are organized collections of data of diverse nature that can be retrieved using different inputs. The management of this information is done through various software and hardware, whose retrieval and organization is performed as quickly and efficiently as possible [168]. Considering biological data, information can be classified into: (i) primary (sequences), (ii) secondary (structure, expression, metabolic pathways, types of drugs, among others) and (iii) specialized, e.g., containing information on a species or class of protein [169]. Within this third group some references to AMPs can be mentioned, such as $CAMP_{R3}$ [156] and APD [159] that compile sequence data and structure retrieved from diverse sources, and also the Defensin knowledgebase [164] and the Cybase [166] which are dedicated to specific classes of peptides (defensins and cyclotides respectively), in addition to PhytAMP [167], a specific database of plant antimicrobial peptides.

Retrieving and annotating sequences from databases

The first step to infer the function of a given sequence (annotation) is to retrieve it in databases. For this purpose, two methodologies have been used mostly: local alignments, especially by using BLAST [170] and FASTA [171], but also by searching for specific patterns with Regular Expression (REGEX) or Hidden Markov Model (HMM) [154]. Since most of the information is available in the databases as sequences, to align them is the best way to compare. Thus the local alignment is the most commonly used approach whereas the BLAST is the primary tool for doing so [172]. This tool splits the sequence into small pieces (words), comparing it with the database. However, this approach has a significant limitation. Small motifs may not be significantly aligned since they comprise small portions of the sequences that can be smaller than 20% of the total size [23,154].

In order to reduce the effects of local alignment limitations, other strategies based on the search for specific patterns were introduced, such as REGEX [173] and HMM [174]. Regular expression (REGEX) is a precise way of describing a pattern in a string where each REGEX position must be set; although ambiguous characters (or wildcards) can also be used. For example, if we want to find a match for both amino acid sequences CAIESSK and WAIESK, we can use the following expression: [CW]AIES{1,2}K, this expression would find a sequence starting with the letter "C" or "W", followed by an "A", an "I" and an "E", one or two "S" and ending with a "K". By the other hand, for HMM, there is a statistic profile inserted in the model, which is calculated from a sequence alignment and a score that is determined site-to-site, with conserved and variable positions defined a priori [154,175].

Predicting antimicrobial activity

The design of new AMPs led to the development of methods for the discovery of new peptides, thus allowing new experiments to be done by researchers. In this way, the new challenge lies in the construction of new prediction models capable of discovering peptides with desired activities.

The APD databank has established a prediction interface based on some parameters defined by the entire set of peptides available in this database. These values are calculated from natural AMPs to consider features as length, net charge, hydrophobicity, amino acid composition, etc. If we take as an example the net load, the AMPs deposited in the APD range from -12 to +30. This is the first parameter incorporated into the prediction algorithm. However, most AMPs have a net load ranging from -5 to +10, which then becomes the alternative prediction condition. Therefore, the same method is applied to the remaining parameters. The

prediction in APD is performed in three main steps. First, the sequence parameters will be calculated and compared. If defined as an AMP, the peptide can then be classified into three groups: (i) rich in given amino acids, (ii) stabilized by disulfide and (iii) linear bridges. Finally, sequence alignments will be conducted to find five peptides of greater similarity [159,176,177].

The advent of machine learning (ML) methods has promoted the field of drug discovery. In ML inferences, both, a positive and a negative dataset, are usually required to train the predictive models. The positive data, in this case, regard preferably experimentally validated AMPs that can be collected in databases, whereas negative data are randomly selected protein sequences that do not have AMP characteristics [157,178]. ML methods based on support vector machine (SVM), random forest (RF) and neural networks (NN) have been the most widely used. SVM is a specific type of supervised method of machine learning, aiming to classify data points by maximizing the margin between classes in a high-dimensional space. RF is a non-parametric tree-based approach that combines the ideas of adaptive neighbors with bagging for efficient adaptive data inference. NN is an information processing paradigm inspired by how a biological nerve system process information. It is composed of highly interconnected processing elements (neurons or nodes) working together to solve specific problems [179–181].

Evaluating Proteomic Data

Regarding the use of AMPs in peptide therapeutics, as an alternative to antibacterial and antimicrobial treatment, new efficient and specific antimicrobials are demanded. As described previously in this review, AMPs are naturally occurring across all classes of life, presenting high active potential as therapeutic agents against various kinds of bacteria [182]. The identification of novel AMPs in databases is primarily dependent on knowing about specific AMPs together with a sufficient sequence similarity [183]. However, orthologs may be divergent in terms of sequence, mainly because they are under strong positive selection for variation in many taxa [184], leading to remarkably lower similarity, even in closely related species. In this scenario, where alignment tools present limited use, one strategy to identify AMPs is related to proteomic approaches.

Proteins and peptides are biomolecules responsible for various biochemical events in living organisms, from formation and composition to regulation and functioning. The search for the understanding of the expression, function, and regulation of the proteins encoded by an organism initiated the so-called ‘Proteomic Era’. The term "proteome" was first used by Marc Wilkins in 1994 [185] and represents the set of proteins encoded by the genome of a biological

system (cell, tissue, organ, biological fluid or organism) at a specific time under certain conditions [185]. Protein extraction, purification, and identification methods have significantly advanced our capacity to elucidate many biological questions using proteomic approaches [186,187]. The wide diversity of proteomic analysis methods makes the choice of the correct methodology dependent mainly on the type of material and compounds that will be analyzed. Thus, proteomic analysis plays an important role in the discovery of new proteins and peptides, as well as providing several other tools to understand the mechanisms involved in the connection between proteins and its functions [188].

Two main tools are used to isolate proteins: (1) the two-dimensional electrophoresis (2-DE) associated with mass spectrometry (MS), and (2) liquid chromatography associated with MS, each one with its limitations [189,190]. Obtaining native proteins is a challenge in proteomics, due to high protein complexity in samples, like the occurrence of post-translational modifications. Alternative strategies applied to extraction, purification, biochemical and functional analyses of these molecules have been proposed, favoring access to structural and functional information of hard-to-reach proteins and peptides [191].

Based in 2D gel, Al Akeel et al. (2017) [192] evaluated 14 spots obtained from seeds of *Foeniculum vulgare* (Apiaceae) aiming at proteomic analyses and isolation of small peptides. Extracted proteins were subjected to 3 kDa dialysis, and separation was carried out by DEAE-ion exchange chromatography while further proteins were identified by 2D gel electrophoresis. One of its spots showed the highest antibacterial activity against *Pseudomonas aeruginosa*, pointing to promising antibacterial effects, but requiring further proteomic research to authenticate the role of the anticipated proteins.

For AMPs, 2-DE is challenging, due to the low concentration of the peptide molecules, their small sizes and their ionic features (strongly cationic). The low number of available specific databases turns their identification through limited proteolysis techniques and MALDI-MS difficult. In addition, the partial hydrophobicity characteristics and surface charges facilitate peptide molecular associations, making analysis difficult by any known proteomic approaches [190]. Additionally, peptides are most often cleaved from larger precursors by various releasing or processing enzymes [193].

However, profiles generated sometimes do not represent integral proteome, since this technique has limitations to detect proteins present in low concentration, with values of extreme molecular masses, pIs and hydrophobic proteins, including those of membranes [194]. Due to these limitations, multidimensional liquid chromatography (MDLC-HPLC) has been

successfully employed as an alternative to two-dimensional gels. Techniques and equipment for the newly developed separation and detection of proteins and peptides, such as nano-HPLC and multidimensional HPLC, have allowed the proteomics improvement [195].

Molecular mass values obtained are used in computational searches in which they are compared with in silico digestion results of proteins in databases, using specific *in silico* approaches, usually by the action of trypsin as a proteolytic agent, generating a set of unique peptides whose masses are determined by mass spectrometry [196,197]. These methodologies are widely adopted for large-scale identification of peptide from MS/MS spectra [198]. Theoretical spectra are generated using fragmentation patterns known for specific series of amino acids. The first two widely used search engines in database searching were SEQUEST [199] and MASCOT [Matrix Science, Boston, MA (www.- matrixscience.com)] [200]. They rank peptide matches based on a cross-correlation to match the hypothetical spectra to the experimental one.

MASCOT is widely used for proteomics analysis, including AMP identification in many organisms or to evaluate the antibacterial efficacy of new antimicrobial peptides. After evaluating new AMP against multidrug-resistant (MDR) *Salmonella enterica*, Tsai et al. (2016) [201] used two-dimensional gel electrophoresis and liquid chromatography-electrospray ionization-quadrupole- time-of-flight tandem mass spectrometry to determine the protein profiles. The protein identification was performed using the MASCOT with trypsin used as the cutting enzyme, whereas NCBI nr protein was set as a reference database. The methodology used in this study indicated that the novel AMP might serve as a potential candidate for antimicrobial drug development against MDR strains, confirming the usability of MASCOT.

Similarly, Umadevi et al. (2018) [202] described the AMP signature profile of black pepper (*Piper nigrum* L.) and their expression upon *Phytophthora* infection using label-free quantitative proteomics strategy. For protein/peptide identification, MS/MS data were searched against the APD database [159] using an in-house MASCOT server, established full tryptic peptides with a maximum of three missed cleavage sites and carbamidomethyl on cysteine, besides an oxidized methionine included as variable modifications. The APD database was used for AMP signature identification [159], together with PhytAMP [167] and $CAMP_{R3}$ [203]. To enrich the characterization parameters, isoelectric point, aliphatic index and grand average of hydropathy were also used [204] (GRAVY) (using ProtParam tool) besides the net charge from PhytAMP database. Based on label-free proteomics strategy, they established for the first time the black pepper proteomics associated with the innate immunity against *Phytophthora*, evidencing de usability of proteomics data for AMP characterization in any taxa, including

plant AMPs, aiming the exploitation of these peptides as next-generation molecules against pathogens [202].

Other search tools are using database searching algorithms, such as X!TANDEM [205], OMSSA [206], ProbID [207], RADARS [208], etc. The search engines are based on database search but use different scoring schemes to determine the top hit for a peptide match. More general information on database search engines, their algorithms and scoring schemes was reviewed by Nesvizhskii et al. (2007) [209]. Despite its efficient ability to identify peptides, the method presents several drawbacks, like false positive identifications because overly noisy spectra and lower quality peptides score related to short peptides. So, the identification is strongly influenced by the amount of protein in the sample, the degree of post-translational modification, the quality of automatic searches and the presence of the protein in the databases [210,211]. In this scenario, the knowledge about the genome from a specific organism has importance to allow the identification of the exact pattern of a given peptide. If an organism has not been sequenced, it is not searchable using these methods [193,198].

More recently a free interactive web software platform, MixProTool, was developed, aiming to process multigroup proteomics data sets. This tool is compiled in R (www.r-project.org), providing integrated data analysis workflow for quality control assessment, statistics, gene ontology enrichment, and other facilities. The MixProTool is compatible with identification and quantification results from other programs, such as MaxQuant and MASCOT, where results may be visualized as vector graphs and tables for further analysis, in contrast to existing softwares, such as GiaPronto [212]. According to the authors, the web tool can be conveniently operated, even by users without bioinformatics training, and it is beneficial for mining the most relevant features among different samples [16].

AMP modeling and simulation in molecular dynamics

The central tenet of structural biology is that structure determines function. For proteins, it is often said the *"function follows form"* and *"form defines function"*. Therefore, to understand protein function in detail at the molecular level it is mandatory to know its tertiary structure [213]. Experimental techniques for determining structures, such as X-ray crystallography, NMR, electron paramagnetic resonance and electron microscopy, require significant effort and investments [214].

All methods mentioned have their limitations, and the gap between the number of known proteins and the number of known structures is still substantial. Thus, there is a need for

computational framework methods to predict protein structures based on the knowledge of the sequence [213]. In addition, in recent years there has been impressive progress in the development of algorithms for protein folding that may aid in the prediction of protein structures from amino acid sequence information [215].

Historically, the prediction of a protein structure has been classified into three categories: comparative modeling, threading and *ab initio*. The first two approaches construct protein models by aligning the query sequences with already solved model structures. If the models are absent in the Protein Data Bank, the models must be constructed from scratch, i.e., by *ab initio* modeling, considered the most challenging way to predict protein structures.

In the case of comparative modeling methods, when inserting a target sequence, the programs identify evolutionarily related models of solved structures based on their sequence or profile comparison, thus constructing structure models supported by these previously resolved models [216]. This approach consists of four main steps: (i) fold assignment, which identifies similarity between the target and the structure of the solved model, (ii) alignment of the target sequence to the model, (iii) generation of a model based on alignment with the chosen template and (iv) analysis of errors considering the generated model [217].

There are several servers and computer models that automate the comparative modeling process, with SWISS-MODEL and MODELLER figuring as the most used [218,219]. Although automation makes comparative modeling accessible to experts and beginners, some adjustments are still needed in most cases to maximize model accuracy, especially in the case of more complex proteins [219]. Therefore, some caution must be taken regarding the generated models, taking into account the resolution and quality of the model used, as well as homology between the model and the protein of interest.

Threading modeling methods are based on the observation that known protein structures appear to comprise a limited set of stable folds, allowing the identification of similarities among elements in evolutionarily distant or unrelated proteins. The most used servers based on this approach are MUSTER [220], SPARKS-X [221], RaptorX [216], ProSa-II [222] and most notably the I-TASSER [223]. In some cases, the incorporation of structural information to combine the sequence used in the search with possible models allows the detection of similarity in the fold, even in the absence of an explicit evolutionary relation.

The prediction of structures from known protein models is, at first sight, a more straightforward task than the prediction of protein structures from available sequences. Therefore, when no solved model is available, another approach is recommended, namely the

ab initio modeling. This method is intended to predict the structure only from the sequence information, without any direct assistance from previously known structures. The *ab initio* modeling aims to predict the best model, based on the minimum energy for a potential energy function by sampling the potential energy surface using various searchable information [224,225]. Such approaches turn it challenging to produce high-resolution modeling, essential for determining the native protein folding and its biochemical interpretation. On the other hand, later resolved structures and comparisons with previously predicted proteins point to a higher successful modeling generated by *ab initio* methods than those generated by pure energy minimization methods, classical or even pure methods [213].

Among the most used servers and programs for *ab initio* modeling, we highlight the ROSETTA [214], and TOUCHSTONE II [224]. The accuracy of the models calculated by many of these methods is evaluated by CAMEO (Continuous Automated Model Evaluation) [226] and by CASP (Critical Assessment of protein Structure Prediction) experiment [215]. Probably the first reasonably accurate *ab initio* model was built in CASP4. Since then, sustained progress was achieved in *ab initio* prediction, but mainly for small proteins (120 residues or less). In CASP11, for the first time, a novel 256-residue protein with a sequence identity with known structures lower than 5% was constructed with high precision for sequences of this size [227].

Molecular Dynamics Simulation

Molecular dynamics (MD) is a computational simulation technique that predicts the changes in the positions and velocities of the constituent atoms of a system under a given time and condition. This calculation is done through a classical approximation of empirical parameters, called “force field” [228]. If, on the one hand, this approximation makes the dynamics of a system containing thousands of atoms numerically accessible, it obviously limits the nature of the processes that can be observed during the simulations. No quantum effect is visualized in a molecular dynamics simulation, just as no chemical bond is broken, no interactions occur between orbitals, resonance, polarization or charge transfer effects [229]. However, the molecules go beyond a static system. Thus, MD is a computational technique that can be used for predicting or refining structures, dynamics of molecular complexes, drug development and action of molecular biological systems [230]. MD simulation is widely used for protein research, aiming to extract information about the physical properties of individual proteins. The results of such simulations are then compared with experimental results. Since these experiments are generally carried out in solvents, it is necessary to simulate molecular

systems of protein in water. These simulations have a variety of applications, such as determining the folding of a structure to a native structure and analyzing the dynamic stability of this structure [231].

The use of MD to simulate protein folding processes is one of the most challenging applications and should be relatively long (in the order of microseconds to milliseconds) to allow observing a single fold event. In addition, the force field used must correctly describe the relative energies of a wide variety of shapes, including unfolding and poorly folded shapes that may occur during the simulation [229]. The considerable application potential led to the implementation of MD simulation in many software packages, including GROMACS [232–234], AMBER [235], NAMD [236], CHARMM [237], LAMMPS [238] and Desmond [239]. In addition to the above mentioned, there are other types of simulation available, such as the Monte Carlo Method, Stochastic Dynamics and Brownian Dynamics [234].

In the last decades, molecular dynamics simulation has become a standard tool in theoretical studies of large biomolecular systems, including DNA or proteins, in environments with near realistic solvents. Historically the computational complexity of this type of computation has been extremely high, and much research has focused on algorithms to achieve unique simulations that are as long or as large as possible [232].

Pathogen interaction studies and molecular docking

Currently, the control of bacterial pathogens is a great challenge, due to the high microbial resistance to antibiotic treatment. Most market available antimicrobial drugs act under the synthesis of DNA, RNA and proteins, disruption of the membrane, and inhibition of pathogen-specific metabolic pathways [240–242]. Despite different strategies, bacteria became adapted to these drug targets and developed mechanisms to overcome available drugs. Among the main mechanisms of resistance to antibacterial agents, we can mention antibiotic inactivation, target modification, resistance and plasmid efflux [243–245]. The acquisition of these mechanisms has led to a worsening of clinical conditions, and new alternative methods have emerged to improve antibiotics efficacy [246]. In this context, plant-derived AMPs have great potential for combating pathogens [247], especially because plants have unique AMP classes, not present in other organisms [12]. The differences of repertoire and composition in an amino acid of plant AMPs reveal structural and functional alterations of their protein families [248] that allow a new perspective since they have a broad-spectrum antimicrobial target. Although AMPs

have not yet obtained authorization for clinical use in humans, they are a promising future alternative [249].

As a perspective for this field, systems biology stands out because it is capable of correlating omics data and promoting studies of plant-pathogen interaction. The construction of plant-pathogen interaction network includes the reconstruction of metabolic pathways of these organisms, identification of the degree of pathogenicity, besides expression of genes and proteins from both, plant and pathogen. The networks can be classified into five types: (i) regulatory, (ii) metabolic, (iii) protein-protein interaction, (iv) signaling and regulatory and (v) signaling, regulatory and metabolic [250]. Each of these networks can be plotted according to computational approaches.

Also, further studies are required to contemplate the construction of evolutionary *in silico* models and the characterization of these molecular targets *in vitro* [251,252]. Studies of protein-protein interactions to understand the regulatory process are essential [253] and new computational methods are necessary for this purpose with more optimized algorithms, also to remove the false positives detected nowadays in most softwares. Thus, in-depth studies on the orientation of molecules and their linkages to the formation of a stable complex are of great importance for understanding plant-pathogen studies and also to develop new drugs [254].

Molecular docking

The understanding of the regulatory principles by which protein receptors recognize, interact and associate with molecular substrates or inhibitors is of paramount importance to generate new therapeutic strategies [255]. In modern drug discovery, docking plays an important role in predicting the orientation of the binder when it is attached to a protein receptor or enzyme, using forms and electrostatic interactions, van der Walls, Colombic and hydrogen bond as parameters to quantify or predict a given interaction [256,257]. Protein-protein interactions are essential for cellular and immune function and, in many cases, due to the absence of an experimentally determined structure of the complex, these interactions must be modeled to obtain an understanding about their molecular basis [258].

Drug research based on structure is a powerful technique for the rapid identification of small molecules against the three-dimensional structure of available macromolecular targets, usually by X-ray crystallography, NMR structures or homology models. Due to abundant information on protein sequences and structures, the structural information on specific proteins and their interactions have become very important for current pharmacological research [259,260].

Even in the absence of knowledge about the binding site and limited backbone movements, a variety of algorithms have been developed for docking over the past two decades. Although the ZDOCK [258], the rDOCK [261] and the HEX [262] have provided results with high coupling precision, the complexes provided are not very useful for designing inhibitors for protein interfaces due to constraints on rigid body docking [257]. In this context, more flexible approaches have been developed which generally examine very limited conformations compared to rigid body methods. These docking methods predict that binding is more likely to occur in broad surface regions and then defines the sites in complex structures of high affinity [263].

The best example is the HADDOCK software [260], which has been successful in solving a large number of precise models for protein-protein complexes. A good example of its use is the study of the complex formed between plectasin, a member of the innate immune system, and a precursor lipid of bacterial cell wall II. The study identified the residues involved in the binding site between the two proteins, providing valuable information for planning new antibiotics [264].

However, the absolute energies associated with intermolecular interaction are not estimated with satisfactory accuracy by the current algorithms. Some significant issues as solvent effects, entropic effects, and receptor flexibility still need to be addressed. However, some methods such as MOE-Dock [265], GOLD [266], Glide [267], FlexX [255] and Surflex [268], which deal with lateral chain flexibility, have proven to be effective and adequate in most cases. Realistic interactions between small molecules and receptors still depend on experimental wet-lab validation [257,269].

Despite the current difficulties, there is a growing interest in the mechanisms and prediction of small molecules such as peptides, since they bind to proteins in a highly selective and conserved manner, being promising as new medicinal and biological agents [270]. While both ‘small molecule docking methods’ and ‘custom protocols’ can be used, short peptides are difficult targets because of their high torsional flexibility [269]. Protein-peptide docking is generally more challenging than those related to other small molecules, and a variety of methods have been applied so far. However, few of these approaches have been published in a way that can be reproduced with ease [256,271,272]. Although it is difficult to use peptide docking, a recent focus of basic and pharmacological research has used computational tools with modified peptides to predict the selective disruption of protein-protein interactions. These

studies are based on the involvement of some critical amino acid residues that contribute most to the binding affinity of a given interaction, also called hot-spots [273,274].

Despite the number of existing docking programs, the algorithms need to be improved. However, approaches are being developed to improve all issues related to punctuation, protein flexibility, interaction with plain water, among other issues [275]. In this context, the CAPRI (Critical Assessment of Predicted Interactions) is a community that provides a quality assessment of different docking approaches. It started in 2001 and since then has aided the development and improvement of the methodologies applied for docking [276].

An evaluation was carried out for CAPRI in 2016 resulting in an improvement in the integration of different modeling tools with docking procedures, as well as the use of more sophisticated evolutionary information to classify models. However, adequate modeling of conformational flexibility in interacting proteins remains an essential demand with a crucial need for improvement [275]. Different docking programs are currently available, and new alternatives continue to appear. Some of these alternatives will disappear, just as others will become the top choices among field users.

Plant biotechnology involving AMPs

Several works reporting the overexpression of AMPs in transgenic plants have been published in the last decades mainly involving plant defensins (**Table 1**), but also regarding other plant AMPs (**Table 2**). The first transgenic plant expressing a defensin was generated by Gao et al. (2000) [277] producing a transgenic potato carrying the alfalfa alfAFP defensin. The transformed plants did not exhibit phenotypic differences while the level of infection by *Verticillium dahliae* fungus was reduced by six-fold when compared to untransformed controls. In addition, the results of greenhouse experiments were consistent with the field analyzes, highlighting the *in vivo* activity of defensin.

Another experiment carried out by Kanzaki et al. (2002) [278] generated transgenic rice plants expressing a defensin isolated from *Eutrema japonicum* (Brassicaceae) and observed that the transformed plants presented quite variable levels of resistance to the fungus *Magnaporthe grisea* when compared to the controls. The authors suggested that this variation could be related to events of gene silencing or to post-transcriptional changes.

Furthermore, Chen et al. (2006) [279] transformed tomato plants with three vector constructs using: (1) the *alfAFP* gene encoding an alfalfa defensin, (2) the *GLU* gene encoding a tobacco defensin, and (3) both genes *alfAFP* + *GLU*. Interestingly the levels of resistance to

Ralstonia solanacearum were similar in transgenic plants carrying a single defensin gene, regardless of the type. However, transgenic plants carrying both genes exhibited a more pronounced resistance, evidencing the synergistic effect of their coexpression.

Anuradha et al. (2008) [280] transformed tobacco and peanut plants with the mustard defensin gene *BjD*, generating individuals with high levels of resistance to fungal infections (*Phytophthora parasitica, Fusarium moniliforme, Pheaoisariopsis personata,* and *Cercospora arachidicola*). In another assay, Abdallah et al. (2010) [281] evaluated transgenic tomato plants expressing the *Msdef1* (*Medicago sativa* defensin) gene. High levels of resistance to *F. oxysporum* were observed. In the inoculation bioassays, 70% of the transgenic plants showed no fusarium wilt symptoms while 7% were asymptomatic.

The first transgenic cotton cv. Coker was generated by Gaspar et al. (2014) [282], using class II defensin. Greenhouse tests and field bioassays confirmed the resistance level increase to *V. dahliae* and *F. oxysporum* in three times when compared to untransformed plants. In addition, the productivity of plants constitutively expressing defensins was similar to that of untransformed plants in the absence of the pathogen. This fact is possibly related to the storage of defensin in the vacuole, which promotes lower phytotoxicity.

Transgenic rice plants expressing the defensin gene *NmDef02* (from *Nicotiana megalosiphon*) acquired resistance to the fungus *Sarocladium oryzae* [283]. This same defensin gene was tested in transgenic tobacco and potato plants that acquired resistance to *P. infestans*, *P. parasitica var. nicotianae*, *Alternaria solani*, *F. oxysporum* and *V. dahlia*. Interestingly, the antifungal activity raised no toxic effects for host plants, highlighting the efficacy of defensin in conferring resistance to different pathogens [284].

Thao et al. (2017) [285] transformed tobacco plants with a defensin gene from *Vigna radiata* (*VrDEF1*; Fabaceae) related to inhibition of weevil alpha-amylase. After an alpha-amylase enzymatic assay with total seed proteins of transgenic and control plants, a reduction of 18% in enzyme activity was observed, demonstrating the potential of defensin as a candidate for biotechnological purposes related to insect resistance or tolerance. Overexpression of defensin J1-1 (from pepper) in transgenic tobacco was carried out using two constructs: (i) the constitutive 35S promoter and (ii) root-specific promoter pPRP3. The resistance to *P. parasitica* var. *nicotianae* was similar for both promoters tested. In addition, overexpression of exogenous J1-1 promoted increased expression of endogenous PR2 and PR10 genes, evidencing its potential in altering signaling pathways and increasing resistance to phytopathogens [286].

Biotechnological approaches related to plant transformation with genes encoding other AMPs (such as snakins, LTPs, alpha-thionin, and heveins) are still poorly represented in the literature when compared to the use of defensin genes. The only exception involves thaumatins, which are extensively characterized and have been researched since the late 1990s (**Table 1**).

Regarding snakins, Almasia et al. (2008) [287] developed a transgenic potato overexpressing snakin-1, which promoted increased resistance to fungal infection by *Rhizoctonia solani*, achieving survival rates of 75% in transgenic plants versus 17% in wild plants. In addition, the transformed plants were tested for resistance to *Erwinia carotovora* resulting in a reduction of 88% of the symptoms. Balaji and Smart (2012) [288] used a snakin-2 gene to generate cisgenic tomato plants. Transformed plants infected with the bacterium *Clavibacter michiganensis* ssp. *michiganensis* presented a delay in the emergence of the disease symptoms, lesion size, and reduction of colonizing bacteria.

Scientific reports related to transformation with LTPs are still scarce. The study by Patkar and Chattoo (2006) [289] is worth mentioning. The authors used the *Ace-AMP1*, an ns-LTP gene from onion to generate transgenic rice plants with a constitutive maize ubiquitin (UbI) promoter or by the pathogen-induced Phenylalanine Ammonia-lyase (PAL). The bioassays involved blast fungus (*Magnaporthe grisea*), sheath blight fungus (*R. solani*) and leaf blight bacterium (*Xanthomonas oryzae*). A significant reduction in the lesion size caused by the three phytopathogens was observed, as well as a delay in the appearance of the symptoms. However, no transgenic lineage acquired total resistance to any of the pathogens, even considering the different promoters used.

Concerning alpha-thionines, the first record of a transformed plant was carried out by Carmona et al. (1993) [290] that overexpressed alpha-thionines (derived from barley and wheat) in tobacco. Transgenic plants bearing the barley gene increased resistance to *Pseudomonas syringae* pv. *syringae* and presented a drastic reduction in the lesion area. In contrast, when wheat alpha-thionin was used, no difference in transformed plants was observed relative to controls, possibly due to the low level of gene expression in transgenic tobacco plants. Transgenic rice transformed with an oat alpha-thionin (*Asthi1*) was resistant against *Burkholderia plantarii* and *B. glumae*, with transformed plants showing similar growth to healthy controls [291].

The alpha-thionin *Thi2.1* was used for transformation of tomato and resistance evaluation of symptoms after inoculation with *F. oxysporum* and *R. solanacearum* [292]. Some transformed plants submitted to infection by *F. oxysporum* were as resistant as the resistant

wild strain. In turn, the plants infected with *R. solanacearum* showed higher resistance than the untransformed ones. However, the tomato strain naturally resistant to *R. solanacearum* presented lower symptoms severity when compared to the transformed ones.

Muramoto et al. (2012) [293] developed a transgenic sweet potato overexpressing barley alpha-hordothionin and found increased resistance to the fungus *Ceratocystis fimbriata* in leaves and roots.

The use of hevein in plant transformation was reported by Koo et al. (2002) [294] that generated transgenic tobacco plants overexpressing the gene *Pn-AMP-h2* from *Pharbitis nil* (Convolvulaceae). Transgenic plants showed increased resistance to fungus *P. parasitica*, presenting a reduction in the symptoms and severity of the disease.

Later, the *in vivo* activity of the hevein *SmAMP1* gene from *Stellaria media* (Caryophyllaceae) was evaluated in transgenic tobacco and in Arabidopsis, both inoculated with the fungus *Bipolaris sorokiniana* [295]. The untransformed plants presented smaller size and lower number of flowers when compared to the transgenic lines that grew similarly to the inoculated wild-type. In an additional trial, the authors evaluated the survival rate of transformed tobacco after inoculation with the fungus *Thielaviopsis basicola* and found that one of the transgenic lines had superior survival (89%) than the wild-type (48%). In contrast, another transformed strain showed high susceptibility to the pathogen, with a survival rate of only 15% compared to 48% in the control.

The first record of genetic transformation using thaumatin was performed by Datta et al. (1999) [296] who developed transgenic rice by overexpressing an endogenous thaumatin. In a bioassay for sheath blight (*R. solani*) a reduction in the area and number of lesions caused by the phytopathogen was observed in the transformed plants. In the same year, [297] verified that transgenic wheat plants overexpressing a rice thaumatin (*tlp*) showed retardation and decreased symptoms of fusarium ear blight caused by *F. graminearum*. However, Anand et al. (2003) [298] reported opposite results in a field bioassay, with transformed individuals showing more intense symptoms than infected wild plants. In the following year, Velazhahan and Muthukrishnan (2004) [299] demonstrated that transgenic tobacco plants carrying *Tlp* inoculated with the fungus *Alternaria alternata* showed a similar number of lesions to the wild type, even though the number of necrotic lesions was significantly lower in the transformed plants.

Recently, He et al. (2017) [300] isolated and overexpressed a thaumatin from *Vitis amurensis* (VaTLP) in *V. vinifera* aiming resistance to Downy mildew caused by *Plasmopara viticola*, reporting a reduction of the lesion area and in the number of spores in leaves infected

with the pathogen. Histological analyses also confirmed disease resistance, demonstrating inhibition and malformation in the development of hyphae in leaf tissues of transgenic plants.

Aghazadeh et al. (2017) [301] developed transgenic canola overexpressing a rice thaumatin (*Ostlp*) and found that the diameter of the lesions caused by *S. sclerotiorum* was reduced by half when compared to the wild plant. *Ostlp* was also used in the transformation of cassava (*Manihot esculenta*, Euphorbiaceae) infected with *Colletotrichum gloeosporioides*. The transformed plants had a delay in the onset of symptoms, as well as a reduction in the lesion diameter by more than 50% compared to controls [302].

Gaspar et al. (2014) [282] mentioned more than 100 publications reporting the expression of genes with antifungal properties in transgenic plants. However, few reports present validated greenhouse data on field bioassays. This fact must be related to the complexity of reproducing laboratory results in the field or to the difficulty of meeting the regulatory requirements necessary for this type of experiment in most countries. To date, no transgenic plant with antimicrobial peptide was introduced commercially, probably due to bureaucratic procedures for GMOs (Genetically Modified Organisms) testing or the cost of resistance to yield.

Zeller et al. (2013) [303] comment that in 88 studies related to transgenic plants with resistance to pathogens, about half of the resistant plants had lower productivity than their respective controls. The authors evidenced these data in a study addressing resistance to pathogenic fungi in genetically modified wheat, in which four transformants carrying resistance genes to powdery mildew *Blumeria graminis* were evaluated. It was verified that even in the presence of the pathogen, three transformants did not differ in the agronomic performance in relation to the untransformed plants of the same cultivar. The single accession that had higher resistance showed better agronomic performance only when the infection levels were extremely high. However, under natural conditions, it is known that infection levels generally vary yearly according to environmental factors.

It is worth noting that even considering the reported difficulties it is expected that in the near future biotech agricultural products based on AMPs will be essential for increasing agricultural production, accelerating the transition between biotechnological research and field bioproducts [304].

Table 2 - Plant defensins previously used for transformation of various plant species, with their donator species, transformed plants and reported *in vivo* activities.

Name	Origin	Transformed plants	*in vivo* activity	Reference
BrD1	*Brassica rapa*	Rice	Insecticide against nymphs and adult females of the brown spittlebug (*Nilaparvata lugens*)	[305]
alfAFP (MsDef1)	*Medicago sativa*	Potato and tomato	Antifungal action against *Verticillium dahliae* (potato*), Fusarium oxysporum* (tomate) and antibacterial against *Ralstonia solanacearum* (tomato)	[277; 279; 281]
Wasabi defensin	*Wasabia japonica*	Rice, tabacco, eggplant, tomato, *Phalaenopsis*, melon 'Egusi' (*Colocynthis citrullus*)	Antifungal action against *Magnaporthe grisea* (rice), *Botrytis cinerea* (tabacco and tomato), *Alternaria solani* (eggplant and tomato), *Alternaria* leaf spot and *Fusarium* wilt (Melon 'Egusi'), *Fusarium oxysporum* and *Erysiphe lycopersici* (tomato) and antibacterial against *Erwinia carotovora* (*Phalaenopsis*)	[278; 306-310]
BjD	*Brassica japonica*	Tabacco and peanut	Antifungal action against *Phytophthora parasitica* and *Fusarium moniliforme* (tabacco), *Phaeosariopsis personata* and *Cercospora arachidicola* (peanut)	[280]
NaD1	*Nicotiana alata*	Cotton	Antifungal action against *Fusarium oxysporum* and *Verticillium dahliae*	[282]
Sm-AMP-D1	*Stellaria media*	Banana	Antifungal action against *Fusarium oxysporum* f. sp. *cubense* (Foc)	[311]
Rs-AFP2	*Raphanus sativus*	Tabacco, wheat and peanut	Antifungal action against *Alternaria longipes* (tabacco), *F. graminearum* and *Rhizoctonia cerealis* (wheat), *Phaeoisariopsis personata* (peanut)	[312-314]
BSD1	*Brassica campestris*	Tabacco	Antifungal action against *Phytophthora parasitica*	[315]

Table 2 continued...

Name	Origin	Transformed plants	*in vivo* activity	Reference
PhDef1	*Petunia hybrida*	Banana	Antifungal action against *Fusarium oxysporum* f. sp. *cubense* (Foc)	[316]
PhDef2	*Petunia hybrida*	Banana	Antifungal action against *Fusarium oxysporum* f. sp. *cubense* (Foc)	[317]
DDR30	*Pisum sativum*	Canola	Antifungal action against *Leptosphaeria maculans*	[352]
DmAMP1	*Dahlia merckii*	Papaya and rice	Antifungal action against *Phytophthora palmivora* (papaya), *Magnaporthe oryzae* and *Rhizoctonia solani* (rice)	[318; 319]
J1-1	*Capsicum annuum*	Pepper and tobacco	Antifungal action against *Colletotrichum gloeosporioides* (Antracnose) in pepper and *Phytophthora parasítica* in tabacco	[286; 320]
MtDef4.2	*Medicago truncatula*	*Arabidopsis thaliana*	Antifungal action against *Hyaloperonospora arabidopsidis* Noco2 and *Fusarium graminearum*	[321]
NmDef02	*Nicotiana megalosiphon*	Rice, potato and tabacco	Antifungal action against *P. parasitica var. nicotianae* e *P. hyoscyami* f.sp. *tabacina* (tabaco), *A. solani* and *P. infestans* (potato), *Sarocladium oryzae* (rice)	[283-284]
VrDEF1	*Vigna radiata*	Tabacco	Inhibitor insecticide of caruncho α-amylase	[285]

Table 3 - Plant Snakins, LTPs, α-Thionins, Heveins and Thaumatins used for transformation of various plant species, with their donator species, transformed plants and reported *in vivo* activities.

Name	Origin	Transformed plants	*in vivo* activity	Reference
Snakin-1 (SN-1)	*Solanum chacoense*	Potato and Wheat	Antifungal action against *Rhizoctonia solani* (potato), *Blumeria graminis* (wheat) and Antibacterial against *Erwinia carotovora* (potato)	[287; 353]
Snakin-2 (SN-2)	*Solanum lycopersicum*	Tomato	Antibacterial against *Clavibacter michiganensis*	[288]
Ace-AMP1 (ns-LTP)	*Allium cepa*	Rice	Antifungal action against *Magnaporthe grisea, Rhizoctonia solani* and antibacterial against *Xanthomonas oryzae*	[289]
α-Thionin	Cevada	Tabacco	Antibacterial against *Pseudomonas syringae*	[290]
α-Thionin	Aveia	Rice	Antibacterial against *Burkholderia plantarii* and *B. glumae* (rice)	[291]
α-Thionin (Thi2.1)	Arabidopsis	Arabidopsis and tomato	Antifungal action against *Ralstonia solanacearum* (tomato) and Antifungal action against *Fusarium oxysporum* (arabidopsis and tomato)	[292, 322]
α-Hordo Thionin (α-HT)	Cevada	Sweet potato	Antifungal action against *Ceratocystis fimbriata*	[293]
Pn-AMP-h2 (Hevein)	*Pharbitis nil*	Tabacco	Antifungal action against *Phytophthora parasitica*	[294]
SmAMP1 (Hevein)	*Stellaria media*	Tabacco and Arabidopsis	Antifungal action against *Bipolaris sorokiniana* (arabidopsis) and *Thielaviopsis basicola* (tabacco)	[295]

Table 3, continued

Thaumatin	Arroz	Rice, Wheat, Tabacco, Banana and Cassava	Antifungal action against *Rhizoctonia solani* (rice), *Fusarium graminearum* (wheat), Alternaria alternata (tabacco), *Fusarium oxysporum* (banana), *Sclerotinia sclerotiorum* (canola), *Colletotrichum gloeosporioides* (cassava)	[296; 297; 299; 301; 302; 323]
Thaumatin	*Thaumatococcus daniellii*	Tabacco	Antifungal action against *Pythium aphanidermatum* and *Rhizoctonia solani* and increased tolerance to water and saline stress	[324]
VaTLP (Thaumatin)	*Vitis amurensis*	Grape	Antifungal action against *Plasmopara viticola*	[300]
ObTLP1 (Thaumatin)	*Ocimum basilicum*	Arabidopsis	Antifungal action against *Scleretonia sclerotiorum* and *Botrytis cinerea* and increased tolerance to water and saline stress	[325]
CsTLP (Thaumatin)	*Camellia sinensis*	Potato	Antifungal action against *Macrophomina phaseolina* and *Phytophthora infestans*	[326]

Plant AMPs in the development of new drugs and bioactive compounds

Plants play an essential role in animal and human survival as a source of food and oxygen, besides their use as therapeutic agents presenting action against diverse human pathologies. There is a rich and unexplored ethnobotanical diversity especially in tropical regions, with numerous plants traditionally used by human populations [327,328].

Some historical examples include *Cinchona pubescens* quinidine for the treatment of cardioarrhythmia and vinblastine of *Catharanthus roseus* for the treatment of various cancer types from a drug derived from a symbiotic organism associated with the plant (Craik et al. 2018). Another example is the paclitaxel (Taxol) a drug used to treat breast cancer, is derived from symbiotic fungi and the gymnosperm tree *Taxos brevifolia* [330].

Many new drugs discovered come from bioactive molecules that are responsible or participate in the therapeutic action of medicinal plants, as is the case of AMPs, which stand out for being active against a wide range of pathogenic microorganisms that attack plants and animals [331]. Also called peptidic antibiotics, AMPs present a new generation of biocidal agents for plant protection, as well as for the treatment of microbial diseases in humans and animals [332]. Different applications have been reported on the action of these peptides, demonstrating broad-spectrum activity, such as (i) action against cancer cells [333,334], (ii) production and accumulation of immune cells, (iii) wound healing and (iv) angiogenesis stimulation [335,336].

In general, the AMPs exhibit low cytotoxicity to mammalian cells and have a particular mode of action [337]. However, the interaction of these proteins with pathogens has not yet been fully unveiled. The proposed modes of action for AMPs involve the interaction of peptides with microbial membranes and the formation of pores leading to one or more processes including micellization, depolarization of membranes, leakage of cytoplasmic material, internalization of biocidal peptides or damage to intracellular macromolecules [338,339]. The action also depends on the peptide structural conformation (α-helices, β-sheets, disordered loops) and their interaction with the microbial membrane through hydrophobic or electrostatic forces [337].

As mentioned before, with the advent of bioinformatics, a number of tools and databases have provided relevant information on natural and synthetic AMPs and are useful to screen and predict functional activities, helping to drive the process of discovery and design of AMPs with therapeutic potential or antimicrobial properties [337]. Laverty et al. (2011) [340] estimate that more than 1700 natural AMPs were identified and that thousands of derivatives and analogs were designed or generated synthetically using these molecules as a model.

Over the years, modern medicine has been dealing with incidences of antimicrobial resistance, and the current and inadequate use of antibiotics are essential factors to generate the great crisis of drug resistance, considered worldwide as a public health problem recognized by the World Health Organization (WHO) and the United Nations [341, 342] that launched a campaign to encourage and prioritize research for the development of new drugs in the fight against resistant bacteria.

In this scenario plant AMPs figure as promising candidates, offering a broad spectrum of activity against the most diverse microorganisms, as well as presenting low or no side effects [343–345].

Limitations to the large-scale commercial production of peptide-derived drugs include the lack of suitable production platforms in terms of yield, cost, and product purity. Techniques such as heterologous expression and solid phase chemical synthesis are the most used. Therefore, the interest in producing large-scale AMPs using plants has increased significantly during the last decade [346,347]. Biotechnological advances in the last decade allowed the use of plant bioreactors as attractive platforms for the large-scale production of peptides, proteins, and drugs [348–351].

Concluding remarks and perspectives

As emphasized in this review, plant antimicrobial peptides (AMPs) show greater diversity and abundance, when compared to other kingdoms. It can be speculated that plants shelter yet undescribed AMP classes, given their huge abundance and isoform diversity.

The genomic and peptidic structure of AMPs can be variable, with few key residues conserved, what turns their identification, classification and comparison challenging even in the omics age. Nevertheless, advances in the generation of new bioinformatics tools and specialized databases have led to new and more efficient approaches for both the identification of primary sequences and molecular modeling, besides the analysis of the stability of generated models.

Plants transformed with AMPs generally have greater resistance to pathogens, with an emphasis on fungi, generally not affecting productivity, although a low number of field assays are available. Despite this, no transgenic or cisgenic (transformed with a gene from the same species) plants expressing AMPs have been commercially launched, possibly due to logistic

difficulties or the lack of interest of large biotech companies that are also generally suppliers of pesticides.

The most significant potential has been recognized in the production of next-generation antibiotics due to the bactericidal and fungicidal action of AMPs with low toxicity to mammals. In this particular, the greatest limitation has been the large-scale production of AMPs by heterologous expression systems (which generally use bacteria and yeasts), since the growth of these organisms is affected by AMPs, requiring immobilization techniques and posterior purification. Their potential as new drugs has been recognized not only individually, but as an adjuvant, in synergism with traditional antibiotic treatment.

Abbreviation list

2-DE	Two-dimensional electrophoresis
2D	Two-dimensional
3D	Three-dimensional
AMP	Antimicrobial peptides
aa	Amino acids
APD	Antimicrobial Peptide Database
At	*Arabidopsis thaliana*
BAM	Barely any meristem
BLAST	Basic local alignment search tool
BP	Bioactive peptides
CAMP	Collection of Antimicrobial Peptides
CAPRI	Critical assessment of predicted interactions
CASP	Critical assessment of protein structure prediction
CCK	Cyclic cystine knot
CD	Circular dichroism
cDNA	Complementary DNA
CLE25	Clavata3/embryo-surrounding region-related 25
CRPs	Cysteine-rich peptides
CSB	Cystine stabilized β-sheet
CSαβ	Cysteine-stabilized αβ motif
Cys	Cysteine
DB	Databanks
DBAASP	Database of Antimicrobial Activity and Structure of Peptides
GASA	Gibberellic acid stimulated in Arabidopsis
GlcNAc	N-acetylglucosamine
GMOs	Genetically modified organisms
HMM	Hidden Markov Model
HPLC	High-performance liquid chromatography
IC_{50}	The half maximal Inhibitory Concentration
LAMP	Database linking antimicrobial peptides
LTP	Lipid transfer protein
MALDI-MS	Mass spectrometry, matrix-assisted laser desorption/ionization
MD	Molecular dynamics
MDR	Multidrug-resistant
MIC	Minimal inhibitory concentration
ML	Machine learning
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information
NMR	Nuclear magnetic resonance
NN	Neural networks
NOEs	Nuclear overhauser enhancement

NR	Non-redundant
nsLTPs	Non-specific lipid transfer proteins
OMSSA	Open mass spectrometry search algorithm
PDB	Protein data bank
pI	Isoelectric point
PINs	Puroindolines
PLS	Polaris
PR-4	Pathogenesis-related protein 4
PR-5	Pathogenesis-related protein 5
PR5K	PR5-like receptor kinases
REGEX	Regular expression
RF	Random forest
sORFs	Small open read frames
sTLPs	Small TLPs
SVM	Support vector machine
SYST	Systemin
TLPs	Thaumatin-like proteins
UbI	Ubiquitin
WHO	World Health Organization

Acknowledgments

The authors are very grateful to CAPES (Coordination for the Improvement of Higher Education Personnel, Brazil) and CNPq (Brazilian National Council for Scientific and Technological Development, Brazil) and FACEPE (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco, Brazil) for valuable financial support and fellowships. We thank Prof. Dr. Palmiro Poltronieri (Italian National Research Council) for the critical review, valuable suggestions and interesting discussions.

REFERENCES

[1] Marmiroli, N.; Maestri, E. Plant Peptides in Defense and Signaling. *Peptides* **2014**, *56*, 30–44. https://doi.org/10.1016/j.peptides.2014.03.013.

[2] Germain, H.; Chevalier, E.; Matton, D. P. Plant Bioactive Peptides: An Expanding Class of Signaling Molecules. *Can. J. Bot.* **2006**, *84* (1), 1–19. https://doi.org/10.1139/b05-162.

[3] Higgins, C. F.; Payne, J. W. Plant Peptides. In *Nucleic Acids and Proteins in Plants I*; Springer, 1982; pp 438–458.

[4] Tavormina, P.; De Coninck, B.; Nikonorova, N.; De Smet, I.; Cammue, B. P. A. The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions. *Plant Cell*

2015, *27* (8), 2095–2118. https://doi.org/10.1105/tpc.15.00440.

[5] Farrokhi, N.; Whitelegge, J. P.; Brusslan, J. A. Plant Peptides and Peptidomics. *Plant Biotechnol. J.* **2008**, *6* (2), 105–134. https://doi.org/10.1111/j.1467-7652.2007.00315.x.

[6] Takahashi, F.; Suzuki, T.; Osakabe, Y.; Betsuyaku, S.; Kondo, Y.; Dohmae, N.; Fukuda, H.; Yamaguchi-Shinozaki, K.; Shinozaki, K. A Small Peptide Modulates Stomatal Control via Abscisic Acid in Long-Distance Signalling. *Nature* **2018**, *556* (7700), 235–238. https://doi.org/10.1038/s41586-018-0009-2.

[7] Liu, J.; Mehdi, S.; Topping, J.; Friml, J.; Lindsey, K. Interaction of PLS and PIN and Hormonal Crosstalk in *Arabidopsis* Root Development. *Front. Plant Sci.* **2013**, *4*. https://doi.org/10.3389/fpls.2013.00075.

[8] Pearce, G.; Bhattacharya, R.; Chen, Y.-C. Peptide Signals for Plant Defense Display a More Universal Role. *Plant Signal. Behav.* **2008**, *3* (12), 1091–1092.

[9] Ryan, C. A. Protease Inhibitors in Plants: Genes for Improving Defenses Against Insects and Pathogens. *Annu. Rev. Phytopathol.* **1990**, *28* (1), 425–449. https://doi.org/10.1146/annurev.py.28.090190.002233.

[10] Stratmann, J. Long Distance Run in the Wound Response – Jasmonic Acid Is Pulling Ahead. *Trends Plant Sci.* **2003**, *8* (6), 247–250. https://doi.org/10.1016/S1360-1385(03)00106-7.

[11] García-Olmedo, F.; Molina, A.; Alamillo, J. M.; Rodríguez-Palenzuéla, P. Plant Defense Peptides. *Biopolymers* **1998**, *47* (6), 479–491. https://doi.org/10.1002/(SICI)1097-0282(1998)47:6<479::AID-BIP6>3.0.CO;2-K.

[12] Benko-Iseppon, A. M.; Galdino, S. L.; Calsa, T.; Kido, E. A.; Tossi, A.; Belarmino, L. C.; Crovella, S. Overview on Plant Antimicrobial Peptides. *Curr. Protein Pept. Sci.* **2010**, *11* (3), 181–188.

[13] Kaas, Q.; Westermann, J.-C.; Craik, D. J. Conopeptide Characterization and Classifications: An Analysis Using ConoServer. *Toxicon* **2010**, *55* (8), 1491–1509. https://doi.org/10.1016/j.toxicon.2010.03.002.

[14] Yu, J.; Kan, Y.; Rapp, M.; Danner, E.; Wei, W.; Das, S.; Miller, D. R.; Chen, Y.; Waite, J. H.; Israelachvili, J. N. Adaptive Hydrophobic and Hydrophilic Interactions of Mussel Foot Proteins with Organic Thin Films. *Proc. Natl. Acad. Sci.* **2013**, *110* (39), 15680–15685. https://doi.org/10.1073/pnas.1315015110.

[15] Zanetti, M. Cathelicidins, Multifunctional Peptides of the Innate Immunity. *J. Leukoc. Biol.* **2004**, *75* (1), 39–48. https://doi.org/10.1189/jlb.0403147.

[16] *Antimicrobial Peptides: Discovery, Design and Novel Therapeutic Strategies*; Wang, G., Ed.; CABI: Wallingford, 2010. https://doi.org/10.1079/9781845936570.0000.

[17] Murzin, A. G.; Brenner, S. E.; Hubbard, T.; Chothia, C. SCOP: A Structural Classification of Proteins Database for the Investigation of Sequences and Structures. *J. Mol. Biol.* **1995**, *247* (4), 536–540. https://doi.org/10.1006/jmbi.1995.0159.

[18] Wang, C. K.; Wacklin, H. P.; Craik, D. J. Cyclotides Insert into Lipid Bilayers to Form Membrane Pores and Destabilize the Membrane through Hydrophobic and Phosphoethanolamine-Specific Interactions. *J. Biol. Chem.* **2012**, *287* (52), 43884–43898. https://doi.org/10.1074/jbc.M112.421198.

[19] Terras, F. R.; Schoofs, H. M.; De Bolle, M. F.; Van Leuven, F.; Rees, S. B.; Vanderleyden, J.; Cammue, B. P.; Broekaert, W. F. Analysis of Two Novel Classes of Plant Antifungal Proteins from Radish (*Raphanus Sativus* L.) Seeds. *J. Biol. Chem.* **1992**, *267* (22), 15301–15309.

[20] Wang, G. Post-Translational Modifications of Natural Antimicrobial Peptides and Strategies for Peptide Engineering. *Curr. Biotechnol. E* **2012**, *1* (1), 72–79. https://doi.org/10.2174/2211550111201010072.

[21] Vriens, K.; Cammue, B.; Thevissen, K. Antifungal Plant Defensins: Mechanisms of Action and Production. *Molecules* **2014**, *19* (8), 12280–12303. https://doi.org/10.3390/molecules190812280.

[22] Bruix, M.; González, C.; Santoro, J.; Soriano, F.; Rocher, A.; Méndez, E.; Rico, M. ^{1} H-Nmr Studies on the Structure of a New Thionin from Barley Endosperm: Structure of a New Thionin. *Biopolymers* **1995**, *36* (6), 751–763. https://doi.org/10.1002/bip.360360608.

[23] Tam, J.; Wang, S.; Wong, K.; Tan, W. Antimicrobial Peptides from Plants. *Pharmaceuticals* **2015**, *8* (4), 711–757. https://doi.org/10.3390/ph8040711.

[24] Colilla, F. J.; Rocher, A.; Mendez, E. γ-Purothionins: Amino Acid Sequence of Two Polypeptides of a New Family of Thionins from Wheat Endosperm. *FEBS Lett.* **1990**, *270* (1–2), 191–194. https://doi.org/10.1016/0014-5793(90)81265-P.

[25] Mendez, E.; Moreno, A.; Colilla, F.; Pelaez, F.; Limas, G. G.; Mendez, R.; Soriano, F.; Salinas, M.; Haro, C. Primary Structure and Inhibition of Protein Synthesis in Eukaryotic Cell-Free System of a Novel Thionin, Gamma-Hordothionin, from Barley Endosperm. *Eur. J. Biochem.* **1990**, *194* (2), 533–539. https://doi.org/10.1111/j.1432-1033.1990.tb15649.x.

[26] Broekaert, W. F.; Terras, F. R.; Cammue, B. P.; Osborn, R. W. Plant Defensins: Novel Antimicrobial Peptides as Components of the Host Defense System. *Plant Physiol.* **1995**, *108* (4), 1353–1358.

[27] Parisi, K.; Shafee, T. M. A.; Quimbar, P.; van der Weerden, N. L.; Bleackley, M. R.; Anderson, M. A. The Evolution, Function and Mechanisms of Action for Plant Defensins. *Semin. Cell Dev. Biol.* **2018**. https://doi.org/10.1016/j.semcdb.2018.02.004.

[28] Pelegrini, P. B.; Franco, O. L. Plant γ-Thionins: Novel Insights on the Mechanism of Action of a Multi-Functional Class of Defense Proteins. *Int. J. Biochem. Cell Biol.* **2005**, *37* (11), 2239–2253. https://doi.org/10.1016/j.biocel.2005.06.011.

[29] Shafee, T. M. A.; Lay, F. T.; Phan, T. K.; Anderson, M. A.; Hulett, M. D. Convergent Evolution of Defensin Sequence, Structure and Function. *Cell. Mol. Life Sci.* **2017**, *74* (4), 663–682. https://doi.org/10.1007/s00018-016-2344-5.

[30] Kraszewska, J.; Beckett, M. C.; James, T. C.; Bond, U. Comparative Analysis of the Antimicrobial Activities of Plant Defensin-Like and Ultrashort Peptides against Food-Spoiling Bacteria. *Appl. Environ. Microbiol.* **2016**, *82* (14), 4288–4298. https://doi.org/10.1128/AEM.00558-16.

[31] Oddepally, R.; Guruprasad, L. Isolation, Purification, and Characterization of a Stable Defensin-like Antifungal Peptide from *Trigonella foenum-graecum* (Fenugreek) Seeds. *Biochem. Mosc.* **2015**, *80* (3), 332–342. https://doi.org/10.1134/S0006297915030086.

[32] Brogden, K. A. Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria? *Nat. Rev. Microbiol.* **2005**, *3* (3), 238–250. https://doi.org/10.1038/nrmicro1098.

[33] Carvalho, A. de O.; Gomes, V. M. Plant Defensins—Prospects for the Biological Functions and Biotechnological Properties. *Peptides* **2009**, *30* (5), 1007–1020. https://doi.org/10.1016/j.peptides.2009.01.018.

[34] Mazliak, P.; Douady, D.; Demandre, C.; Kader, J. C. *Recent Advances in the Chemistry and Biochemistry of Plant Lipids. Ed. T. Gailliard and EJ Mercer*; Academic Press, New York, 1975.

[35] Edstam, M. M.; Viitanen, L.; Salminen, T. A.; Edqvist, J. Evolutionary History of the Non-Specific Lipid Transfer Proteins. *Mol. Plant* **2011**, *4* (6), 947–964.

[36] Salminen, T. A.; Blomqvist, K.; Edqvist, J. Lipid Transfer Proteins: Classification, Nomenclature, Structure, and Function. *Planta* **2016**, *244* (5), 971–997. https://doi.org/10.1007/s00425-016-2585-4.

[37] Castro, M. S.; Gerhardt, I. R.; Orrù, S.; Pucci, P.; Bloch, C. Purification and Characterization of a Small (7.3 KDa) Putative Lipid Transfer Protein from Maize Seeds. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* **2003**, *794* (1), 109–114.

[38] Kader, J.-C. Lipid-Transfer Proteins in Plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 627–654. https://doi.org/10.1146/annurev.arplant.47.1.627.

[39] Salminen, T. A.; Blomqvist, K.; Edqvist, J. Lipid Transfer Proteins: Classification, Nomenclature, Structure, and Function. *Planta* **2016**, *244* (5), 971–997. https://doi.org/10.1007/s00425-016-2585-4.

[40] Lindorff-Larsen, K.; Winther, J. R. Surprisingly High Stability of Barley Lipid Transfer Protein, LTP1, towards Denaturant, Heat and Proteases. *FEBS Lett.* **2001**, *488* (3), 145–148.

[41] Berecz, B.; Mills, E. N. C.; Tamás, L.; Láng, F.; Shewry, P. R.; Mackie, A. R. Structural Stability and Surface Activity of Sunflower 2S Albumins and Nonspecific Lipid Transfer Protein. *J. Agric. Food Chem.* **2010**, *58* (10), 6490–6497. https://doi.org/10.1021/jf100554d.

[42] Edstam, M. M.; Edqvist, J. Involvement of GPI-Anchored Lipid Transfer Proteins in the Development of Seed Coats and Pollen in *Arabidopsis thaliana*. *Physiol. Plant.* **2014**, *152* (1), 32–42. https://doi.org/10.1111/ppl.12156.

[43] Goyal, R. K.; Mattoo, A. K. Plant Antimicrobial Peptides. In *Host Defense Peptides and Their Potential as Therapeutic Agents*; Springer, 2016; pp 111–136.

[44] Simorre, J. P.; Caille, A.; Marion, D.; Marion, D.; Ptak, M. Two- and Three-Dimensional 1H NMR Studies of a Wheat Phospholipid Transfer Protein: Sequential Resonance Assignments and Secondary Structure. *Biochemistry* **1991**, *30* (49), 11600–11608.

[45] Gincel, E.; Simorre, J. P.; Caille, A.; Marion, D.; Ptak, M.; Vovelle, F. Three-Dimensional Structure in Solution of a Wheat Lipid-Transfer Protein from Multidimensional 1H-NMR Data. A New Folding for Lipid Carriers. *Eur. J. Biochem.* **1994**, *226* (2), 413–422.

[46] Peumans, W. J.; De Ley, M.; Broekaert, W. F. An Unusual Lectin from Stinging Nettle (*Urtica dioica*) Rhizomes. *FEBS Lett.* **1984**, *177* (1), 99–103. https://doi.org/10.1016/0014-5793(84)80989-8.

[47] Slavokhotova, A. A.; Shelenkov, A. A.; Andreev, Y. A.; Odintsova, T. I. Hevein-like Antimicrobial Peptides of Plants. *Biochem. Mosc.* **2017**, *82* (13), 1659–1674. https://doi.org/10.1134/S0006297917130065.

[48] Slavokhotova, A. A.; Shelenkov, A. A.; Andreev, Y. A.; Odintsova, T. I. Hevein-like Antimicrobial Peptides of Plants. *Biochem. Mosc.* **2017**, *82* (13), 1659–1674. https://doi.org/10.1134/S0006297917130065.

[49] Asensio, J. L.; Cañada, F. J.; Siebert, H.-C.; Laynez, J.; Poveda, A.; Nieto, P. M.; Soedjanaamadja, U.; Gabius, H.-J.; Jiménez-Barbero, J. Structural Basis for Chitin Recognition by Defense Proteins: GlcNAc Residues Are Bound in a Multivalent Fashion by Extended Binding Sites in Hevein Domains. *Chem. Biol.* **2000**, *7* (7), 529–543. https://doi.org/10.1016/S1074-5521(00)00136-8.

[50] Wong, K. H.; Tan, W. L.; Serra, A.; Xiao, T.; Sze, S. K.; Yang, D.; Tam, J. P. Ginkgotides: Proline-Rich Hevein-Like Peptides from Gymnosperm Ginkgo Biloba. *Front. Plant Sci.* **2016**, *7*. https://doi.org/10.3389/fpls.2016.01639.

[51] Raikhel, N. V.; Lee, H. I.; Broekaert, W. F. Structure and Function of Chitin-Binding

Proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1993**, *44* (1), 591–615. https://doi.org/10.1146/annurev.pp.44.060193.003111.

[52] Beintema, J. J. Structural Features of Plant Chitinases and Chitin-Binding Proteins. *FEBS Lett.* **1994**, *350* (2–3), 159–163.

[53] Rogozhin, E. A.; Slezina, M. P.; Slavokhotova, A. A.; Istomina, E. A.; Korostyleva, T. V.; Smirnov, A. N.; Grishin, E. V.; Egorov, T. A.; Odintsova, T. I. A Novel Antifungal Peptide from Leaves of the Weed Stellaria Media L. *Biochimie* **2015**, *116*, 125–132. https://doi.org/10.1016/j.biochi.2015.07.014.

[54] Beintema, J. J. Structural Features of Plant Chitinases and Chitin-Binding Proteins. *FEBS Lett.* **1994**, *350* (2–3), 159–163.

[55] Broekaert, W. F.; Marien, W.; Terras, F. R. G.; De Bolle, M. F. C.; Proost, P.; Van Damme, J.; Dillen, L.; Claeys, M.; Rees, S. B. Antimicrobial Peptides from *Amaranthus caudatus* Seeds with Sequence Homology to the Cysteine/Glycine-Rich Domain of Chitin-Binding Proteins. *Biochemistry* **1992**, *31* (17), 4308–4314. https://doi.org/10.1021/bi00132a023.

[56] Iseli, B.; Boller, T.; Neuhaus, J. M. The N-Terminal Cysteine-Rich Domain of Tobacco Class I Chitinase Is Essential for Chitin Binding but Not for Catalytic or Antifungal Activity. *Plant Physiol.* **1993**, *103* (1), 221–226. https://doi.org/10.1104/pp.103.1.221.

[57] Broekaert, W. F.; Van Parijs, J.; Leyns, F.; Joos, H.; Peumans, W. J. A Chitin-Binding Lectin from Stinging Nettle Rhizomes with Antifungal Properties. *Science* **1989**, *245* (4922), 1100–1102. https://doi.org/10.1126/science.245.4922.1100.

[58] Leah, R.; Tommerup, H.; Svendsen, I.; Mundy, J. Biochemical and Molecular Characterization of Three Barley Seed Proteins with Antifungal Properties. *J. Biol. Chem.* **1991**, *266* (3), 1564–1573.

[59] Van Parijs, J.; Broekaert, W. F.; Goldstein, I. J.; Peumans, W. J. Hevein: An Antifungal Protein from Rubber-Tree (*Hevea brasiliensis*) Latex. *Planta* **1991**, *183* (2), 258–264. https://doi.org/10.1007/BF00197797.

[60] Koo, J. C.; Lee, S. Y.; Chun, H. J.; Cheong, Y. H.; Choi, J. S.; Kawabata, S.; Miyagi, M.; Tsunasawa, S.; Ha, K. S.; Bae, D. W.; et al. Two Hevein Homologs Isolated from the Seed of *Pharbitis nil* L. Exhibit Potent Antifungal Activity. *Biochim. Biophys. Acta BBA - Protein Struct. Mol. Enzymol.* **1998**, *1382* (1), 80–90. https://doi.org/10.1016/S0167-4838(97)00148-9.

[61] Lipkin, A.; Anisimova, V.; Nikonorova, A.; Babakov, A.; Krause, E.; Bienert, M.; Grishin, E.; Egorov, T. An Antimicrobial Peptide Ar-AMP from Amaranth (*Amaranthus retroflexus* L.) Seeds. *Phytochemistry* **2005**, *66* (20), 2426–2431. https://doi.org/10.1016/j.phytochem.2005.07.015.

[62] Molesini, B.; Treggiari, D.; Dalbeni, A.; Minuz, P.; Pandolfini, T. Plant Cystine-Knot Peptides: Pharmacological Perspectives: Plant Cystine-Knot Proteins in Pharmacology. *Br. J. Clin. Pharmacol.* **2017**, *83* (1), 63–70. https://doi.org/10.1111/bcp.12932.

[63] Rees, D. C.; Lipscomb, W. N. Refined Crystal Structure of the Potato Inhibitor Complex of Carboxypeptidase A at 2.5 Å Resolution. *J. Mol. Biol.* **1982**, *160* (3), 475–498. https://doi.org/10.1016/0022-2836(82)90309-6.

[64] Molesini, B.; Treggiari, D.; Dalbeni, A.; Minuz, P.; Pandolfini, T. Plant Cystine-Knot Peptides: Pharmacological Perspectives: Plant Cystine-Knot Proteins in Pharmacology. *Br. J. Clin. Pharmacol.* **2017**, *83* (1), 63–70. https://doi.org/10.1111/bcp.12932.

[65] Chiche, L.; Heitz, A.; Gelly, J.-C.; Gracy, J.; Chau, P. T. T.; Ha, P. T.; Hernandez, J.-F.; Le-Nguyen, D. Squash Inhibitors: From Structural Motifs to Macrocyclic Knottins. *Curr. Protein Pept. Sci.* **2004**, *5* (5), 341–349.

[66] Murphy, E.; Smith, S.; De Smet, I. Small Signaling Peptides in *Arabidopsis* Development: How Cells Communicate Over a Short Distance. *Plant Cell* **2012**, *24* (8), 3198–3217. https://doi.org/10.1105/tpc.112.099010.

[67] Li, G.; Asiegbu, F. O. Use of Scots Pine Seedling Roots as an Experimental Model to Investigate Gene Expression during Interaction with the Conifer Pathogen *Heterobasidion annosum* (P-Type). *J. Plant Res.* **2004**, *117* (2), 155–162. https://doi.org/10.1007/s10265-003-0140-4.

[68] Iyer, S.; Acharya, K. R. Tying the Knot: The Cystine Signature and Molecular-Recognition Processes of the Vascular Endothelial Growth Factor Family of Angiogenic Cytokines: Cystine-Knot Growth Factors and Angiogenesis. *FEBS J.* **2011**, *278* (22), 4304–4322. https://doi.org/10.1111/j.1742-4658.2011.08350.x.

[69] Aboye, T. L.; Strömstedt, A. A.; Gunasekera, S.; Bruhn, J. G.; El-Seedi, H.; Rosengren, K. J.; Göransson, U. A Cactus-Derived Toxin-Like Cystine Knot Peptide with Selective Antimicrobial Activity. *ChemBioChem* **2015**, *16* (7), 1068–1077. https://doi.org/10.1002/cbic.201402704.

[70] Göransson, U.; Burman, R.; Gunasekera, S.; Strömstedt, A. A.; Rosengren, K. J. Circular Proteins from Plants and Fungi. *J. Biol. Chem.* **2012**, *287* (32), 27001–27006. https://doi.org/10.1074/jbc.R111.300129.

[71] Gustafson, K. R.; Sowder, R. C.; Henderson, L. E.; Parsons, I. C.; Kashman, Y.; Cardellina, J. H.; McMahon, J. B.; Buckheit, R. W.; Pannell, L. K.; Boyd, M. R. Circulins A and B. Novel Human Immunodeficiency Virus (HIV)-Inhibitory Macrocyclic Peptides from the Tropical Tree *Chassalia parvifolia*. *J. Am. Chem. Soc.* **1994**, *116* (20), 9337–9338. https://doi.org/10.1021/ja00099a064.

[72] Jennings, C. V.; Rosengren, K. J.; Daly, N. L.; Plan, M.; Stevens, J.; Scanlon, M. J.; Waine, C.; Norman, D. G.; Anderson, M. A.; Craik, D. J. Isolation, Solution Structure, and Insecticidal Activity of Kalata B2, a Circular Protein with a Twist: Do Möbius Strips Exist in Nature? †, ‡. *Biochemistry* **2005**, *44* (3), 851–860. https://doi.org/10.1021/bi047837h.

[73] Göransson, U.; Burman, R.; Gunasekera, S.; Strömstedt, A. A.; Rosengren, K. J. Circular Proteins from Plants and Fungi. *J. Biol. Chem.* **2012**, *287* (32), 27001–27006. https://doi.org/10.1074/jbc.R111.300129.

[74] Burman, R.; Strömstedt, A. A.; Malmsten, M.; Göransson, U. Cyclotide–Membrane Interactions: Defining Factors of Membrane Binding, Depletion and Disruption. *Biochim. Biophys. Acta BBA - Biomembr.* **2011**, *1808* (11), 2665–2673. https://doi.org/10.1016/j.bbamem.2011.07.004.

[75] Marcus, J. P.; Goulter, K. C.; Green, J. L.; Harrison, S. J.; Manners, J. M. Purification, Characterisation and CDNA Cloning of an Antimicrobial Peptide from *Macadamia integrifolia*. *Eur. J. Biochem.* **1997**, *244* (3), 743–749.

[76] McManus, A. M.; Nielsen, K. J.; Marcus, J. P.; Harrison, S. J.; Green, J. L.; Manners, J. M.; Craik, D. J. MiAMP1, a Novel Protein from *Macadamia integrifolia* Adopts a Greek Key β-Barrel Fold Unique amongst Plant Antimicrobial Proteins 1 1Edited by P. E. Wright. *J. Mol. Biol.* **1999**, *293* (3), 629–638. https://doi.org/10.1006/jmbi.1999.3163.

[77] Ohno, A.; Tate, S.; Seeram, S. S.; Hiraga, K.; Swindells, M. B.; Oda, K.; Kainosho, M. NMR Structure of the *Streptomyces metalloproteinase* Inhibitor, SMPI, Isolated from *Streptomyces nigrescens* TK-23: Another Example of an Ancestral Bγ-Crystallin Precursor Structure 1 1Edited by P. E. Wright. *J. Mol. Biol.* **1998**, *282* (2), 421–433. https://doi.org/10.1006/jmbi.1998.2022.

[78] Antuch, W.; Güntert, P.; Wüthrich, K. Ancestral Beta Gamma-Crystallin Precursor Structure in a Yeast Killer Toxin. *Nat. Struct. Biol.* **1996**, *3* (8), 662–665.

[79] Kazan, K.; Rusu, A.; Marcus, J. P.; Goulter, K. C.; Manners, J. M. Enhanced Quantitative Resistance to *Leptosphaeria maculans* Conferred by Expression of a Novel Antimicrobial Peptide in Canola (*Brassica napus* L.). *Mol. Breed.* **2002**, *10* (1–2), 63–70.

[80] Manners, J. M. Primitive Defence: The MiAMP1 Antimicrobial Peptide Family. *Plant Mol. Biol. Report.* **2009**, *27* (3), 237–242. https://doi.org/10.1007/s11105-008-0083-y.

[81] Tailor, R. H.; Acland, D. P.; Attenborough, S.; Cammue, B. P.; Evans, I. J.; Osborn, R. W.; Ray, J. A.; Rees, S. B.; Broekaert, W. F. A Novel Family of Small Cysteine-Rich Antimicrobial Peptides from Seed of *Impatiens balsamina* Is Derived from a Single Precursor Protein. *J. Biol. Chem.* **1997**, *272* (39), 24480–24487.

[82] Patel, S. U.; Osborn, R.; Rees, S.; Thornton, J. M. Structural Studies of *Impatiens balsamina* Antimicrobial Protein (Ib-AMP1). *Biochemistry* **1998**, *37* (4), 983–990. https://doi.org/10.1021/bi971747d.

[83] Lee, D. G.; Shin, S. Y.; Kim, D.-H.; Seo, M. Y.; Kang, J. H.; Lee, Y.; Kim, K. L.; Hahm, K.-S. Antifungal Mechanism of a Cysteine-Rich Antimicrobial Peptide, Ib-AMP1, from *Impatiens balsamina* against *Candida albicans*. *Biotechnol. Lett.* **1999**, *21* (12), 1047–1050.

[84] Thevissen, K.; François, I. E. J. A.; Sijtsma, L.; van Amerongen, A.; Schaaper, W. M. M.; Meloen, R.; Posthuma-Trumpie, T.; Broekaert, W. F.; Cammue, B. P. A. Antifungal Activity of Synthetic Peptides Derived from *Impatiens balsamina* Antimicrobial Peptides Ib-AMP1 and Ib-AMP4. *Peptides* **2005**, *26* (7), 1113–1119. https://doi.org/10.1016/j.peptides.2005.01.008.

[85] Wang, P.; Bang, J.-K.; Kim, H. J.; Kim, J.-K.; Kim, Y.; Shin, S. Y. Antimicrobial Specificity and Mechanism of Action of Disulfide-Removed Linear Analogs of the Plant-Derived Cys-Rich Antimicrobial Peptide Ib-AMP1. *Peptides* **2009**, *30* (12), 2144–2149. https://doi.org/10.1016/j.peptides.2009.09.020.

[86] Gautier, M. F.; Aleman, M. E.; Guirao, A.; Marion, D.; Joudrier, P. *Triticum aestivum* Puroindolines, Two Basic Cystine-Rich Seed Proteins: CDNA Sequence Analysis and Developmental Gene Expression. *Plant Mol. Biol.* **1994**, *25* (1), 43–57.

[87] Le Bihan, T.; Blochet, J. E.; Désormeaux, A.; Marion, D.; Pézolet, M. Determination of the Secondary Structure and Conformation of Puroindolines by Infrared and Raman Spectroscopy. *Biochemistry* **1996**, *35* (39), 12712–12722. https://doi.org/10.1021/bi960869n.

[88] Giroux, M. J.; Sripo, T.; Gerhardt, S.; Sherwood, J. Puroindolines: Their Role in Grain Hardness and Plant Defence. *Biotechnol. Genet. Eng. Rev.* **2003**, *20* (1), 277–290. https://doi.org/10.1080/02648725.2003.10648047.

[89] Bhave, M.; Morris, C. F. Molecular Genetics of Puroindolines and Related Genes: Allelic Diversity in Wheat and Other Grasses. *Plant Mol. Biol.* **2008**, *66* (3), 205–219. https://doi.org/10.1007/s11103-007-9263-7.

[90] Dhatwalia, V. K.; Sati, O. P.; Tripathi, M. K.; Kumar, A. Isolation, Characterization and Antimicrobial Activity at Diverse Dilution of Wheat Puroindoline Protein. *World J. Agric. Sci.* **2009**, *5* (3), 297–300.

[91] Zhang, J.; Martin, J. M.; Balint-Kurti, P.; Huang, L.; Giroux, M. J. The Wheat Puroindoline Genes Confer Fungal Resistance in Transgenic Corn: The Puroindolines Confer Corn SLB Resistance. *J. Phytopathol.* **2011**, *159* (3), 188–190. https://doi.org/10.1111/j.1439-0434.2010.01744.x.

[92] Douliez, J.-P.; Michon, T.; Elmorjani, K.; Marion, D. Mini Review: Structure, Biological and Technological Functions of Lipid Transfer Proteins and Indolines, the Major Lipid Binding Proteins from Cereal Kernels. *J. Cereal Sci.* **2000**, *32* (1), 1–20. https://doi.org/10.1006/jcrs.2000.0315.

[93] Morris, C. F. Puroindolines: The Molecular Genetic Basis of Wheat Grain Hardness. *Plant Mol. Biol.* **2002**, *48* (5–6), 633–647.

[94] Marion, D.; Bakan, B.; Elmorjani, K. Plant Lipid Binding Proteins: Properties and Applications. *Biotechnol. Adv.* **2007**, *25* (2), 195–197. https://doi.org/10.1016/j.biotechadv.2006.11.003.

[95] Charnet, P.; Molle, G.; Marion, D.; Rousset, M.; Lullien-Pellerin, V. Puroindolines Form Ion Channels in Biological Membranes. *Biophys. J.* **2003**, *84* (4), 2416–2426. https://doi.org/10.1016/S0006-3495(03)75046-2.

[96] Segura, A.; Moreno, M.; Madueño, F.; Molina, A.; García-Olmedo, F. Snakin-1, a Peptide from Potato That Is Active Against Plant Pathogens. *Mol. Plant. Microbe Interact.* **1999**, *12* (1), 16–23. https://doi.org/10.1094/MPMI.1999.12.1.16.

[97] Berrocal-Lobo, M.; Segura, A.; Moreno, M.; Lopez, G.; Garcia-Olmedo, F.; Molina, A. Snakin-2, an Antimicrobial Peptide from Potato Whose Gene Is Locally Induced by Wounding and Responds to Pathogen Infection. *PLANT Physiol.* **2002**, *128* (3), 951–961. https://doi.org/10.1104/pp.010685.

[98] Oliveira-Lima, M.; Benko-Iseppon, A.; Neto, J.; Rodriguez-Decuadro, S.; Kido, E.; Crovella, S.; Pandolfi, V. Snakin: Structure, Roles and Applications of a Plant Antimicrobial Peptide. *Curr. Protein Pept. Sci.* **2017**, *18* (4), 368–374. https://doi.org/10.2174/1389203717666160619183140.

[99] Mao, Z.; Zheng, J.; Wang, Y.; Chen, G.; Yang, Y.; Feng, D.; Xie, B. The New CaSn Gene Belonging to the Snakin Family Induces Resistance against Root-Knot Nematode Infection in Pepper. *Phytoparasitica* **2011**, *39* (2), 151–164. https://doi.org/10.1007/s12600-011-0149-5.

[100] Yeung, H.; Squire, C. J.; Yosaatmadja, Y.; Panjikar, S.; López, G.; Molina, A.; Baker, E. N.; Harris, P. W. R.; Brimble, M. A. Radiation Damage and Racemic Protein Crystallography Reveal the Unique Structure of the GASA/Snakin Protein Superfamily. *Angew. Chem. Int. Ed.* **2016**, *55* (28), 7930–7933. https://doi.org/10.1002/anie.201602719.

[101] Almasia, N. I.; Narhirñak, V.; Hopp, H. E.; Vazquez-Rovere, C. Isolation and Characterization of the Tissue and Development-Specific Potato Snakin-1 Promoter Inducible by Temperature and Wounding. *Electron. J. Biotechnol.* **2010**, *13* (5), 0–0. https://doi.org/10.2225/vol13-issue5-fulltext-12.

[102] Zimmermann, R.; Sakai, H.; Hochholdinger, F. The Gibberellic Acid Stimulated-Like Gene Family in Maize and Its Role in Lateral Root Development. *PLANT Physiol.* **2010**, *152* (1), 356–365. https://doi.org/10.1104/pp.109.149054.

[103] Guzmán-Rodríguez, J. J.; Ibarra-Laclette, E.; Herrera-Estrella, L.; Ochoa-Zarzosa, A.; Suárez-Rodríguez, L. M.; Rodríguez-Zapata, L. C.; Salgado-Garciglia, R.; Jimenez-Moraila, B.; López-Meza, J. E.; López-Gómez, R. Analysis of Expressed Sequence Tags (ESTs) from Avocado Seed (*Persea americana* Var. Drymifolia) Reveals Abundant Expression of the Gene Encoding the Antimicrobial Peptide Snakin. *Plant Physiol. Biochem.* **2013**, *70*, 318–324. https://doi.org/10.1016/j.plaphy.2013.05.045.

[104] Zhang, S.; Yang, C.; Peng, J.; Sun, S.; Wang, X. GASA5, a Regulator of Flowering Time and Stem Growth in *Arabidopsis thaliana*. *Plant Mol. Biol.* **2009**, *69* (6), 745–759. https://doi.org/10.1007/s11103-009-9452-7.

[105] Herbel, V.; Schäfer, H.; Wink, M. Recombinant Production of Snakin-2 (an Antimicrobial Peptide from Tomato) in *E. coli* and Analysis of Its Bioactivity. *Molecules* **2015**, *20* (8), 14889–14901. https://doi.org/10.3390/molecules200814889.

[106] Faccio, P.; Vazquez-Rovere, C.; Hopp, E.; Gonzalez, G.; Decima-Oneto, C.; Favret, E.; Paleo, A. D.; Franzone, P. Increased Tolerance to Wheat Powdery Mildew by Heterologous Constitutive Expression of the *Solanum chacoense* Snakin-1 Gene. *Czech J. Genet. Plant Breed.* **2011**, *47* (Special Issue).

[107] Furukawa, T.; Sakaguchi, N.; Shimada, H. Two OsGASR Genes, Rice GAST Homologue Genes That Are Abundant in Proliferating Tissues, Show Different Expression Patterns in Developing Panicles. *Genes Genet. Syst.* **2006**, *81* (3), 171–180.

[108] Kotilainen, null; Helariutta, null; Mehto, null; Pollanen, null; Albert, null; Elomaa, null; Teeri, null. GEG Participates in the Regulation of Cell and Organ Shape during Corolla and Carpel Development in Gerbera Hybrida. *Plant Cell* **1999**, *11* (6), 1093–1104.

[109] Roxrud, I.; Lid, S. E.; Fletcher, J. C.; Schmidt, E. D. L.; Opsahl-Sorteberg, H.-G. GASA4, One of the 14-Member *Arabidopsis* GASA Family of Small Polypeptides, Regulates Flowering and Seed Development. *Plant Cell Physiol.* **2007**, *48* (3), 471–483. https://doi.org/10.1093/pcp/pcm016.

[110] Lucau-Danila, A.; Laborde, L.; Legrand, S.; Huot, L.; Hot, D.; Lemoine, Y.; Hilbert, J.-L.; Hawkins, S.; Quillet, M.-C.; Hendriks, T.; et al. Identification of Novel Genes Potentially Involved in Somatic Embryogenesis in Chicory (*Cichorium intybus* L.). *BMC Plant Biol.* **2010**, *10* (1), 122. https://doi.org/10.1186/1471-2229-10-122.

[111] Craik, D. J.; Daly, N. L.; Bond, T.; Waine, C. Plant Cyclotides: A Unique Family of Cyclic and Knotted Proteins That Defines the Cyclic Cystine Knot Structural Motif. *J. Mol. Biol.* **1999**, *294* (5), 1327–1336. https://doi.org/10.1006/jmbi.1999.3383.

[112] da Silva Lima, S. C. B.; Benko-Iseppon, A. M.; Bezerra-Neto, J. P.; Amorim, L. L. B.; Neto, J. R. C. F.; Crovella, S.; Pandolfi, V. Plants Defense-Related Cyclic Peptides: Diversity, Structure and Applications. *Curr. Protein Pept. Sci.* **2016**.

[113] Weidmann, J.; Craik, D. J. Discovery, Structure, Function, and Applications of Cyclotides: Circular Proteins from Plants. *J. Exp. Bot.* **2016**, *67* (16), 4801–4812. https://doi.org/10.1093/jxb/erw210.

[114] Park, S.; Yoo, K.-O.; Marcussen, T.; Backlund, A.; Jacobsson, E.; Rosengren, K. J.; Doo, I.; Göransson, U. Cyclotide Evolution: Insights from the Analyses of Their Precursor Sequences, Structures and Distribution in Violets (Viola). *Front. Plant Sci.* **2017**, *8*. https://doi.org/10.3389/fpls.2017.02058.

[115] Gran, L. On the Effect of a Polypeptide Isolated from "Kalata-Kalata" (*Oldenlandia affinis* DC) on the Oestrogen Dominated Uterus. *Acta Pharmacol. Toxicol. (Copenh.)* **1973**, *33* (5), 400–408.

[116] Claeson, P.; Göransson, U.; Johansson, S.; Luijendijk, T.; Bohlin, L. Fractionation Protocol for the Isolation of Polypeptides from Plant Biomass. *J. Nat. Prod.* **1998**, *61* (1), 77–81. https://doi.org/10.1021/np970342r.

[117] Poth, A. G.; Colgrave, M. L.; Philip, R.; Kerenga, B.; Daly, N. L.; Anderson, M. A.; Craik, D. J. Discovery of Cyclotides in the Fabaceae Plant Family Provides New Insights into the Cyclization, Evolution, and Distribution of Circular Proteins. *ACS Chem. Biol.* **2011**, *6* (4), 345–355. https://doi.org/10.1021/cb100388j.

[118] Poth, A. G.; Mylne, J. S.; Grassl, J.; Lyons, R. E.; Millar, A. H.; Colgrave, M. L.; Craik, D. J. Cyclotides Associate with Leaf Vasculature and Are the Products of a Novel Precursor in *Petunia* (Solanaceae). *J. Biol. Chem.* **2012**, *287* (32), 27033–27046. https://doi.org/10.1074/jbc.M112.370841.

[119] Nguyen, G. K. T.; Zhang, S.; Nguyen, N. T. K.; Nguyen, P. Q. T.; Chiu, M. S.; Hardjojo, A.; Tam, J. P. Discovery and Characterization of Novel Cyclotides Originated from Chimeric Precursors Consisting of Albumin-1 Chain a and Cyclotide Domains in the Fabaceae Family. *J. Biol. Chem.* **2011**, *286* (27), 24275–24287. https://doi.org/10.1074/jbc.M111.229922.

[120] Pranting, M.; Loov, C.; Burman, R.; Goransson, U.; Andersson, D. I. The Cyclotide Cycloviolacin O2 from Viola Odorata Has Potent Bactericidal Activity against Gram-Negative Bacteria. *J. Antimicrob. Chemother.* **2010**, *65* (9), 1964–1971. https://doi.org/10.1093/jac/dkq220.

[121] Lindholm, P.; Göransson, U.; Johansson, S.; Claeson, P.; Gullbo, J.; Larsson, R.; Bohlin, L.; Backlund, A. Cyclotides: A Novel Type of Cytotoxic Agents 1 P. L. and U. G. Contributed Equally to This Manuscript. *Mol. Cancer Ther.* **2002**, *1* (6), 365.

[122] Craik, D. J.; Clark, R. J.; Daly, N. L. Potential Therapeutic Applications of the Cyclotides and

Related Cystine Knot Mini-Proteins. *Expert Opin. Investig. Drugs* **2007**, *16* (5), 595–604. https://doi.org/10.1517/13543784.16.5.595.

[123] Northfield, S. E.; Wang, C. K.; Schroeder, C. I.; Durek, T.; Kan, M.-W.; Swedberg, J. E.; Craik, D. J. Disulfide-Rich Macrocyclic Peptides as Templates in Drug Design. *Eur. J. Med. Chem.* **2014**, *77*, 248–257. https://doi.org/10.1016/j.ejmech.2014.03.011.

[124] Liu, J.-J.; Sturrock, R.; Ekramoddoullah, A. K. M. The Superfamily of Thaumatin-like Proteins: Its Origin, Evolution, and Expression towards Biological Function. *Plant Cell Rep.* **2010**, *29* (5), 419–436. https://doi.org/10.1007/s00299-010-0826-8.

[125] Grenier, J.; Potvin, C.; Asselin, A. Some Fungi Express Beta-1,3-Glucanases Similar to Thaumatin-like Proteins. *Mycologia* **2000**, *92* (5), 841. https://doi.org/10.2307/3761579.

[126] Sakamoto, Y.; Watanabe, H.; Nagai, M.; Nakade, K.; Takahashi, M. Lentinula Edode s Tlg1 Encodes a Thaumatin-Like Protein That Is Involved in Lentinan Degradation and Fruiting Body Senescence. **2006**, *141* (June), 793–801. https://doi.org/10.1104/pp.106.076679.ture.

[127] Brandazza, A.; Angeli, S.; Tegoni, M.; Cambillau, C.; Pelosi, P. Plant Stress Proteins of the Thaumatin-like Family Discovered in Animals. **2004**, *572*, 3–7. https://doi.org/10.1016/j.febslet.2004.07.003.

[128] Kitajima, S.; Sato, F. Plant Pathogenesis-Related Proteins : Molecular Mechanisms of Gene Expression and Protein Function 1. **1999**, *8*, 1–8.

[129] Batalia, M. A.; Monzingo, A. F.; Ernst, S.; Roberts, W.; Robertus, J. D. The Crystal Structure of the Antifungal Protein Zeamatin, a Member of the Thaumatin-like, PR-5 Protein Family. *Nat. Struct. Biol.* **1996**, *3* (1), 19–23. https://doi.org/10.1038/nsb0196-19.

[130] Trudel, J.; Grenier, J.; Potvin, C.; Asselin, A. Several Thaumatin-like Proteins Bind to Beta-1,3-Glucans. *Plant Physiol.* **1998**, *118* (4), 1431–1438.

[131] Grenier, J.; Potvin, C.; Trudel, J.; Asselin, A. Some Thaumatin-like Proteins Hydrolyse Polymeric β-1,3-Glucans. *Plant J.* **1999**, *19* (4), 473–480. https://doi.org/10.1046/j.1365-313X.1999.00551.x.

[132] Fierens, E.; Rombouts, S.; Gebruers, K.; Goesaert, H.; Brijs, K.; Beaugrand, J.; Volckaert, G.; Campenhout, S. V. A. N.; Proost, P.; Courtin, C. M.; et al. TLXI , a Novel Type of Xylanase Inhibitor from Wheat (*Triticum aestivum*) Belonging to the Thaumatin Family. **2007**, *591*, 583–591. https://doi.org/10.1042/BJ20061291.

[133] Rourke, R. S.; Richardson, M. Zeamatin Inhibits Trypsin and -Amylase Activities. **2001**, *67* (5), 2365–2366. https://doi.org/10.1128/AEM.67.5.2365.

[134] Jung, Y. C.; Lee, H. J.; Yum, S. S.; Soh, W. Y.; Cho, D. Y.; Auh, C. K.; Lee, T. K.; Soh, H. C.; Kim, Y. S.; Lee, S. C. Drought-Inducible - but ABA-Independent - Thaumatin-like Protein from Carrot (*Daucus carota* L.). *Plant Cell Rep.* **2005**, *24* (6), 366–373. https://doi.org/10.1007/s00299-005-0944-x.

[135] Ruperti, B.; Cattivelli, L.; Pagni, S.; Ramina, A. Ethylene-Responsive Genes Are Differentially Regulated during Abscission, Organ Senescence and Wounding in Peach (*Prunus persica*). *J. Exp. Bot.* **2002**, *53* (368), 429–37. https://doi.org/10.1093/jexbot/53.368.429.

[136] Hon, W. C.; Griffith, M.; Mlynarz, A.; Kwok, Y. C.; Yang, D. Antifreeze Proteins in Winter Rye Are Similar to Pathogenesis-Related Proteins. *Plant Physiol.* **1995**, *109* (3), 879–889. https://doi.org/10.1104/pp.109.3.879.

[137] Kumar, K. R. R.; Kirti, P. B. Differential Gene Expression in Arachis Diogoi upon Interaction with Peanut Late Leaf Spot Pathogen, *Phaeoisariopsis personata* and Characterization of a Pathogen Induced Cyclophilin. *Plant Mol. Biol.* **2011**, *75* (4–5), 497–513. https://doi.org/10.1007/s11103-011-

9747-3.

[138] Rudd, J. J.; Kanyuka, K.; Hassani-pak, K.; Derbyshire, M.; Andongabo, A.; Devonshire, J.; Lysenko, A.; Saqi, M.; Desai, N. M.; Powers, S. J.; et al. Transcriptome and Metabolite Pro Fi Ling of the Infection Cycle of *Zymoseptoria tritici* on Wheat Reveals a Biphasic Interaction with Plant Immunity Involving Differential Pathogen Chromosomal Contributions and a Variation on the Hemibiotrophic Lifestyle D. **2015**, *167* (March), 1158–1185. https://doi.org/10.1104/pp.114.255927.

[139] Breiteneder, H.; Radauer, C. A Classification of Plant Food Allergens☆. *J. Allergy Clin. Immunol.* **2004**, *113* (5), 821–830. https://doi.org/10.1016/j.jaci.2004.01.779.

[140] Jami, S. K.; Anuradha, T. S.; Guruprasad, L. Molecular , Biochemical and Structural Characterization of Osmotin-like Protein from Black Nightshade (*Solanum nigrum*). **2007**, *164*, 238–252. https://doi.org/10.1016/j.jplph.2006.01.006.

[141] Tachi, H.; Fukuda-Yamada, K.; Kojima, T.; Shiraiwa, M.; Takahara, H. Molecular Characterization of a Novel Soybean Gene Encoding a Neutral PR-5 Protein Induced by High-Salt Stress. *Plant Physiol. Biochem.* **2009**, *47* (1), 73–79. https://doi.org/10.1016/j.plaphy.2008.09.012.

[142] Breiteneder, H. Thaumatin-like Proteins – a New Family of Pollen and Fruit Allergens. **2004**, No. 16, 479–481.

[143] Fierens, E.; Gebruers, K.; Voet, A. R. D.; Maeyer, M. D. E.; Courtin, C. M.; Delcour, J. A. N. A. Biochemical and Structural Characterization of TLXI , the *Triticum aestivum* L . Thaumatin-like Xylanase Inhibitor. **2009**, *24* (June), 646–654. https://doi.org/10.1080/14756360802321831.

[144] Leone, P.; Menu-Bouaouiche, L.; Peumans, W. J.; Payan, F.; Barre, A.; Roussel, A.; Van Damme, E. J. M.; Rougé, P. Resolution of the Structure of the Allergenic and Antifungal Banana Fruit Thaumatin-like Protein at 1.7-\AA. *Biochimie* **2006**, *88* (1), 45–52. https://doi.org/10.1016/j.biochi.2005.07.001.

[145] Koiwa, H.; Kato, H.; Nakatsu, T.; Oda, J.; Yamada, Y.; Sato, F. Crystal Structure of Tobacco PR-5d Protein at 1.8 \AA Resolution Reveals a Conserved Acidic Cleft Structure in Antifungal Thaumatin-like Proteins 1 1Edited by R. Huber. *J. Mol. Biol.* **1999**, *286* (4), 1137–1145. https://doi.org/10.1006/jmbi.1998.2540.

[146] Min, K.; Ha, S. C.; Hasegawa, P. M.; Bressan, R. A.; Yun, D.; Kim, K. K. Crystal Structure of Osmotin , a Plant Antifungal Protein. **2004**, *173* (June 2003), 170–173.

[147] Ogata, C. M.; Gordon, P. F.; Vos, M. D. Crystal Structure of a Swe + Tasting Protein Thaumatin I , at 165 A Resolution. **1992**, 893–908.

[148] Antonia, Y. D. Crystallization Communications Crystallization and Preliminary Structure Determination of the Plant Food Allergen Pru Av 2 Crystallization Communications. **2005**, 186–188. https://doi.org/10.1107/S1744309104033822.

[149] Osherov, N.; Mathew, J.; Romans, A.; May, G. S. Identification of Conidial-Enriched Transcripts in *Aspergillus nidulans* Using Suppression Subtractive Hybridization. **2002**, *37*, 197–204.

[150] Greenstein, S.; Shadkchan, Y.; Jadoun, J.; Sharon, C.; Markovich, S.; Osherov, N. Analysis of the *Aspergillus nidulans* Thaumatin-like CetA Gene and Evidence for Transcriptional Repression of Pyr4 Expression in the CetA -Disrupted Strain. **2006**, *43*, 42–53. https://doi.org/10.1016/j.fgb.2005.10.001.

[151] Wang, X.; Zafian, P.; Choudhary, M.; Lawton, M. The PR5K Receptor Protein Kinase from *Arabidopsis thaliana* Is Structurally Related to a Family of Plant Defense Proteins. **1996**, *93* (March), 2598–2602.

[152] Apweiler, R. UniProt: The Universal Protein Knowledgebase. *Nucleic Acids Res.* **2004**, *32* (90001), 115D – 119. https://doi.org/10.1093/nar/gkh131.

[153] Torrent, M.; Victoria Nogues, M.; Boix, E. Discovering New *In Silico* Tools for Antimicrobial Peptide Prediction. *Curr. Drug Targets* **2012**, *13* (9), 1148–1157. https://doi.org/10.2174/138945012802002311.

[154] Porto, W. F.; Pires, A. S.; Franco, O. L. Computational Tools for Exploring Sequence Databases as a Resource for Antimicrobial Peptides. *Biotechnol. Adv.* **2017**, *35* (3), 337–349. https://doi.org/10.1016/j.biotechadv.2017.02.001.

[155] Thomas, S.; Karnik, S.; Barai, R. S.; Jayaraman, V. K.; Idicula-Thomas, S. CAMP: A Useful Resource for Research on Antimicrobial Peptides. *Nucleic Acids Res.* **2010**, *38* (suppl_1), D774–D780. https://doi.org/10.1093/nar/gkp1021.

[156] Waghu, F. H.; Gopi, L.; Barai, R. S.; Ramteke, P.; Nizami, B.; Idicula-Thomas, S. CAMP: Collection of Sequences and Structures of Antimicrobial Peptides. *Nucleic Acids Res.* **2014**, *42* (D1), D1154–D1158. https://doi.org/10.1093/nar/gkt1157.

[157] Waghu, F. H.; Barai, R. S.; Gurung, P.; Idicula-Thomas, S. CAMP $_{R3}$: A Database on Sequences, Structures and Signatures of Antimicrobial Peptides: Table 1. *Nucleic Acids Res.* **2016**, *44* (D1), D1094–D1097. https://doi.org/10.1093/nar/gkv1051.

[158] Wang, Z. APD: The Antimicrobial Peptide Database. *Nucleic Acids Res.* **2004**, *32* (90001), 590D – 592. https://doi.org/10.1093/nar/gkh025.

[159] Wang, G.; Li, X.; Wang, Z. APD3: The Antimicrobial Peptide Database as a Tool for Research and Education. *Nucleic Acids Res.* **2016**, *44* (D1), D1087–D1093. https://doi.org/10.1093/nar/gkv1278.

[160] Zhao, X.; Wu, H.; Lu, H.; Li, G.; Huang, Q. LAMP: A Database Linking Antimicrobial Peptides. *Plos One* **2013**, *8* (6), e66557. https://doi.org/10.1371/journal.pone.0066557.

[161] Gogoladze, G.; Grigolava, M.; Vishnepolsky, B.; Chubinidze, M.; Duroux, P.; Lefranc, M.-P.; Pirtskhalava, M. DBAASP : Database of Antimicrobial Activity and Structure of Peptides. *FEMS Microbiol. Lett.* **2014**, *357* (1), 63–68. https://doi.org/10.1111/1574-6968.12489.

[162] Jorge, P.; Lourenço, A.; Pereira, M. O. New Trends in Peptide-Based Anti-Biofilm Strategies: A Review of Recent Achievements and Bioinformatic Approaches. *Biofouling* **2012**, *28* (10), 1033–1061. https://doi.org/10.1080/08927014.2012.728210.

[163] Lee, H.-T.; Lee, C.-C.; Yang, J.-R.; Lai, J. Z. C.; Chang, K. Y. A Large-Scale Structural Classification of Antimicrobial Peptides. *BioMed Res. Int.* **2015**, *2015*, 1–6. https://doi.org/10.1155/2015/475062.

[164] Seebah, S.; Suresh, A.; Zhuo, S.; Choong, Y. H.; Chua, H.; Chuon, D.; Beuerman, R.; Verma, C. Defensins Knowledgebase: A Manually Curated Database and Information Source Focused on the Defensins Family of Antimicrobial Peptides. *Nucleic Acids Res.* **2007**, *35* (Database), D265–D268. https://doi.org/10.1093/nar/gkl866.

[165] Liu, S.; Fan, L.; Sun, J.; Lao, X.; Zheng, H. Computational Resources and Tools for Antimicrobial Peptides: Computational Resources and Tools for Antimicrobial Peptides. *J. Pept. Sci.* **2017**, *23* (1), 4–12. https://doi.org/10.1002/psc.2947.

[166] Mulvenna, J. P. CyBase: A Database of Cyclic Protein Sequence and Structure. *Nucleic Acids Res.* **2006**, *34* (90001), D192–D194. https://doi.org/10.1093/nar/gkj005.

[167] Hammami, R.; Ben Hamida, J.; Vergoten, G.; Fliss, I. PhytAMP: A Database Dedicated to Antimicrobial Plant Peptides. *Nucleic Acids Res.* **2009**, *37* (Database), D963–D968. https://doi.org/10.1093/nar/gkn655.

[168] Eltabakh, M. Y.; Ouzzani, M.; Aref, W. G. BDBMS--a Database Management System for Biological Data. *ArXiv Prepr. Cs0612127* **2006**.

[169] Mehboob-ur-Rahman, T. S.; Mahmood-ur-Rahman, M. A. I.; Zafar, Y. Bioinformatics: A Way Forward to Explore “Plant Omics.” *Bioinforma.-Updat. Featur. Appl.* **2016**, 203.

[170] Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic Local Alignment Search Tool. *J. Mol. Biol.* **1990**, *215* (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

[171] Pearson, W. R. [5] Rapid and Sensitive Sequence Comparison with FASTP and FASTA. In *Methods in Enzymology*; Elsevier, 1990; Vol. 183, pp 63–98. https://doi.org/10.1016/0076-6879(90)83007-V.

[172] Polyanovsky, V. O.; Roytberg, M. A.; Tumanyan, V. G. Comparative Analysis of the Quality of a Global Algorithm and a Local Algorithm for Alignment of Two Sequences. *Algorithms Mol. Biol.* **2011**, *6* (1), 25. https://doi.org/10.1186/1748-7188-6-25.

[173] Thompson, K. Programming Techniques: Regular Expression Search Algorithm. *Commun. ACM* **1968**, *11* (6), 419–422. https://doi.org/10.1145/363347.363387.

[174] Eddy, S. R. Profile Hidden Markov Models. *Bioinforma. Oxf. Engl.* **1998**, *14* (9), 755–763.

[175] Yoon, B.-J. Hidden Markov Models and Their Applications in Biological Sequence Analysis. *Curr. Genomics* **2009**, *10* (6), 402–415. https://doi.org/10.2174/138920209789177575.

[176] Zhou, P.; Yang, C.; Ren, Y.; Wang, C.; Tian, F. What Are the Ideal Properties for Functional Food Peptides with Antihypertensive Effect? A Computational Peptidology Approach. *Food Chem.* **2013**, *141* (3), 2967–2973. https://doi.org/10.1016/j.foodchem.2013.05.140.

[177] *Computational Peptidology*; Zhou, P., Huang, J., Eds.; Methods in Molecular Biology; Springer New York: New York, NY, 2015; Vol. 1268. https://doi.org/10.1007/978-1-4939-2285-7.

[178] Niarchou, A.; Alexandridou, A.; Athanasiadis, E.; Spyrou, G. C-PAmP: Large Scale Analysis and Database Construction Containing High Scoring Computationally Predicted Antimicrobial Peptides for All the Available Plant Species. *Plos One* **2013**, *8* (11), e79728. https://doi.org/10.1371/journal.pone.0079728.

[179] Lin, Y.; Jeon, Y. Random Forests and Adaptive Nearest Neighbors. *J. Am. Stat. Assoc.* **2006**, *101* (474), 578–590. https://doi.org/10.1198/016214505000001230.

[180] Karasuyama, M.; Takeuchi, I. Multiple Incremental Decremental Learning of Support Vector Machines. *IEEE Trans. Neural Netw.* **2010**, *21* (7), 1048–1059. https://doi.org/10.1109/TNN.2010.2048039.

[181] Ding, S.; Li, H.; Su, C.; Yu, J.; Jin, F. Evolutionary Artificial Neural Networks: A Review. *Artif. Intell. Rev.* **2013**, *39* (3), 251–260. https://doi.org/10.1007/s10462-011-9270-6.

[182] Dobslaff, K.; Kreisig, T.; Berthold, N.; Hoffmann, R.; Zuchner, T. Novel Peptide–Protein Assay for Identification of Antimicrobial Peptides by Fluorescence Quenching. *Anal. Bioanal. Chem.* **2012**, *403* (9), 2725–2731. https://doi.org/10.1007/s00216-012-6050-3.

[183] Fedders, H.; Leippe, M. A Reverse Search for Antimicrobial Peptides in Ciona Intestinalis: Identification of a Gene Family Expressed in Hemocytes and Evaluation of Activity. *Dev. Comp. Immunol.* **2008**, *32* (3), 286–298. https://doi.org/10.1016/j.dci.2007.06.003.

[184] Tennessen, J. A. Positive Selection Drives a Correlation between Non-Synonymous/ Synonymous Divergence and Functional Divergence. *Bioinformatics* **2008**, *24* (12), 1421–1425. https://doi.org/10.1093/bioinformatics/btn205.

[185] Wilkins, M. R.; Sanchez, J. C.; Gooley, A. A.; Appel, R. D.; Humphery-Smith, I.; Hochstrasser, D. F.; Williams, K. L. Progress with Proteome Projects: Why All Proteins Expressed by a Genome Should Be Identified and How to Do It. *Biotechnol. Genet. Eng. Rev.* **1996**, *13* (1), 19–50. https://doi.org/10.1080/02648725.1996.10647923.

[186] Wasinger, V. C.; Corthals, G. L. Proteomic Tools for Biomedicine. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* **2002**, *771* (1–2), 33–48. https://doi.org/10.1016/S1570-0232(02)00125-3.

[187] Sheoran, I. S.; Ross, A. R. S.; Olson, D. J. H.; Sawhney, V. K. Compatibility of Plant Protein Extraction Methods with Mass Spectrometry for Proteome Analysis. *Plant Sci.* **2009**, *176* (1), 99–104. https://doi.org/10.1016/j.plantsci.2008.09.015.

[188] Thompson, J. D.; Schaeffer-Reiss, C.; Ueffing, M. *Functional Proteomics : Methods and Protocols*; 2008.

[189] Cho, W. C. S. Proteomics Technologies and Challenges. *Genomics Proteomics Bioinformatics* **2007**, *5* (2), 77–85. https://doi.org/10.1016/S1672-0229(07)60018-7.

[190] Baracat-Pereira, M. C.; de Oliveira Barbosa, M.; Magalhães, M. J.; Carrijo, L. C.; Games, P. D.; Almeida, H. O.; Netto, J. F. S.; Pereira, M. R.; de Barros, E. G. Separomics Applied to the Proteomics and Peptidomics of Low-Abundance Proteins: Choice of Methods and Challenges - A Review. *Genet. Mol. Biol.* **2012**, *35* (SUPPL.1), 283–291. https://doi.org/10.1590/S1415-47572012000200009.

[191] Kolodziejek, I.; van der Hoorn, R. A. Mining the Active Proteome in Plant Science and Biotechnology. *Curr. Opin. Biotechnol.* **2010**, *21* (2), 225–233. https://doi.org/10.1016/j.copbio.2010.02.003.

[192] Al Akeel, R.; Mateen, A.; Syed, R.; Alyousef, A. A.; Shaik, M. R. Screening, Purification and Characterization of Anionic Antimicrobial Proteins from *Foeniculum vulgare*. *Molecules* **2017**, *22* (4). https://doi.org/10.3390/molecules22040602.

[193] Menschaert, G.; Vandekerckhove, T. T. M. Peptidomics Coming of Age : A Review of Contributions from a Bioinf ... Peptidomics Coming of Age : A Review of Contributions from a Bioinf ... Peptidomics Coming of Age : A Review of Contributions from a Bioinformatics Angle. **2010**, 9–10. https://doi.org/10.1021/pr900929m.

[194] Nägele, E.; Vollmer, M.; Hörth, P.; Vad, C. 2D-LC/MS Techniques for the Identification of Proteins in Highly Complex Mixtures. *Expert Rev. Proteomics* **2004**, *1* (1), 37–46. https://doi.org/10.1586/14789450.1.1.37.

[195] Mitulovic, G.; Mechtler, K. HPLC Techniques for Proteomics Analysis--a Short Overview of Latest Developments. *Brief. Funct. Genomic. Proteomic.* **2006**, *5* (4), 249–260. https://doi.org/10.1093/bfgp/ell034.

[196] Blueggel, M.; Chamrad, D.; Meyer, H. E. Bioinformatics in Proteomics. *Curr. Pharm. Biotechnol.* **2004**, *5* (1), 79–88.

[197] McHugh, L.; Arthur, J. W. Computational Methods for Protein Identification from Mass Spectrometry Data. *PLoS Comput. Biol.* **2008**, *4* (2). https://doi.org/10.1371/journal.pcbi.0040012.

[198] Hughes, C.; Ma, B.; Lajoie, G. A. De Novo Sequencing Methods in Proteomics. In *Proteome Bioinformatics*; Hubbard, S. J., Jones, A. R., Eds.; Humana Press: Totowa, NJ, 2010; Vol. 604, pp 105–121. https://doi.org/10.1007/978-1-60761-444-9_8.

[199] Eng, J. K.; Fischer, B.; Grossmann, J.; Maccoss, M. J. A Fast SEQUEST Cross Correlation Algorithm. **2008**, 4598–4602.

[200] Perkins, D. N.; Pappin, D. J. C.; Creasy, D. M.; Cottrell, J. S. Probability-Based Protein Identification by Searching Sequence Databases Using Mass Spectrometry Data. *Electrophoresis* **1999**, *20* (18), 3551–3567. https://doi.org/10.1002/(sici)1522-2683(19991201)20:18<3551::aid-elps3551>3.0.co;2-2.

[201] Tsai, W.-C.; Zhuang, Z.-J.; Lin, C.-Y.; Chen, W.-J. Novel antimicrobial peptides showed promising activity against multidrug resistant *Salmonella enterica* serovar Choleraesuis and its stress

response mechanism revealed. *J. Appl. Microbiol.* **2016**, *121* (4), 952–965. https://doi.org/10.1111/jam.13203.

[202] Umadevi, P.; Soumya, M.; George, J. K.; Anandaraj, M. Proteomics Assisted Profiling of Antimicrobial Peptide Signatures from Black Pepper (*Piper nigrum* L.). *Physiol. Mol. Biol. Plants* **2018**, *24* (3), 379–387. https://doi.org/10.1007/s12298-018-0524-5.

[203] Waghu, F. H.; Barai, R. S.; Gurung, P.; Idicula-Thomas, S. CAMPR3: A Database on Sequences, Structures and Signatures of Antimicrobial Peptides. *Nucleic Acids Res.* **2016**, *44* (D1), D1094–D1097. https://doi.org/10.1093/nar/gkv1051.

[204] Gasteiger, E.; Hoogland, C.; Gattiker, a; Duvaud, S.; Wilkins, M.; Appel, R.; Bairoch, A. Protein Identification and Analysis Tools on the ExPASy Server. *Proteomics Protoc. Handb.* **2005**, 571–607. https://doi.org/10.1385/1-59259-890-0:571.

[205] Craig, R.; Beavis, R. C. TANDEM: Matching Proteins with Tandem Mass Spectra. *Bioinformatics* **2004**, *20* (9), 1466–1467. https://doi.org/10.1093/bioinformatics/bth092.

[206] Geer, L. Y.; Markey, S. P.; Kowalak, J. A.; Wagner, L.; Xu, M.; Maynard, D. M.; Yang, X.; Shi, W.; Bryant, S. H. Open Mass Spectrometry Search Algorithm. *J. Proteome Res.* **2004**, *3* (5), 958–964. https://doi.org/10.1021/pr0499491.

[207] Zhang, N.; Aebersold, R.; Schwikowski, B. ProbID: A Probabilistic Algorithm to Identify Peptides through Sequence Database Searching Using Tandem Mass Spectral Data. *Proteomics* **2002**, *2* (10), 1406–1412. https://doi.org/10.1002/1615-9861(200210)2:10<1406::AID-PROT1406>3.0.CO;2-9.

[208] Field, H. I.; Fenyö, D.; Beavis, R. C. RADARS, a Bioinformatics Solution That Automates Proteome Mass Spectral Analysis, Optimises Protein Identification, and Archives Data in a Relational Database. *Proteomics* **2002**, *2* (1), 36–47. https://doi.org/10.1002/1615-9861(200201)2:1<36::AID-PROT36>3.0.CO;2-W.

[209] Nesvizhskii, A. I.; Vitek, O.; Aebersold, R. Analysis and Validation of Proteomic Data Generated by Tandem Mass Spectrometry. *Nat Methods* **2007**, *4*, 787–797.

[210] Gazzana, G.; Borlak, J. Improved Method for Proteome Mapping of the Liver by 2-DE MALDI-TOF MS. *J. Proteome Res.* **2007**, *6* (8), 3143–3151. https://doi.org/10.1021/pr070097p.

[211] Pestana-Calsa, M. C.; Ribeiro, I. L.; Calsa Jr., T. Bioinformatics-Coupled Molecular Approaches for Unravelling Potential Antimicrobial Peptides Coding Genes in Brazilian Native and Crop Plant Species. *Curr. Protein Pept. Sci.* **2010**, *11* (3), 199–209. https://doi.org/CPPS-38 [pii].

[212] Weiner, A. K.; Sidoli, S.; Diskin, S. J.; Garcia, B. GiaPronto: a one-click graph visualization software for proteomics datasets. *Mol. Cell. Proteomics* **2017**, mcp.TIR117.000438. https://doi.org/10.1074/mcp.TIR117.000438.

[213] Jothi, A. Principles, Challenges and Advances in Ab Initio Protein Structure Prediction. *Protein Pept. Lett.* **2012**, *19* (11), 1194–1204.

[214] Kaufmann, K. W.; Lemmon, G. H.; DeLuca, S. L.; Sheehan, J. H.; Meiler, J. Practically Useful: What the ROSETTA Protein Modeling Suite Can Do for You. *Biochemistry* **2010**, *49* (14), 2987–2998. https://doi.org/10.1021/bi902153g.

[215] Moult, J.; Fidelis, K.; Kryshtafovych, A.; Schwede, T.; Tramontano, A. Critical Assessment of Methods of Protein Structure Prediction (CASP)-Round XII. *Proteins Struct. Funct. Bioinforma.* **2018**, *86*, 7–15. https://doi.org/10.1002/prot.25415.

[216] Källberg, M.; Wang, H.; Wang, S.; Peng, J.; Wang, Z.; Lu, H.; Xu, J. Template-Based Protein Structure Modeling Using the RaptorX Web Server. *Nat. Protoc.* **2012**, *7* (8), 1511–1522. https://doi.org/10.1038/nprot.2012.085.

[217] Martí-Renom, M. A.; Stuart, A. C.; Fiser, A.; Sánchez, R.; Melo, F.; Šali, A. Comparative Protein Structure Modeling of Genes and Genomes. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29* (1), 291–325. https://doi.org/10.1146/annurev.biophys.29.1.291.

[218] Biasini, M.; Bienert, S.; Waterhouse, A.; Arnold, K.; Studer, G.; Schmidt, T.; Kiefer, F.; Cassarino, T. G.; Bertoni, M.; Bordoli, L.; et al. SWISS-MODEL: Modelling Protein Tertiary and Quaternary Structure Using Evolutionary Information. *Nucleic Acids Res.* **2014**, *42* (W1), W252–W258. https://doi.org/10.1093/nar/gku340.

[219] Webb, B.; Sali, A. Comparative Protein Structure Modeling Using MODELLER: Comparative Protein Structure Modeling Using Modeller. In *Current Protocols in Bioinformatics*; Bateman, A., Pearson, W. R., Stein, L. D., Stormo, G. D., Yates, J. R., Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2016; pp 5.6.1-5.6.37. https://doi.org/10.1002/cpbi.3.

[220] Wu, S.; Zhang, Y. MUSTER: Improving Protein Sequence Profile-Profile Alignments by Using Multiple Sources of Structure Information. *Proteins Struct. Funct. Bioinforma.* **2008**, *72* (2), 547–556. https://doi.org/10.1002/prot.21945.

[221] Yang, Y.; Faraggi, E.; Zhao, H.; Zhou, Y. Improving Protein Fold Recognition and Template-Based Modeling by Employing Probabilistic-Based Matching between Predicted One-Dimensional Structural Properties of Query and Corresponding Native Properties of Templates. *Bioinformatics* **2011**, *27* (15), 2076–2082. https://doi.org/10.1093/bioinformatics/btr350.

[222] Wiederstein, M.; Sippl, M. J. ProSA-Web: Interactive Web Service for the Recognition of Errors in Three-Dimensional Structures of Proteins. *Nucleic Acids Res.* **2007**, *35* (Web Server), W407–W410. https://doi.org/10.1093/nar/gkm290.

[223] Zhang, Y. I-TASSER Server for Protein 3D Structure Prediction. *BMC Bioinformatics* **2008**, *9* (1), 40. https://doi.org/10.1186/1471-2105-9-40.

[224] Zhang, Y.; Kolinski, A.; Skolnick, J. TOUCHSTONE II: A New Approach to *Ab Initio* Protein Structure Prediction. *Biophys. J.* **2003**, *85* (2), 1145–1164. https://doi.org/10.1016/S0006-3495(03)74551-2.

[225] Wu, S.; Skolnick, J.; Zhang, Y. Ab Initio Modeling of Small Proteins by Iterative TASSER Simulations. *BMC Biol.* **2007**, *5* (1), 17. https://doi.org/10.1186/1741-7007-5-17.

[226] Haas, J.; Barbato, A.; Behringer, D.; Studer, G.; Roth, S.; Bertoni, M.; Mostaguir, K.; Gumienny, R.; Schwede, T. Continuous Automated Model EvaluatiOn (CAMEO) Complementing the Critical Assessment of Structure Prediction in CASP12. *Proteins Struct. Funct. Bioinforma.* **2018**, *86*, 387–398. https://doi.org/10.1002/prot.25431.

[227] Moult, J.; Fidelis, K.; Kryshtafovych, A.; Schwede, T.; Tramontano, A. Critical Assessment of Methods of Protein Structure Prediction: Progress and New Directions in Round XI: Progress in CASP XI. *Proteins Struct. Funct. Bioinforma.* **2016**, *84*, 4–14. https://doi.org/10.1002/prot.25064.

[228] Robustelli, P.; Piana, S.; Shaw, D. E. Developing a Molecular Dynamics Force Field for Both Folded and Disordered Protein States. *Proc. Natl. Acad. Sci.* **2018**, *115* (21), E4758–E4766. https://doi.org/10.1073/pnas.1800690115.

[229] Freddolino, P. L.; Harrison, C. B.; Liu, Y.; Schulten, K. Challenges in Protein-Folding Simulations. *Nat. Phys.* **2010**, *6* (10), 751–758. https://doi.org/10.1038/nphys1713.

[230] Karplus, M.; McCammon, J. A. Molecular Dynamics Simulations of Biomolecules. *Nat. Struct. Biol.* **2002**, *9* (9), 646–652. https://doi.org/10.1038/nsb0902-646.

[231] Mitsutake, A.; Takano, H. Relaxation Mode Analysis for Molecular Dynamics Simulations of Proteins. *Biophys. Rev.* **2018**, *10* (2), 375–389. https://doi.org/10.1007/s12551-018-0406-7.

[232] Hess, B.; Kutzner, C.; van der Spoel, D.; Lindahl, E. GROMACS 4: Algorithms for Highly

Efficient, Load-Balanced, and Scalable Molecular Simulation. *J. Chem. Theory Comput.* **2008**, *4* (3), 435–447. https://doi.org/10.1021/ct700301q.

[233] Pronk, S.; Páll, S.; Schulz, R.; Larsson, P.; Bjelkmar, P.; Apostolov, R.; Shirts, M. R.; Smith, J. C.; Kasson, P. M.; van der Spoel, D.; et al. GROMACS 4.5: A High-Throughput and Highly Parallel Open Source Molecular Simulation Toolkit. *Bioinformatics* **2013**, *29* (7), 845–854. https://doi.org/10.1093/bioinformatics/btt055.

[234] Abraham, M. J.; Murtola, T.; Schulz, R.; Páll, S.; Smith, J. C.; Hess, B.; Lindahl, E. GROMACS: High Performance Molecular Simulations through Multi-Level Parallelism from Laptops to Supercomputers. *SoftwareX* **2015**, *1–2*, 19–25. https://doi.org/10.1016/j.softx.2015.06.001.

[235] Case, D. A.; Cheatham, T. E.; Darden, T.; Gohlke, H.; Luo, R.; Merz, K. M.; Onufriev, A.; Simmerling, C.; Wang, B.; Woods, R. J. The Amber Biomolecular Simulation Programs. *J. Comput. Chem.* **2005**, *26* (16), 1668–1688. https://doi.org/10.1002/jcc.20290.

[236] Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kalé, L.; Schulten, K. Scalable Molecular Dynamics with NAMD. *J. Comput. Chem.* **2005**, *26* (16), 1781–1802. https://doi.org/10.1002/jcc.20289.

[237] Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. CHARMM: A Program for Macromolecular Energy, Minimization, and Dynamics Calculations. *J. Comput. Chem.* **1983**, *4* (2), 187–217. https://doi.org/10.1002/jcc.540040211.

[238] Plimpton, S. Fast Parallel Algorithms for Short-Range Molecular Dynamics. *J. Comput. Phys.* **1995**, *117* (1), 1–19. https://doi.org/10.1006/jcph.1995.1039.

[239] Bowers, K.; Chow, E.; Xu, H.; Dror, R.; Eastwood, M.; Gregersen, B.; Klepeis, J.; Kolossvary, I.; Moraes, M.; Sacerdoti, F.; et al. Scalable Algorithms for Molecular Dynamics Simulations on Commodity Clusters; IEEE, 2006; pp 43–43. https://doi.org/10.1109/SC.2006.54.

[240] Neu, H. C.; Gootz, T. D. Antimicrobial Chemotherapy. In *Medical Microbiology*; Baron, S., Ed.; University of Texas Medical Branch at Galveston: Galveston (TX), 1996.

[241] Hong, W.; Zeng, J.; Xie, J. Antibiotic Drugs Targeting Bacterial RNAs. *Acta Pharm. Sin. B* **2014**, *4* (4), 258–265. https://doi.org/10.1016/j.apsb.2014.06.012.

[242] Cheng, G.; Dai, M.; Ahmed, S.; Hao, H.; Wang, X.; Yuan, Z. Antimicrobial Drugs in Fighting against Antimicrobial Resistance. *Front. Microbiol.* **2016**, *7*. https://doi.org/10.3389/fmicb.2016.00470.

[243] Sun, J.; Deng, Z.; Yan, A. Bacterial Multidrug Efflux Pumps: Mechanisms, Physiology and Pharmacological Exploitations. *Biochem. Biophys. Res. Commun.* **2014**, *453* (2), 254–267. https://doi.org/10.1016/j.bbrc.2014.05.090.

[244] Munita, J. M.; Arias, C. A. Mechanisms of Antibiotic Resistance. *Microbiol. Spectr.* **2016**, *4* (2). https://doi.org/10.1128/microbiolspec.VMBF-0016-2015.

[245] Nikaido, H. Prevention of Drug Access to Bacterial Targets: Permeability Barriers and Active Efflux. *Science* **1994**, *264* (5157), 382–388. https://doi.org/10.1126/science.8153625.

[246] Tam, J. P.; Wang, S.; Wong, K. H.; Tan, W. L. Antimicrobial Peptides from Plants. *Pharmaceuticals* **2015**, *8* (4), 711–757. https://doi.org/10.3390/ph8040711.

[247] Chandra, H.; Bishnoi, P.; Yadav, A.; Patni, B.; Mishra, A. P.; Nautiyal, A. R. Antimicrobial Resistance and the Alternative Resources with Special Emphasis on Plant-Based Antimicrobials - A Review. *Plants* **2017**, *6* (2). https://doi.org/10.3390/plants6020016.

[248] Bolouri Moghaddam, M. R.; Vilcinskas, A.; Rahnamaeian, M. Cooperative Interaction of Antimicrobial Peptides with the Interrelated Immune Pathways in Plants: AMPs in Plant Immunity.

Mol. Plant Pathol. **2016**, *17* (3), 464–471. https://doi.org/10.1111/mpp.12299.

[249] Nawrot, R.; Barylski, J.; Nowicki, G.; Broniarczyk, J.; Buchwald, W.; Goździcka-Józefiak, A. Plant Antimicrobial Peptides. *Folia Microbiol. (Praha)* **2014**, *59* (3), 181–196. https://doi.org/10.1007/s12223-013-0280-4.

[250] Botero, D.; Alvarado, C.; Bernal, A.; Danies, G.; Restrepo, S. Network Analyses in Plant Pathogens. *Front. Microbiol.* **2018**, *9*. https://doi.org/10.3389/fmicb.2018.00035.

[251] Peyraud, R.; Dubiella, U.; Barbacci, A.; Genin, S.; Raffaele, S.; Roby, D. Advances on Plant–Pathogen Interactions from Molecular toward Systems Biology Perspectives. *Plant J.* **2016**, *90* (4), 720–737. https://doi.org/10.1111/tpj.13429.

[252] Schneider, D. J.; Collmer, A. Studying Plant-Pathogen Interactions in the Genomics Era: Beyond Molecular Koch's Postulates to Systems Biology. *Annu. Rev. Phytopathol.* **2010**, *48*, 457–479. https://doi.org/10.1146/annurev-phyto-073009-114411.

[253] Ramakrishnan, G.; Chandra, N. R.; Srinivasan, N. From Workstations to Workbenches: Towards Predicting Physicochemically Viable Protein–Protein Interactions across a Host and a Pathogen. *IUBMB Life* **2014**, *66* (11), 759–774. https://doi.org/10.1002/iub.1331.

[254] Hammami, R.; Fliss, I. Current Trends in Antimicrobial Agent Research : Chemo- and Bioinformatics Approaches. *Drug Discov. Today* **2010**, *15* (13–14), 540–546. https://doi.org/10.1016/j.drudis.2010.05.002.

[255] Sousa, S. F.; Fernandes, P. A.; Ramos, M. J. Protein-Ligand Docking: Current Status and Future Challenges. *Proteins Struct. Funct. Bioinforma.* **2006**, *65* (1), 15–26. https://doi.org/10.1002/prot.21082.

[256] London, N.; Raveh, B.; Schueler-Furman, O. Peptide Docking and Structure-Based Characterization of Peptide Binding: From Knowledge to Know-How. *Curr. Opin. Struct. Biol.* **2013**, *23* (6), 894–902. https://doi.org/10.1016/j.sbi.2013.07.006.

[257] Pagadala, N. S.; Syed, K.; Tuszynski, J. Software for Molecular Docking: A Review. *Biophys. Rev.* **2017**, *9* (2), 91–102. https://doi.org/10.1007/s12551-016-0247-1.

[258] Pierce, B. G.; Wiehe, K.; Hwang, H.; Kim, B.-H.; Vreven, T.; Weng, Z. ZDOCK Server: Interactive Docking Prediction of Protein-Protein Complexes and Symmetric Multimers. *Bioinformatics* **2014**, *30* (12), 1771–1773. https://doi.org/10.1093/bioinformatics/btu097.

[259] Petrey, D.; Honig, B. Structural Bioinformatics of the Interactome. *Annu. Rev. Biophys.* **2014**, *43* (1), 193–210. https://doi.org/10.1146/annurev-biophys-051013-022726.

[260] van Zundert, G. C. P.; Rodrigues, J. P. G. L. M.; Trellet, M.; Schmitz, C.; Kastritis, P. L.; Karaca, E.; Melquiond, A. S. J.; van Dijk, M.; de Vries, S. J.; Bonvin, A. M. J. J. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. *J. Mol. Biol.* **2016**, *428* (4), 720–725. https://doi.org/10.1016/j.jmb.2015.09.014.

[261] Ruiz-Carmona, S.; Alvarez-Garcia, D.; Foloppe, N.; Garmendia-Doval, A. B.; Juhos, S.; Schmidtke, P.; Barril, X.; Hubbard, R. E.; Morley, S. D. RDock: A Fast, Versatile and Open Source Program for Docking Ligands to Proteins and Nucleic Acids. *PLoS Comput. Biol.* **2014**, *10* (4), e1003571. https://doi.org/10.1371/journal.pcbi.1003571.

[262] Ghoorah, A. W.; Devignes, M.-D.; Smaïl-Tabbone, M.; Ritchie, D. W. Protein Docking Using Case-Based Reasoning: Protein Docking Using Case-Based Reasoning. *Proteins Struct. Funct. Bioinforma.* **2013**, *81* (12), 2150–2158. https://doi.org/10.1002/prot.24433.

[263] Andrusier, N.; Mashiach, E.; Nussinov, R.; Wolfson, H. J. Principles of Flexible Protein-Protein Docking. *Proteins Struct. Funct. Bioinforma.* **2008**, *73* (2), 271–289. https://doi.org/10.1002/prot.22170.

[264] Schneider, T.; Kruse, T.; Wimmer, R.; Wiedemann, I.; Sass, V.; Pag, U.; Jansen, A.; Nielsen, A. K.; Mygind, P. H.; Raventos, D. S.; et al. Plectasin, a Fungal Defensin, Targets the Bacterial Cell Wall Precursor Lipid II. *Science* **2010**, *328* (5982), 1168–1172. https://doi.org/10.1126/science.1185723.

[265] Corbeil, C. R.; Williams, C. I.; Labute, P. Variability in Docking Success Rates Due to Dataset Preparation. *J. Comput. Aided Mol. Des.* **2012**, *26* (6), 775–786. https://doi.org/10.1007/s10822-012-9570-1.

[266] Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and Validation of a Genetic Algorithm for Flexible Docking 1 1Edited by F. E. Cohen. *J. Mol. Biol.* **1997**, *267* (3), 727–748. https://doi.org/10.1006/jmbi.1996.0897.

[267] Friesner, R. A.; Murphy, R. B.; Repasky, M. P.; Frye, L. L.; Greenwood, J. R.; Halgren, T. A.; Sanschagrin, P. C.; Mainz, D. T. Extra Precision Glide: Docking and Scoring Incorporating a Model of Hydrophobic Enclosure for Protein−Ligand Complexes. *J. Med. Chem.* **2006**, *49* (21), 6177–6196. https://doi.org/10.1021/jm051256o.

[268] Spitzer, R.; Jain, A. N. Surflex-Dock: Docking Benchmarks and Real-World Application. *J. Comput. Aided Mol. Des.* **2012**, *26* (6), 687–699. https://doi.org/10.1007/s10822-011-9533-y.

[269] Rentzsch, R.; Renard, B. Y. Docking Small Peptides Remains a Great Challenge: An Assessment Using AutoDock Vina. *Brief. Bioinform.* **2015**, *16* (6), 1045–1056. https://doi.org/10.1093/bib/bbv008.

[270] Audie, J.; Swanson, J. Advances in the Prediction of Protein-Peptide Binding Affinities: Implications for Peptide-Based Drug Discovery: **Protein-Peptide Binding Affinities**. *Chem. Biol. Drug Des.* **2013**, *81* (1), 50–60. https://doi.org/10.1111/cbdd.12076.

[271] Audie, J.; Swanson, J. Recent Work in the Development and Application of Protein–Peptide Docking. *Future Med. Chem.* **2012**, *4* (12), 1619–1644. https://doi.org/10.4155/fmc.12.99.

[272] Sousa, S. F.; Ribeiro, A. J. M.; Coimbra, J. T. S.; Neves, R. P. P.; Martins, S. A.; Moorthy, N. S. H. N.; Fernandes, P. A.; Ramos, M. J. Protein-Ligand Docking in the New Millennium--a Retrospective of 10 Years in the Field. *Curr. Med. Chem.* **2013**, *20* (18), 2296–2314.

[273] J. Wilson, A. Inhibition of Protein–Protein Interactions Using Designed Molecules. *Chem. Soc. Rev.* **2009**, *38* (12), 3289. https://doi.org/10.1039/b807197g.

[274] Unal, E. B.; Gursoy, A.; Erman, B. VitAL: Viterbi Algorithm for *de Novo* Peptide Design. *Plos One* **2010**, *5* (6), e10926. https://doi.org/10.1371/journal.pone.0010926.

[275] Lensink, M. F.; Velankar, S.; Wodak, S. J. Modeling Protein-Protein and Protein-Peptide Complexes: CAPRI 6th Edition: Modeling Protein-Protein and Protein-Peptide Complexes. *Proteins Struct. Funct. Bioinforma.* **2017**, *85* (3), 359–377. https://doi.org/10.1002/prot.25215.

[276] Lensink, M. F.; Wodak, S. J. Docking, Scoring, and Affinity Prediction in CAPRI: Docking, Scoring, and Affinity Prediction in CAPRI. *Proteins Struct. Funct. Bioinforma.* **2013**, *81* (12), 2082–2095. https://doi.org/10.1002/prot.24428.

[277] Gao, A.-G.; Hakimi, S. M.; Mittanck, C. A.; Wu, Y.; Woerner, B. M.; Stark, D. M.; Shah, D. M.; Liang, J.; Rommens, C. M. T. Fungal Pathogen Protection in Potato by Expression of a Plant Defensin Peptide. *Nat. Biotechnol.* **2000**, *18* (12), 1307–1310. https://doi.org/10.1038/82436.

[278] Kanzaki, H.; Nirasawa, S.; Saitoh, H.; Ito, M.; Nishihara, M.; Terauchi, R.; Nakamura, I. Overexpression of the Wasabi Defensin Gene Confers Enhanced Resistance to Blast Fungus (*Magnaporthe grisea*) in Transgenic Rice. *Theor. Appl. Genet.* **2002**, *105* (6–7), 809–814.

[279] Chen, S.-C.; Liu, A.-R.; Zou, Z.-R. Overexpression of Glucanase Gene and Defensin Gene in Transgenic Tomato Enhances Resistance to *Ralstonia solanacearum*. *Russ. J. Plant Physiol.* **2006**, *53*

(5), 671–677. https://doi.org/10.1134/S1021443706050116.

[280] Anuradha, T. S.; Divya, K.; Jami, S. K.; Kirti, P. B. Transgenic Tobacco and Peanut Plants Expressing a Mustard Defensin Show Resistance to Fungal Pathogens. *Plant Cell Rep.* **2008**, *27* (11), 1777–1786. https://doi.org/10.1007/s00299-008-0596-8.

[281] Abdallah, N. A.; Shah, D.; Abbas, D.; Madkour, M. Stable Integration and Expression of a Plant Defensin in Tomato Confers Resistance to *Fusarium* Wilt. *GM Crops* **2010**, *1* (5), 344–350. https://doi.org/10.4161/gmcr.1.5.15091.

[282] Gaspar, Y. M.; McKenna, J. A.; McGinness, B. S.; Hinch, J.; Poon, S.; Connelly, A. A.; Anderson, M. A.; Heath, R. L. Field Resistance to *Fusarium oxysporum* and *Verticillium dahliae* in Transgenic Cotton Expressing the Plant Defensin NaD1. *J. Exp. Bot.* **2014**, *65* (6), 1541–1550. https://doi.org/10.1093/jxb/eru021.

[283] Pérez-Bernal, M.; Delgado, M.; Cruz, A.; Abreu, D.; Valdivia, O.; Armas, R. Marker-Free Transgenic Rice Lines with a Defensin Gene Are Potentially Active against Phytopathogenic Fungus *Sarocladium oryzae*. *Acta Phytopathol. Entomol. Hung.* **2017**, *52* (2), 135–144. https://doi.org/10.1556/038.52.2017.021.

[284] Portieles, R.; Ayra, C.; Gonzalez, E.; Gallo, A.; Rodriguez, R.; Chacón, O.; López, Y.; Rodriguez, M.; Castillo, J.; Pujol, M.; et al. NmDef02, a Novel Antimicrobial Gene Isolated from *Nicotiana megalosiphon* Confers High-Level Pathogen Resistance under Greenhouse and Field Conditions: NmDef02, a Novel Antimicrobial Protein. *Plant Biotechnol. J.* **2010**, *8* (6), 678–690. https://doi.org/10.1111/j.1467-7652.2010.00501.x.

[285] Thao, H.; Lan, N.; Tuong, H.; Thanh, N.; Son, L.; Mau, C. Expression Analysis of Recombinant *Vigna radiata* Plant Defensin 1 Protein in Transgenic Tobacco Plants. *J. Appl. Biol. Biotechnol.* **2017**, 070–075. https://doi.org/10.7324/JABB.2017.50112.

[286] Lee, H.-H.; Kim, J.-S.; Hoang, Q. T. N.; Kim, J.-I.; Kim, Y. S. Root-Specific Expression of Defensin in Transgenic Tobacco Results in Enhanced Resistance against *Phytophthora parasitica* Var. Nicotianae. *Eur. J. Plant Pathol.* **2018**, *151* (3), 811–823. https://doi.org/10.1007/s10658-018-1419-6.

[287] Almasia, N. I.; Bazzini, A. A.; Hopp, H. E.; Vazquez-Rovere, C. Overexpression of Snakin-1 Gene Enhances Resistance to *Rhizoctonia solani* and *Erwinia carotovora* in Transgenic Potato Plants. *Mol. Plant Pathol.* **2008**, *9* (3), 329–338. https://doi.org/10.1111/j.1364-3703.2008.00469.x.

[288] Balaji, V.; Smart, C. D. Over-Expression of Snakin-2 and Extensin-like Protein Genes Restricts Pathogen Invasiveness and Enhances Tolerance to *Clavibacter michiganensis* Subsp. Michiganensis in Transgenic Tomato (*Solanum lycopersicum*). *Transgenic Res.* **2012**, *21* (1), 23–37. https://doi.org/10.1007/s11248-011-9506-x.

[289] Patkar, R. N.; Chattoo, B. B. Transgenic Indica Rice Expressing Ns-LTP-Like Protein Shows Enhanced Resistance to Both Fungal and Bacterial Pathogens. *Mol. Breed.* **2006**, *17* (2), 159–171. https://doi.org/10.1007/s11032-005-4736-3.

[290] Carmona, M. J.; Molina, A.; Fernández, J. A.; López-Fando, J. J.; García-Olmedo, F. Expression of the Alpha-Thionin Gene from Barley in Tobacco Confers Enhanced Resistance to Bacterial Pathogens. *Plant J. Cell Mol. Biol.* **1993**, *3* (3), 457–462.

[291] Iwai, T.; Kaku, H.; Honkura, R.; Nakamura, S.; Ochiai, H.; Sasaki, T.; Ohashi, Y. Enhanced Resistance to Seed-Transmitted Bacterial Diseases in Transgenic Rice Plants Overproducing an Oat Cell-Wall-Bound Thionin. *Mol. Plant. Microbe Interact.* **2002**, *15* (6), 515–521. https://doi.org/10.1094/MPMI.2002.15.6.515.

[292] Chan, Y.-L.; Prasad, V.; Sanjaya; Chen, K. H.; Liu, P. C.; Chan, M.-T.; Cheng, C.-P. Transgenic Tomato Plants Expressing an *Arabidopsis* Thionin (Thi2.1) Driven by Fruit-Inactive Promoter Battle against Phytopathogenic Attack. *Planta* **2005**, *221* (3), 386–393.

https://doi.org/10.1007/s00425-004-1459-3.

[293] Muramoto, N.; Tanaka, T.; Shimamura, T.; Mitsukawa, N.; Hori, E.; Koda, K.; Otani, M.; Hirai, M.; Nakamura, K.; Imaeda, T. Transgenic Sweet Potato Expressing Thionin from Barley Gives Resistance to Black Rot Disease Caused by *Ceratocystis fimbriata* in Leaves and Storage Roots. *Plant Cell Rep.* **2012**, *31* (6), 987–997. https://doi.org/10.1007/s00299-011-1217-5.

[294] Koo, J. C.; Chun, H. J.; Park, H. C.; Kim, M. C.; Koo, Y. D.; Koo, S. C.; Ok, H. M.; Park, S. J.; Lee, S.-H.; Yun, D.-J.; et al. Over-Expression of a Seed Specific Hevein-like Antimicrobial Peptide from *Pharbitis nil* Enhances Resistance to a Fungal Pathogen in Transgenic Tobacco Plants. *Plant Mol. Biol.* **2002**, *50* (3), 441–452.

[295] R. Shukurov, R.; D. Voblikova, V.; Nikonorova, A. K.; Komakhin, R. A.; V. Komakhina, V.; A. Egorov, T.; V. Grishin, E.; V. Babakov, A. Transformation of Tobacco and *Arabidopsis* Plants with *Stellaria media* Genes Encoding Novel Hevein-like Peptides Increases Their Resistance to Fungal Pathogens. *Transgenic Res.* **2012**, *21* (2), 313–325. https://doi.org/10.1007/s11248-011-9534-6.

[296] Datta, K.; Velazhahan, R.; Oliva, N.; Ona, I.; Mew, T.; Khush, G. S.; Muthukrishnan, S.; Datta, S. K. Over-Expression of the Cloned Rice Thaumatin-like Protein (PR-5) Gene in Transgenic Rice Plants Enhances Environmental Friendly Resistance to *Rhizoctonia solani* Causing Sheath Blight Disease. *Theor. Appl. Genet.* **1999**, *98* (6–7), 1138–1145. https://doi.org/10.1007/s001220051178.

[297] Chen, W. P.; Chen, P. D.; Liu, D. J.; Kynast, R.; Friebe, B.; Velazhahan, R.; Muthukrishnan, S.; Gill, B. S. Development of Wheat Scab Symptoms Is Delayed in Transgenic Wheat Plants That Constitutively Express a Rice Thaumatin-like Protein Gene. *Theor. Appl. Genet.* **1999**, *99* (5), 755–760. https://doi.org/10.1007/s001220051294.

[298] Anand, A.; Zhou, T.; Trick, H. N.; Gill, B. S.; Bockus, W. W.; Muthukrishnan, S. Greenhouse and Field Testing of Transgenic Wheat Plants Stably Expressing Genes for Thaumatin-like Protein, Chitinase and Glucanase against *Fusarium graminearum*. *J. Exp. Bot.* **2003**, *54* (384), 1101–1111. https://doi.org/10.1093/jxb/erg110.

[299] Velazhahan, R.; Muthukrishnan, S. Transgenic Tobacco Plants Constitutively Overexpressing a Rice Thaumatin-like Protein (PR-5) Show Enhanced Resistance to *Alternaria alternata*. *Biol. Plant.* **2004**, *47* (3), 347–354. https://doi.org/10.1023/B:BIOP.0000023876.55053.5e.

[300] He, R.; Wu, J.; Zhang, Y.; Agüero, C. B.; Li, X.; Liu, S.; Wang, C.; Walker, M. A.; Lu, J. Overexpression of a Thaumatin-like Protein Gene from *Vitis amurensis* Improves Downy Mildew Resistance in *Vitis vinifera* Grapevine. *Protoplasma* **2017**, *254* (4), 1579–1589. https://doi.org/10.1007/s00709-016-1047-y.

[301] Aghazadeh, R.; Zamani, M.; Motallebi, M.; Moradyar, M. Agrobacterium-Mediated Transformation of the *Oryza sativa* Thaumatin-Like Protein to Canola (R Line Hyola308) for Enhancing Resistance to *Sclerotinia sclerotiorum*. *Iran. J. Biotechnol.* **2017**, *15* (3), 201–207. https://doi.org/10.15171/ijb.1585.

[302] Ojola, P.; Nyaboga, E. N.; Njiru, P. N.; Orinda, G. Overexpression of Rice Thaumatin-like Protein (Ostlp) Gene in Transgenic Cassava Results in Enhanced Tolerance to *Colletotrichum gloeosporioides* f. Sp. *Manihotis*. *J. Genet. Eng. Biotechnol.* **2018**, *16* (1), 125–131. https://doi.org/10.1016/j.jgeb.2017.12.002.

[303] Zeller, S. L.; Kalinina, O.; Schmid, B. Costs of Resistance to Fungal Pathogens in Genetically Modified Wheat. *J. Plant Ecol.* **2013**, *6* (1), 92–100. https://doi.org/10.1093/jpe/rts013.

[304] Lacerda, A. F.; Vasconcelos, Ã. A. R.; Pelegrini, P. B.; Grossi de Sa, M. F. Antifungal Defensins and Their Role in Plant Defense. *Front. Microbiol.* **2014**, *5*. https://doi.org/10.3389/fmicb.2014.00116.

[305] Choi, M.-S.; Kim, Y.-H.; Park, H.-M.; Seo, B.-Y.; Jung, J.-K.; Kim, S.-T.; Kim, M.-C.; Shin,

D.-B.; Yun, H.-T.; Choi, I.-S.; et al. Expression of BrD1, a Plant Defensin from *Brassica rapa*, Confers Resistance against Brown Planthopper (*Nilaparvata lugens*) in Transgenic Rices. *Mol. Cells* **2009**, *28* (2), 131–137. https://doi.org/10.1007/s10059-009-0117-9.

[306] Khan, R. S.; Nishihara, M.; Yamamura, S.; Nakamura, I.; Mii, M. Transgenic Potatoes Expressing Wasabi Defensin Peptide Confer Partial Resistance to Gray Mold (*Botrytis cinerea*). *Plant Biotechnol.* **2006**, *23* (2), 179–183. https://doi.org/10.5511/plantbiotechnology.23.179.

[307] Sjahril, R.; Chin, D. P.; Khan, R. S.; Yamamura, S.; Nakamura, I.; Amemiya, Y.; Mii, M. Transgenic Phalaenopsis Plants with Resistance to *Erwinia carotovora* Produced by Introducing Wasabi Defensin Gene Using Agrobacterium Method. *Plant Biotechnol.* **2006**, *23* (2), 191–194. https://doi.org/10.5511/plantbiotechnology.23.191.

[308] Ntui, V. O.; Thirukkumaran, G.; Azadi, P.; Khan, R. S.; Nakamura, I.; Mii, M. Stable Integration and Expression of Wasabi Defensin Gene in "Egusi" Melon (*Colocynthis citrullus* L.) Confers Resistance to *Fusarium* Wilt and Alternaria Leaf Spot. *Plant Cell Rep.* **2010**, *29* (9), 943–954. https://doi.org/10.1007/s00299-010-0880-2.

[309] Khan, R. S.; Nakamura, I.; Mii, M. Development of Disease-Resistant Marker-Free Tomato by R/RS Site-Specific Recombination. *Plant Cell Rep.* **2011**, *30* (6), 1041–1053. https://doi.org/10.1007/s00299-011-1011-4.

[310] Darwish, N. A.; Khan, R. S.; Ntui, V. O.; Nakamura, I.; Mii, M. Generation of Selectable Marker-Free Transgenic Eggplant Resistant to *Alternaria solani* Using the R/RS Site-Specific Recombination System. *Plant Cell Rep.* **2014**, *33* (3), 411–421. https://doi.org/10.1007/s00299-013-1541-z.

[311] Ghag, S. B.; Shekhawat, U. K. S.; Ganapathi, T. R. Transgenic Banana Plants Expressing a *Stellaria media* Defensin Gene (Sm-AMP-D1) Demonstrate Improved Resistance to *Fusarium oxysporum*. *Plant Cell Tissue Organ Cult. PCTOC* **2014**, *119* (2), 247–255. https://doi.org/10.1007/s11240-014-0529-x.

[312] Terras, F. Small Cysteine-Rich Antifungal Proteins from Radish: Their Role in Host Defense. *Plant Cell online* **1995**, *7* (5), 573–588. https://doi.org/10.1105/tpc.7.5.573.

[313] Li, Z.; Zhou, M.; Zhang, Z.; Ren, L.; Du, L.; Zhang, B.; Xu, H.; Xin, Z. Expression of a Radish Defensin in Transgenic Wheat Confers Increased Resistance to *Fusarium graminearum* and *Rhizoctonia cerealis*. *Funct. Integr. Genomics* **2011**, *11* (1), 63–70. https://doi.org/10.1007/s10142-011-0211-x.

[314] Vasavirama, K.; Kirti, P. B. Increased Resistance to Late Leaf Spot Disease in Transgenic Peanut Using a Combination of PR Genes. *Funct. Integr. Genomics* **2012**, *12* (4), 625–634. https://doi.org/10.1007/s10142-012-0298-8.

[315] Park, H. C.; Kang, Y. H.; Chun, H. J.; Koo, J. C.; Cheong, Y. H.; Kim, C. Y.; Kim, M. C.; Chung, W. S.; Kim, J. C.; Yoo, J. H. Characterization of a Stamen-Specific CDNA Encoding a Novel Plant Defensin in Chinese Cabbage. *Plant Mol. Biol.* **2002**, *50* (1), 57–68.

[316] Ghag, S. B.; Shekhawat, U. K. S.; Ganapathi, T. R. Petunia Floral Defensins with Unique Prodomains as Novel Candidates for Development of *Fusarium* Wilt Resistance in Transgenic Banana Plants. *Plos One* **2012**, *7* (6), e39557. https://doi.org/10.1371/journal.pone.0039557.

[317] Wang, Y.; Nowak, G.; Culley, D.; Hadwiger, L. A.; Fristensky, B. Constitutive Expression of Pea Defense Gene DRR206 Confers Resistance to Blackleg (*Leptosphaeria maculans*) Disease in Transgenic Canola (*Brassica napus*). *Mol. Plant. Microbe Interact.* **1999**, *12* (5), 410–418. https://doi.org/10.1094/MPMI.1999.12.5.410.

[318] Zhu, Y. J.; Agbayani, R.; Moore, P. H. Ectopic Expression of *Dahlia merckii* Defensin DmAMP1 Improves Papaya Resistance to *Phytophthora palmivora* by Reducing Pathogen Vigor.

Planta **2007**, *226* (1), 87–97. https://doi.org/10.1007/s00425-006-0471-1.

[319] Jha, S.; Tank, H. G.; Prasad, B. D.; Chattoo, B. B. Expression of Dm-AMP1 in Rice Confers Resistance to *Magnaporthe oryzae* and *Rhizoctonia solani*. *Transgenic Res.* **2009**, *18* (1), 59–69. https://doi.org/10.1007/s11248-008-9196-1.

320] Seo, H.-H.; Park, S.; Park, S.; Oh, B.-J.; Back, K.; Han, O.; Kim, J.-I.; Kim, Y. S. Overexpression of a Defensin Enhances Resistance to a Fruit-Specific *Anthracnose fungus* in Pepper. *Plos One* **2014**, *9* (5), e97936. https://doi.org/10.1371/journal.pone.0097936.

[321] Kaur, J.; Thokala, M.; Robert-Seilaniantz, A.; Zhao, P.; Peyret, H.; Berg, H.; Pandey, S.; Jones, J.; Shah, D. Subcellular Targeting of an Evolutionarily Conserved Plant Defensin MtDef4.2 Determines the Outcome of Plant-Pathogen Interaction in Transgenic *Arabidopsis*: Defensin-Mediated Resistance to Fungal Pathogens. *Mol. Plant Pathol.* **2012**, *13* (9), 1032–1046. https://doi.org/10.1111/j.1364-3703.2012.00813.x.

[322] Epple, P. Overexpression of an Endogenous Thionin Enhances Resistance of *Arabidopsis* against *Fusarium oxysporum*. *Plant Cell online* **1997**, *9* (4), 509–520. https://doi.org/10.1105/tpc.9.4.509.

[323] Mahdavi, F.; Sariah, M.; Maziah, M. Expression of Rice Thaumatin-Like Protein Gene in Transgenic Banana Plants Enhances Resistance to *Fusarium* Wilt. *Appl. Biochem. Biotechnol.* **2012**, *166* (4), 1008–1019. https://doi.org/10.1007/s12010-011-9489-3.

[324] Rajam, M. V.; Chandola, N.; Saiprasad Goud, P.; Singh, D.; Kashyap, V.; Choudhary, M. L.; Sihachakr, D. Thaumatin Gene Confers Resistance to Fungal Pathogens as Well as Tolerance to Abiotic Stresses in Transgenic Tobacco Plants. *Biol. Plant.* **2007**, *51* (1), 135–141. https://doi.org/10.1007/s10535-007-0026-8.

[325] Misra, R. C.; Sandeep; Kamthan, M.; Kumar, S.; Ghosh, S. A Thaumatin-like Protein of *Ocimum basilicum* Confers Tolerance to Fungal Pathogen and Abiotic Stress in Transgenic *Arabidopsis*. *Sci. Rep.* **2016**, *6* (1). https://doi.org/10.1038/srep25340.

[326] Acharya, K.; Pal, A. K.; Gulati, A.; Kumar, S.; Singh, A. K.; Ahuja, P. S. Overexpression of Camellia Sinensis Thaumatin-Like Protein, CsTLP in Potato Confers Enhanced Resistance to *Macrophomina phaseolina* and *Phytophthora infestans* Infection. *Mol. Biotechnol.* **2013**, *54* (2), 609–622. https://doi.org/10.1007/s12033-012-9603-y.

[327] Benko-Iseppon, A. M.; Crovella, S. Ethnobotanical Bioprospection of Candidates for Potential Antimicrobial Drugs from Brazilian Plants: State of Art and Perspectives. *Curr. Protein Pept. Sci.* **2010**, *11* (3), 189–194.

[328] Benko-Iseppon, A. M.; Pinangé, D. S. B.; Chang, S. C.; Morawetz, W. Ethnobotanical Uses of the Native Flora from Brazilian North-Eastern Region. *Med. Plants Biodivers. Drugs* **2012**, 84.

[329] Craik, D. J.; Lee, M.-H.; Rehm, F. B. H.; Tombling, B.; Doffek, B.; Peacock, H. Ribosomally-Synthesised Cyclic Peptides from Plants as Drug Leads and Pharmaceutical Scaffolds. *Bioorg. Med. Chem.* **2018**, *26* (10), 2727–2737. https://doi.org/10.1016/j.bmc.2017.08.005.

[330] Stierle, A.; Strobel, G.; Stierle, D. Taxol and Taxane Production by *Taxomyces andreanae*, an Endophytic Fungus of Pacific Yew. *Science* **1993**, *260* (5105), 214–216. https://doi.org/10.1126/science.8097061.

[331] Thevissen, K.; Kristensen, H.-H.; Thomma, B. P. H. J.; Cammue, B. P. A.; François, I. E. J. A. Therapeutic Potential of Antifungal Plant and Insect Defensins. *Drug Discov. Today* **2007**, *12* (21–22), 966–971. https://doi.org/10.1016/j.drudis.2007.07.016.

[332] Gerwick, W. H. Plant Sources of Drugs and Chemicals. In *Encyclopedia of Biodiversity*; Elsevier, 2013; pp 129–139. https://doi.org/10.1016/B978-0-12-384719-5.00111-8.

[333] Hoskin, D. W.; Ramamoorthy, A. Studies on Anticancer Activities of Antimicrobial Peptides. *Biochim. Biophys. Acta BBA - Biomembr.* **2008**, *1778* (2), 357–375. https://doi.org/10.1016/j.bbamem.2007.11.008.

[334] Felício, M. R.; Silva, O. N.; Gonçalves, S.; Santos, N. C.; Franco, O. L. Peptides with Dual Antimicrobial and Anticancer Activities. *Front. Chem.* **2017**, *5*. https://doi.org/10.3389/fchem.2017.00005.

[335] Elsbach, P. What Is the Real Role of Antimicrobial Polypeptides That Can Mediate Several Other Inflammatory Responses? *J. Clin. Invest.* **2003**, *111* (11), 1643–1645. https://doi.org/10.1172/JCI18761.

[336] Bowdish, D. M. E.; Davidson, D. J.; Lau, Y. E.; Lee, K.; Scott, M. G.; Hancock, R. E. W. Impact of LL-37 on Anti-Infective Immunity. *J. Leukoc. Biol.* **2005**, *77* (4), 451–459. https://doi.org/10.1189/jlb.0704380.

[337] Holaskova, E.; Galuszka, P.; Frebort, I.; Oz, M. T. Antimicrobial Peptide Production and Plant-Based Expression Systems for Medical and Agricultural Biotechnology. *Biotechnol. Adv.* **2015**, *33* (6), 1005–1023. https://doi.org/10.1016/j.biotechadv.2015.03.007.

[338] Straus, S. K.; Hancock, R. E. Mode of Action of the New Antibiotic for Gram-Positive Pathogens Daptomycin: Comparison with Cationic Antimicrobial Peptides and Lipopeptides. *Biochim. Biophys. Acta BBA-Biomembr.* **2006**, *1758* (9), 1215–1223.

[339] Melo, M. N.; Ferre, R.; Castanho, M. A. Antimicrobial Peptides: Linking Partition, Activity and High Membrane-Bound Concentrations. *Nat. Rev. Microbiol.* **2009**, *7* (3), 245.

[340] Laverty, G.; Gorman, S. P.; Gilmore, B. F. The Potential of Antimicrobial Peptides as Biocides. *Int. J. Mol. Sci.* **2011**, *12* (10), 6566–6596.

[341] Silva, O. N. O.; Mulder, K. C.; Barbosa, A. A.; Otero-Gonzalez, A. J.; Lopez-Abarrategui, C.; Dias, S. C.; Rezende, T.; Franco, O. L. Exploring the Pharmacological Potential of Promiscuous Host-Defense Peptides: From Natural Screenings to Biotechnological Applications. *Front. Microbiol.* **2011**, *2*, 232.

[342] Organization, W. H. Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics. *Geneva World Health Organ.* **2017**.

[343] Hancock, R. E. Cationic Peptides: Effectors in Innate Immunity and Novel Antimicrobials. *Lancet Infect. Dis.* **2001**, *1* (3), 156–164.

[344] Peters, B. M.; Shirtliff, M. E.; Jabra-Rizk, M. A. Antimicrobial Peptides: Primeval Molecules or Future Drugs? *PLoS Pathog.* **2010**, *6* (10), e1001067.

[345] Galdino da Rocha Pitta, M.; Galdino da Rocha Pitta, M.; Lins Galdino, S. Development of Novel Therapeutic Drugs in Humans from Plant Antimicrobial Peptides. *Curr. Protein Pept. Sci.* **2010**, *11* (3), 236–247.

[346] Nadal, A.; Montero, M.; Badosa, E.; Messeguer, J.; Montesinos, L.; Montesinos, E.; Pla, M. Constitutive Expression of Transgenes Encoding Derivatives of the Synthetic Antimicrobial Peptide BP100: Impact on Rice Host Plant Fitness. *BMC Plant Biol.* **2012**, *12* (1), 159.

[347] Cabanos, C.; Ekyo, A.; Amari, Y.; Kato, N.; Kuroda, M.; Nagaoka, S.; Takaiwa, F.; Utsumi, S.; Maruyama, N. High-Level Production of Lactostatin, a Hypocholesterolemic Peptide, in Transgenic Rice Using Soybean A1aB1b as Carrier. *Transgenic Res.* **2013**, *22* (3), 621–629.

[348] Fukuzawa, N.; Tabayashi, N.; Okinaka, Y.; Furusawa, R.; Furuta, K.; Kagaya, U.; Matsumura, T. Production of Biologically Active Atlantic Salmon Interferon in Transgenic Potato and Rice Plants. *J. Biosci. Bioeng.* **2010**, *110* (2), 201–207.

[349] Sathish, K.; Sriraman, R.; Subramanian, B. M.; Rao, N. H.; Balaji, K.; Narasu, M. L.; Srinivasan, V. A. Plant Expressed EtMIC2 Is an Effective Immunogen in Conferring Protection against Chicken *Coccidiosis*. *Vaccine* **2011**, *29* (49), 9201–9208.

[350] Rubio-Infante, N.; Govea-Alonso, D. O.; Alpuche-Solís, Á. G.; García-Hernández, A. L.; Soria-Guerra, R. E.; Paz-Maldonado, L. T.; Ilhuicatzi-Alvarado, D.; Varona-Santos, J. T.; Verdín-Terán, L.; Korban, S. S. A Chloroplast-Derived C4V3 Polypeptide from the Human Immunodeficiency Virus (HIV) Is Orally Immunogenic in Mice. *Plant Mol. Biol.* **2012**, *78* (4–5), 337–349.

[351] Cabanos, C.; Ekyo, A.; Amari, Y.; Kato, N.; Kuroda, M.; Nagaoka, S.; Takaiwa, F.; Utsumi, S.; Maruyama, N. High-Level Production of Lactostatin, a Hypocholesterolemic Peptide, in Transgenic Rice Using Soybean A1aB1b as Carrier. *Transgenic Res.* **2013**, *22* (3), 621–629.

[352] Wang, Y.; Nowak, G.; Culley, D.; Hadwiger, L. A.; Fristensky, B. Constitutive Expression of Pea Defense Gene DRR206 Confers Resistance to Blackleg (*Leptosphaeria maculans*) Disease in Transgenic Canola (*Brassica napus*). *Mol. Plant. Microbe Interact.* **1999**, *12* (5), 410–418. https://doi.org/10.1094/MPMI.1999.12.5.410.

[353] Faccio, P.; Vazquez-Rovere, C.; Hopp, E.; Gonzalez, G.; Decima-Oneto, C.; Favret, E.; Paleo, A. D.; Franzone, P. Increased Tolerance to Wheat Powdery Mildew by Heterologous Constitutive Expression of the *Solanum chacoense* Snakin-1 Gene. *Czech J. Genet. Plant Breed.* **2011**, *47* (Special Issue).

.

4. Capítulo II

Manuscrito de artigo.
Em preparação para submissão à revista *Peptides*
(Elsevier, Qualis B1)
https://www.journals.elsevier.com/peptides

MANIHOT ESCULENTA DEFENSINS: PROSPECTION, STRUCTURAL ANALYSIS AND EXPRESSION

Carlos André dos Santos Silva[1]; Lívia Maria Batista Vilela[1]; Roberta Lane de Oliveira Silva[1]; João Pacífico Bezerra Neto[1]; Jéssica Barboza da Silva[1]; Sérgio Crovella[1]; Ana Maria Benko-Iseppon[1]*

[1]Departamento de Genética, Centro de Biociências, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, CEP 50.670-423, Recife - PE, Brazil.

*Corresponding Author's e-mail: ana.benko.iseppon@pq.cnpq.br

Abstract

Molecules with various medicinal properties have been identified in plants, including defense proteins with potential biotechnological and medicinal uses. The tissues of *Manihot esculenta* Crantz – popularly known as cassava – also exhibit various medicinal properties, including anti-inflammatory and antimicrobial activities. This work meant to prospect and analyze antimicrobial peptides of the defensin family in *M. esculenta*. Defensin sequences were obtained from databases and used as probes to find candidate sequences in the *M. esculenta* genome, allowing the identification of eight candidate sequences. However, three of these sequences lacked the conserved gamma-thionin domain. The remaining five sequences were characterized, including conserved domain (CD) annotation, predicted disulfide bonds, secondary structure and antimicrobial activity. The characterized sequences were amplified from the genomic DNA and also submitted to comparative modeling. All 3D structures had antiparallel ß-sheet and an α-helix. Furthermore, all the modeled structures presented amphipathic residues distributed throughout the structure with a predominance of cationic surface charge. Moreover, an analysis of the expression of leave cassava defensins with real-time quantitative PCR (RT-qPCR) after inoculation with the fungus *Lasiodiplodia theobromae*. Expression analysis showed that defensins Def-Me1 and Def-Me4 maintained their expression level, indicating their role in the constitutive defense mechanism. In turn, defensins Def-Me2 and Def-Me3 were down-regulated in the leaves, suggesting distinct roles or tissue specificity. These results show that although defensins are present in infections against pathogens, they may act as preformed defense or, still, have tissue or pathogen specificities.

Keywords: Antimicrobial peptides, *M. esculenta*, bioinformatics, RT-qPCR.

Introduction

Cassava (*Manihot esculenta* Crantz), a plant of the family Euphorbiaceae originating in the Amazon [1], is one of the world's major crops whose roots provide basic food for more than 800 million people, especially in countries with a humid tropical climate [2–4]. It is particularly important for the sustainable livelihoods of resource-poor farmers in many parts of Africa, Asia, and South America due to its tolerance to depleted soils, to drought and their ease of cultivation. The variety of processed food types that are made of cassava biomass (including roots and leaves) also justify rapid expansion of its cultivation around the world [5,6].

Besides its economic importance, *M. esculenta* leaves have been used in folk medicine, with identified antifungal properties [7]. Indeed, such molecules represent many millions of years of plant-pathogen coevolution, favoring the development of molecules as secondary metabolites as well as antimicrobial peptides (AMPs), also known as host defense peptides (HDPs) [8].

Peptides fulfill a myriad of functions including plant growth, development, and stress responses. They act as critical components of cell-to-cell communication, interfere with signaling and response pathways, or exhibit antimicrobial activity. Unexpectedly, both the diversity and the number of plant peptides have been largely underestimated [9,10]. AMPs are distinguished by their general basic nature and small size (up to 100 amino acid residues). Most plant AMPs present an "amphipathic design", with a conformation in which hydrophobic and cationic amino acids are grouped into distinct segments of the peptide [11]. They are classified into families based on their sequence similarity, cysteine motifs that determine their disulfide bond and folding patterns of the tertiary structure. These characteristics are well represented by two plant AMP families, thionins and plant defensins [11]. Plant defensins are small cationic peptides with a highly conserved three-dimensional structure. They have been studied extensively in the last decades, and several biological activities have been attributed to plant defensins, whereas their antimicrobial activity is the most reported [12].

As in animals, the expression of such peptides in plants is constitutive or induced and can often be tissue-specific. In addition, their sequences are hypervariable, sharing a conserved skeleton that provides the capacity to recognize

different targets by varying the residues sequence while preserving the cysteines [11,13].

Plants are widely used in traditional medicine, and various plant AMPs have been described as potential alternatives to conventional antibiotics [14]. In this scenario of possibilities, this work meant to prospect and analyze *M. esculenta* genomic defensins, inferring on their diversity, predicted structure, function, and expression in leave tissue. The identified peptides may serve a basis for further studies aimed at their biotechnological use.

Materials and methods

Sequence selection and characterization

Firstly, defensin seed-sequences were obtained from the PhytAMP database (**Supplementary Table 1-1**) [15] and used as a query in the search for new defensins in the genome of *M. esculenta* available in GenBank. For this purpose, a program based on Hidden Markov Model (HMM) was used to recognize defensis patterns taken from the PhytAmp database. An e-value threshold from $1.5.e^{-23}$ to 8.6 (**Supplementary Table 1-2**) was used as cutoff, so that no sequence could be discarded without a previous characterization. Queries regarded full-length defensin cDNA sequences with previously described antimicrobial activity. Signal peptides were identified with the SignalP tool [16], while conserved domains were detected using the CD-Search tool [17]. Disulfide bond prediction was carried out with the aid of the DISULFIND tool [18].

Plant material, genomic DNA extraction, and PCR amplification

Cassava sprouts (*M. esculenta* variety Santo Estevão) were kindly provided by Ph.D. Antonio Félix da Costa of the Instituto Agronômico de Pernambuco. The plants were cultivated in the greenhouse until leaf biomass was sufficient for genomic DNA extraction (ca. 400 - 800 mg), which was based on the procedure of Weising et al. 2005 [19] (with modifications described by Benko-Iseppon et al. 2003 [20]).

Primers were designed for all characterized sequences which contained at least the conserved gamma-thionin domain (**Table 4**). For the primer design, the Primer3Plus tool [21] was used, with the following parameters: size between 18-24 bp,

the difference between the denaturation temperatures of the primer pairs was not greater than 5 °C, melting temperature around 60 °C, guanine and cytosine content (GC Content) between 40% and 60%, avoiding long repetitions of the same nucleotides, besides avoiding self-complementary primers.

Table 4 - Forward and reverse primers for defensin amplification in the genomic DNA of *Manihot esculenta*.

Name	Primers	Expected size
Me1	5'TGGACGGTTCTCGACTTCTGGTTC3' 3'TCCGCCTATTCTCGGCTGTTCTC5'	1265
Me2	5'CGGTCAGGGAACAAGCATTATTAGC3' 3'TGTGTGTTGGACGATGTCTCACG5'	868
Me3	5'CGAGTGATAACAGCACCAGAGAGTGC3' 3'TGGGTTCCATCTGTCAAACCAATC5'	1421
Me4	5'CGGTCAGGGAACAAGCATTATTAGC3' 3'TGTGTGTTGGACGATGTCTCACG5'	2262
Me5	5'ATCCCAGCACCCAGCACTGAAC3' 3'GCGCAGATGAAAGAACGGAGAAC5'	1479

PCR reaction was carried out in Eppendorf tube containing: 2 µL Buffer (10x) with $(NH_4)_2SO_4$, 0.2 µL dNTPs (10 mM), 0.6 µL $MgCl_2$ (50 mM), 0.6 µL of each primer (forward and reverse) (5 mM), 14.9 µL H_2O, 0.1 µl of Taq Polymerase (5 U/µL) and 1 µl of DNA (20 ng/µL) of the material to be amplified, totalizing a volume of 20 µL for each reaction. Genomic DNA was amplified in an automated thermal cycler (Techne) according to the following program: initial denaturation at 94 ^oC for 5 minutes, followed by 35 cycles of: denaturation (1 minute at 94 ^oC), primer annealing (1 minute at 58 ^oC or 60 ^oC) and primer extension (1 minute at 72 ^oC), followed by a final extension at 72 ^oC for 10 min. PCR products were visualized on 1% agarose gel.

DNA sequencing of the PCR products was performed using a Sanger sequencer with the BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 2.0 (Applied Biosystems). DNA sequences were detected and analyzed on an automated ABI Prism 3100 automated analyzer (Applied Biosystems).

Sequence analysis

The sequences were submitted to Bioedit7.1 [22] and CodonCode Aligner (CodonCode Co., USA) for removal of low-quality ends and generation of contigs. Obtained contigs and singlets were annotated via BlastN against the NCBI non-redundant database. After the annotation, the sequences with similarity to defensins (e-value between $2{,}78.e^{-14}$ a $4{,}58.e^{-49}$) were translated into their respective peptide sequences via ORF finder [23] and submitted to a new annotation via BLASTp, followed by three-dimensional modeling.

Comparative modeling

For 3D modeling purposes, LOMETS server [24] was used in order to find the best template for the construction of the comparative models, where the defensins with the highest sequence identity were selected. Using Modeller 9.20 [25] a hundred models were constructed, and the final model was chosen according to the discrete optimized protein energy (DOPE) score that accesses the energy of the model and indicates the most probable structure. Finally, for validation of the models ProSa II [26] and PROCHECK [27] were used to analyze the dubbing quality and stereochemistry of the models.

RNA extraction and cDNA synthesis

Two separate groups of plants were used for this experiment, the control, and the test group. For the control group, three different individuals had one of their leaves stimulated exclusively with mechanical stress (light friction using a gauze). For the test group, three plants were submitted to the same mechanical stress followed by inoculation with the fungus *Lasiodiplodia theobromae*. This is a promiscuous phytopathogenic fungus that has been reported to infect plants of the Euphorbiaceae family such as *Hevea brasiliensis* [28]. The inoculum site was isolated in a humid microenvironment to prevent inoculated leave from falling and to protect from other environmental stresses. After 48 hours after inoculation, the plant material was removed and the leave tissued each individual (of both, control and test group) was stored at -80 °C.

The total RNA was extracted using approximately 200 mg of leaf tissue and an association of the CTAB-Acetate protocol [29] and the SV Total RNA Isolation System Kit (Promega), followed by a DNAse treatment. The samples were quantified using the NanoDrop 2000c spectrophotometer (ThermoFisher Scientific) and Qubit® fluorimeter (Life Technologies). RNA integrity was checked on 1% agarose gel (80 V, 120 A for 40 min) stained with Blue-green Loading Dye (LGC Biotechnology). Total RNA (1 µg) was converted to cDNA using the GoScript™ Reverse Transcription System (Promega) and Oligo (dT) primers, following the manufacturer's instructions, and was resuspended in a final volume of 60 µL. The cDNA synthesis included incubation in thermocycler (Techne) at 70 °C for 5 minutes (initial denaturation), 25 °C for 5 minutes (annealing) and 42 °C for 60 minutes (extension), followed by 70 °C for 15 minutes (inactivation of the enzyme). The samples were stored in a freezer at -20 °C until RT-qPCR reactions.

Quantitative Reverse Transcription PCR (RT-qPCR)

Expression validation by RT-qPCR was performed according to the MIQE (The Minimum Information for Publication of Quantitative RealTime PCR Experiments) guidelines [30] in order to increase the transparency and reliability of the obtained results. The RT-qPCR reactions were performed on CFX96 Touch Real-Time (BioRad) equipment using SYBR Green detection system. In all reactions, biological and technical triplicates were used, as well as a negative control (NTC) for each primer. The amplification efficiency values (E = 10-1 / slope), correlation coefficient (R), interception (y) and slope were calculated by the standard curve method using serial dilutions (1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). The reaction mixture comprised 1 µl of cDNA, 5 µL of Go-Taq SYBR Green qPCR Master Mix 2x (Promega), 3.4 µL ultrapure water and 0.6 µL of each primer (5 µM) in an end volume of 10 µL. The reactions were subjected to an initial denaturation step of 95 °C for 2 min followed by 40 cycles at 95 °C for 15 sec and 60 °C for 60 sec using 96 well plates. Dissociation curves were analyzed at 65-95 °C for 10 min. For the relative expression analyzes, three reference genes were used to normalize the results. The relative gene expression levels were evaluated with the REST tool (version 2.0.13) that relies on the ΔΔCq method [31],

which is based on pairwise comparisons using randomization and bootstrapping techniques - Pairwise Fixed Reallocation Randomization Test [32].

In silico expression

To evaluate the presence of expressed defensins on *M. esculenta*, we conducted a de novo assembly for six experimental paired-end RNA-Seq data (Illumina HiSeq 2500) available on SRA (https://www.ncbi.nlm.nih.gov/sra) database [33]. The experiments included transcripts obtained from roots colonized by *Rhizophagus irregularis* (SRR7447848, SRR7447849 and SRR7447850) and leaves (SRR6748426, SRR6748427 and SRR6748428), totalizing 207,662,384 reads to be assembled. The sequencing quality was assessed using FASTQC (0.11.8). Data was filtered using Trimmomatic v0.30, including removal of adaptor sequences, truncated sequence reads, and reads with pair end quality < 30. All reads were combined to assemble a transcriptome for cassava using Trinity [34], aiming to reconstruct transcript sequences in a manner that reflects the original molecules. The assembly quality was evaluated by N50 parameter.

The screening of coding genes for defensins in *M. esculenta* assembled transcriptome was based on probabilistic models based on the Hidden Markov Model [35], through the HMMER toolkit, an inference that allows greater efficiency in homologues identification [36]. The defensin HMM profiles used on our search were available on CAMP database (Collection of Anti-Microbial Peptides; www.camp.bicnirrh.res.in/), all results under the cut-off were analyzed (< e^{-03}). To enlarge the number of putative defensins identified in our transcriptome a second step was conducted, using the putative defensin identified as a ‘seed sequence’ in a BLASTn [37] against assembled transcriptome (cut-off of e^{-05}). The sequences retrieved after first BLASTn interaction were used to a second round of search, to maximize the number of identified sequences. Then the sequences obtained were characterized in the same method as described above.

Results

Sequence selection and characterization

Search using 55 seed sequences against the *M. esculenta* genome allowed the identification of eight candidate defensin sequences, five of them with similarities ranging from 65.9 - 84.8%, while the remaining three sequences had similarities from 7.5 to 23.5%. Of the eight cassava defensin candidates, three (XP_021622697.1; XP_021611651.1; XP_021607184.1) lacked a conserved domain, being thus removed from the further analyzes. The remaining five sequences showed similarity throughout the characterization of candidate sequences with the same pattern in relation to the amount of disulfide bridges (four), in the location of the cysteine (Cys1 - Cys8, Cys2 - Cys5, Cys3 - Cys6, Cys4 - Cys7) in the secondary structure with an α-helix and three β-sheets, in addition to presenting a single expected gamma-thionin domain (**Figure 12)**.

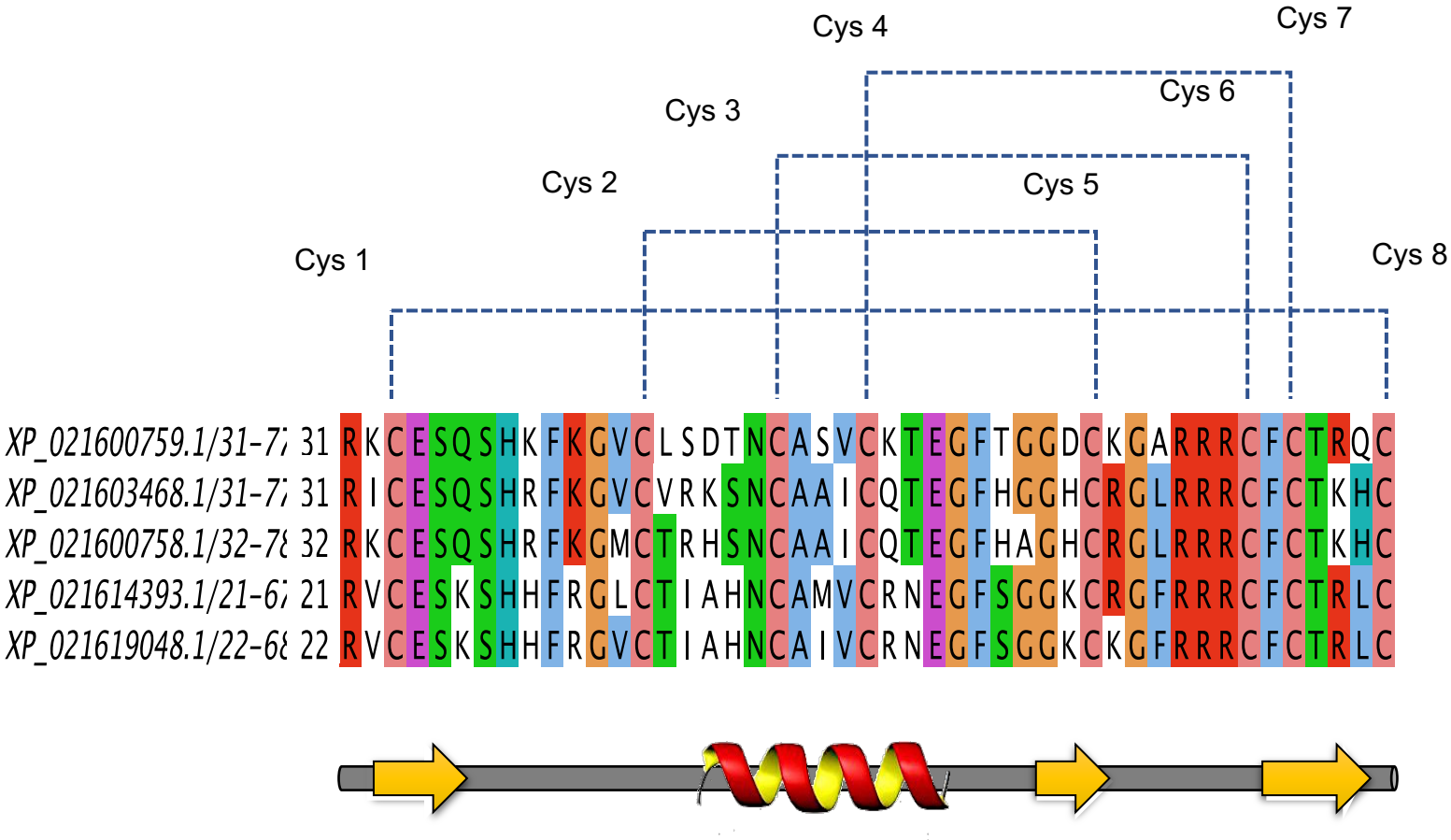

Figure 12 - Alignment of the conserved domain region of five defensin candidates of *M. esculenta* defensins. Dotted lines above the alignment represent the four predicted disulfide bonds. Conserved amino acids are highlighted. Below is the representation of the secondary structure with the yellow arrows representing ß-ribbons and in red/yellow the α-helix. Abbreviation: Cys: cysteine

All five defensin candidates exhibited predicted *in silico* antimicrobial activity based on the independent evaluation using four algorithms (SVM, RFC, ANN, and DAC) as shown in **Table 5**.

Table 5 - Antimicrobial screening of the *M. esculenta* defensin sequences, with the prediction of four different algorithms.

Number	ID	*SVM	*RFC	*ANN	*DAC
1	XP_021600759.1	0.979	0.8065	AMP*	0.995
2	XP_021603468.1	0.972	0.958	AMP*	1.000
3	XP_021600758.1	0.929	0.767	AMP*	0.997
4	XP_021614393.1	0.973	0.884	AMP*	0.999
5	XP_021619048.1	0.986	0.981	AMP*	1.000
6	XP_021622697.1	0.806	0.5375	AMP*	0.918
7	XP_021611651.1	0.015	0.184	AMP*	0.000
8	XP_021607184.1	0.098	0.112	NAMP*	0.120

*Support Vector Machine (SVM), Random Forest Classifier (RFC), Artificial Neural Network (ANN), Discriminant Analysis Classifier (DAC). AMP: Antimicrobial Peptide. NAMP: No Antimicrobial Peptide.

Support Vector Machine is a specific type of supervised method of machine learning, aiming to classify data points by maximizing the margin between classes in a high-dimensional space. Random Forest is a non-parametric tree-based approach that combines the ideas of adaptive neighbors with bagging for efficient adaptive data inference. Neural Network is an information processing paradigm inspired by how a biological nerve system process information. It is composed of highly interconnected processing elements (neurons or nodes) working together to solve specific problems. Discriminant Analysis finds a set of prediction equations based on independent variables that are used to classify individuals into groups. [38–40]. After analysis with the four algorithms, the five candidate sequences obtained values compatible with antimicrobial peptide, where sequence number 5 obtained the best values in all the algorithms when compared to the other four sequences (1-4).

Amplification and analysis of sequences

Of the primers designed and tested (**Table 5**), only Me5 did not amplify even using different annealing temperatures (Def-Me4). After sequence clustering Me2 and Me4 sequences aligned as a single contig (Def-Me1), whereas Me1 (Def-Me2) and Me3 (Def-Me3) remained as singlets. All three remaining sequences had their conserved domain, disulfide bonds and secondary structure analyzed (**Figure 2-2**), as well as prediction of antimicrobial activity (**Table 6**).

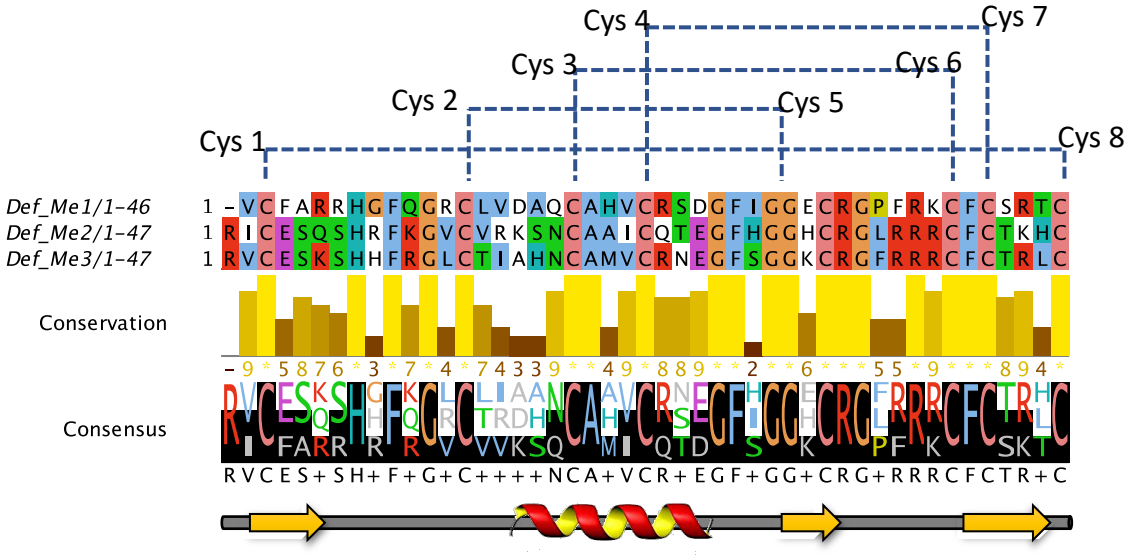

Figure 13 - Alignment of defensins amplified from the genome of *M. esculenta*. The dotted lines above the alignment represent disulfide bonds. Most conserved amino acids sequences of the gamma-thionin domain are highlighted in color. Yellow/brown bars (below the alignment) shows the degree of conservation ranging from 1 to 10 (represented by *). Letters at the bottom of the figure regard amino acid consensus/conservation. The + sign means that no amino acid predominated in that position. The secondary structure is represented at the base of the figure, where yellow arrows represent ß-sheets and the α-helix is highlighted in red/yellow. Abbreviation: Cys: cysteine

Table 6 - Prediction of antimicrobial activity of *M. esculenta* genomic sequences using four algorithms.

Number	ID	*SVM	*RFC	*ANN	*DAC
1	Def-Me1	0.964	0.838	AMP	1.000
2	Def-Me2	0.979	0.8065	AMP	0.995
3	Def-Me3	0.929	0.767	AMP	0.997

*Support Vector Machine (SVM), Random Forest Classifier (RFC), Artificial Neural Network (ANN), Discriminant Analysis Classifier (DAC). AMP: Antimicrobial Peptide.

Results predict antimicrobial activity for all three cassava genomic sequences, with Def-Me1 presenting higher prediction values in relation to Def-Me2 and Def-Me3.

Comparative modeling

Sequence modeling was based on resolved structures available in the Protein Data Bank (PDB) using the program Modeller 9.15 (**Figure 14**). For Def-Me1 the best selected template was a *Triticum aestivum* defensin (PDB code 1GPS), sharing 47.8% of identity. However, Def-Me2 and Def-Me3 were most similar to *Medicago truncatula* defensin (PDB code 2LR3), sharing sharing 68% and 74.5% of identity respectively. After stereochemical analysis of the amino acids, it was possible to observe that 90% of the amino acids were in the favorable positions for all models (**Figure 14**). The Z-score indicated that all the models were within the score range typically found for defensins of similar size.

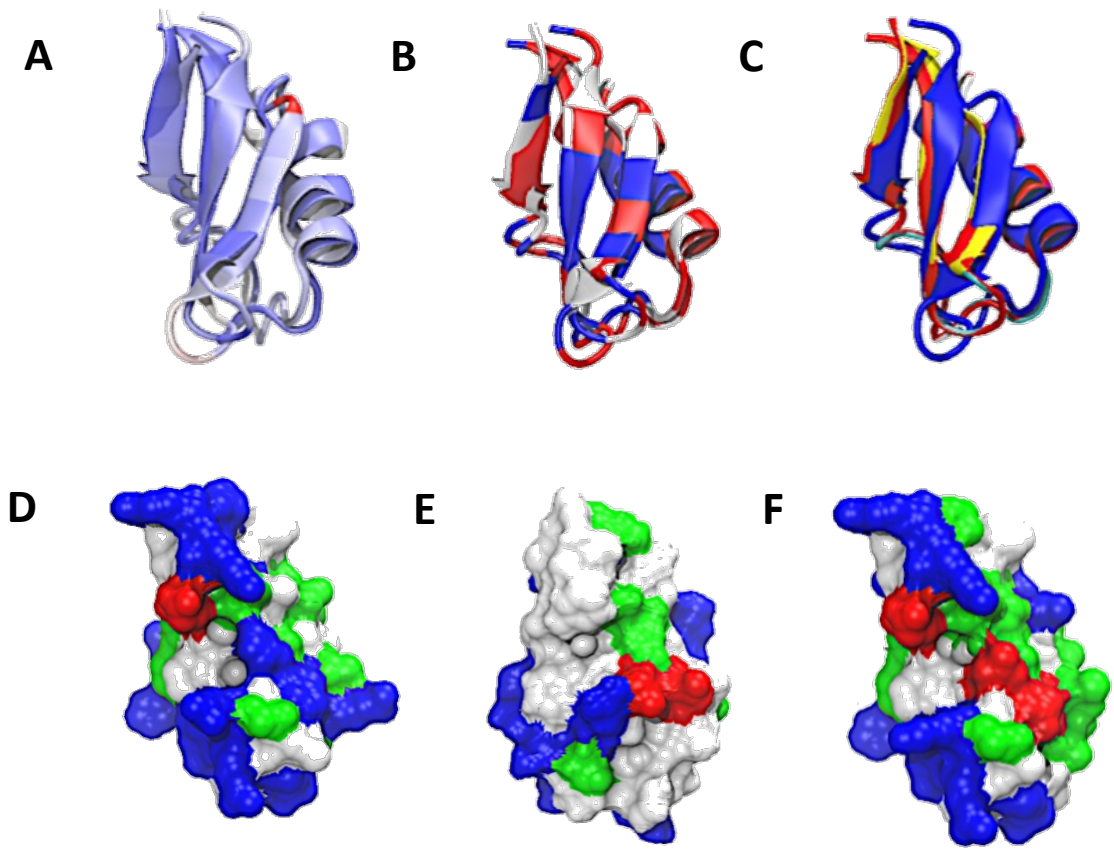

Figure 14 - Three-dimensional models of Def-Me1, Def-Me2, and Def-Me3. **A**, **B** and **C** show the structural alignment of the three *M. esculenta* defensin models with RMSD of 1.53 nm realized in VMD (Visual Molecular Dynamics). **(A)** The color closer to blue means that the regions are more structurally similar, whereas the color closer to the red means the regions are structurally more distinct. **(B)** In blue regions where amino acids are identical, in blank amino acids with physicochemical similarity and in red regions with different amino acids. **(C)** Def-Me1 blue, Def-Me2 red and Def-Me3 yellow. In **D**, **E** and **F** the representation of the aminoacid surface charge of Def-Me1, Def-Me2, and Def-Me3 respectively. Blue represents positively charged residues, red negative charge, white apolar and green neutral polar.

Expression analysis in RT-qPCR

All primer pairs designed (**Table 7**) for the target sequences (Def-Me1, Def-Me2, Def-Me3 and Def-Me4) and reference genes (EF1α, UBQ and VATP16) amplified the cDNA samples used (**Figure S2-1**), with a single peak in the melting curve (**Figure S2-2**), confirming their specificity, as well as negative controls (no template control, NTC) without amplification (**Figure 15**). However, the EF1α gene still showed a small dimer formation in the NTC and had, therefore, the primer concentration reduced. The mean Cqs (Quantification cycle) ranged from 26.07 for the Def-Me2 gene (most

expressed) to 32.99 for the Def-Me4 gene (less expressed) (**Table 7**; **Figure S2-1**). Efficiencies ranged from 90.3% to 107.8%, with correlation coefficients between 0.954 and 1,000 and slope from -3,147 to -3,580 (**Table 7**, **Figure S2-3**). All results obtained are in accordance with the considerations of the MIQE Guidelines.

In the relative expression analyzes Def-Me2 and Def-Me3 genes were down-regulated 0.723 and 0.280 fold relative to their uninoculated controls, respectively (**Figure 15**). Def-Me1 and Def-Me4 genes no altered expression was observed considering the stress applied (**Figure 15**).

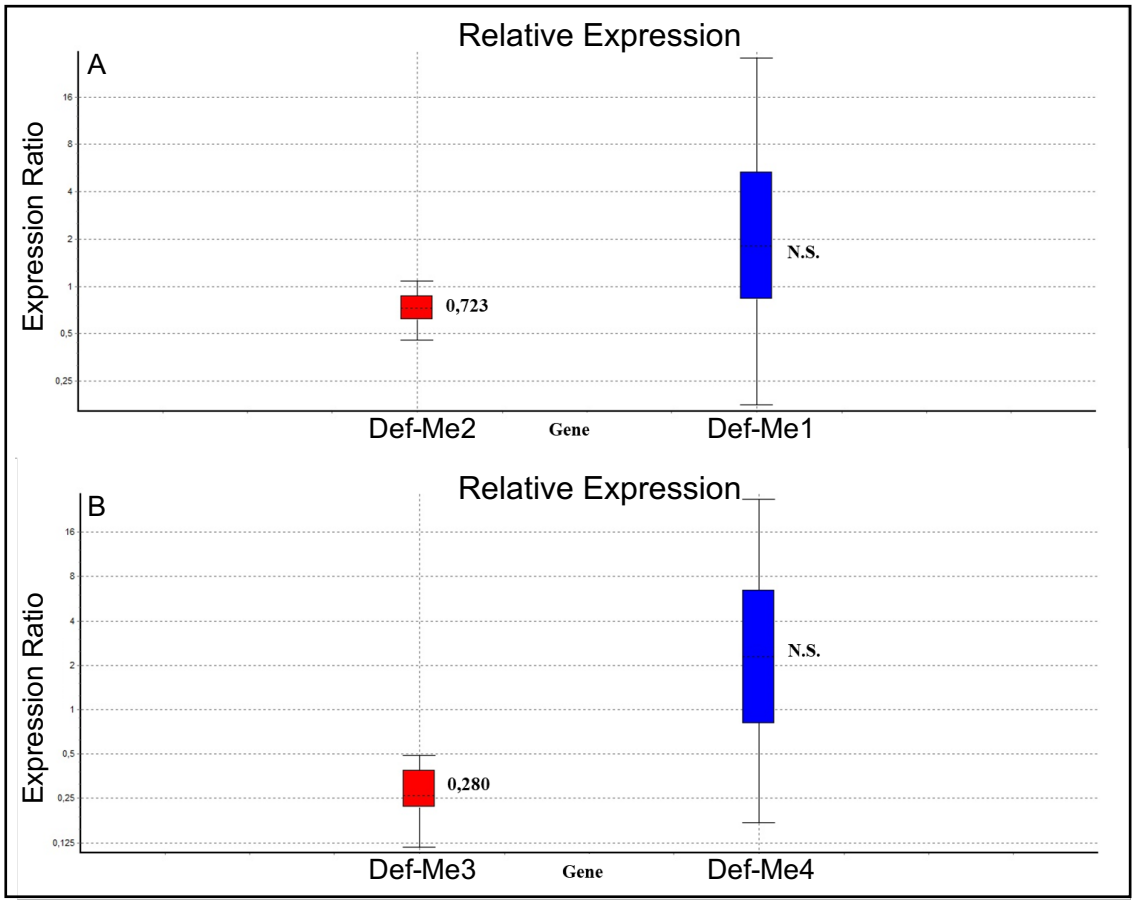

Figure 15 - Relative expression of defensin genes in *M. esculenta* leaves infected by the fungus *L. theobromae*, normalized with reference genes EF1α (U 1Q elongation factor), UBQ (Ubiquitin) and VATP16 (V-type proton ATPase). The relative expression values were calculated by the REST software (v.2.0.13) [32]. (A) Def-Me1 and Def-Me2. (B) Def-Me3 and Def-Me4.

Table 7 - Parameters obtained in standard curve analyzes for genes of interest and reference.

Genes	Primers (forward/reverse)	Accession	Tm (°C)	Amplicon (bp)	Média Cq	E (%)	R^2	Slope	Y intercept
Target genes									
Def-Me1	GCTTGTAGTTGGGCAGCAGGAG (F) TTTCCACAGCATCTCTCCATAGCC (R)	XP_021600759.1	83.0	113	33.11	92.6	0.989	-3.512	22,970
Def-Me2	TGCTGCCCATTGAAGCTAGGG (F) GAAACAGCGGCGACGGAATC (R)	XP_021603468.1	79.5	143	26.07	94.3	0.995	-3.466	19.664
Def-Me3	CCATCGGTTCAAGGGCGTATG (F) AGTGTCCTCCTTGGAAGCCTTCTG (R)	XP_021600758.1	80.5	80	23.96	90.3	1.000	-3.580	21.580
Def-Me4	GGTGCTGCCCATTGAAGCTAGG (F) GAAACAGCGGCGACGGAATC (R)	XP_021619048.1	83.5	145	32.99	107.8	0.991	-3.147	28.587
Reference genes									
EF1α	GGTCATTGGTCATGTCGACTCTG (F) GCACCCAGGCATACTTGAATGACC (R)	XM_021751760.1	81.5	146	27.22	104.7	0.985	-3.213	23,177
UBQ	CCAAGGAACTTCAGATTGCTG (F) GTCATCACCATCATCCATTCC (R)	XM_021743739.1	78.5	93	26.03	105.6	0.983	-3.195	23.350
VATP16	CTTCTCCTGTATGGGAGCTG (F) CCATAACAACTGGTACAATCGAC (R)	XM_021745527.1	80.5	113	30.22	106.9	0.954	-3.166	28.267

Tm: melting temperature (ºC); Cq: Quantification cycle; E: Efficiency of the primers; R^2: Correlation coefficient. Def-Me1; Def-Me2; Def-Me3; Def-Me4; VATP16: V-type proton ATPase; EF1α: Elongation factor 1α; UBQ: Ubiquitin.

***In silico* expression.**

From the raw reads obtained on SRA database (207,662,384), after the removal of adapters, we obtained 198,256,884 clean reads, representing 95,47% of the reads sequenced. The analyzed assembled transcripts presented N50 equal to 1883 bp and average GC content of 39.04%. After assembly of the transcriptome, 228,363 transcripts were obtained, associated to a total of 151,872 unigenes.

The HMMER search using defensin HMM profiles from CAMP, returned one candidate sequence (Me_DN129826_c0_g1_i1) from the assembled cassava transcriptome. This sequence, together with other cassava defensins previously isolated in this work, were used as seeds in a BLASTn against the assembled transcriptome. Three defensin candidates (Me_DN106656_c0_g1_i1, Me_DN251_c1_g1_i1 and Me_DN5718_c0_g1_i1) were retrieved after this step. The second BLAST interaction HMMER search returned just one defensin candidate (Me_DN56765_c0_g1_i1) (**Figure 16**).

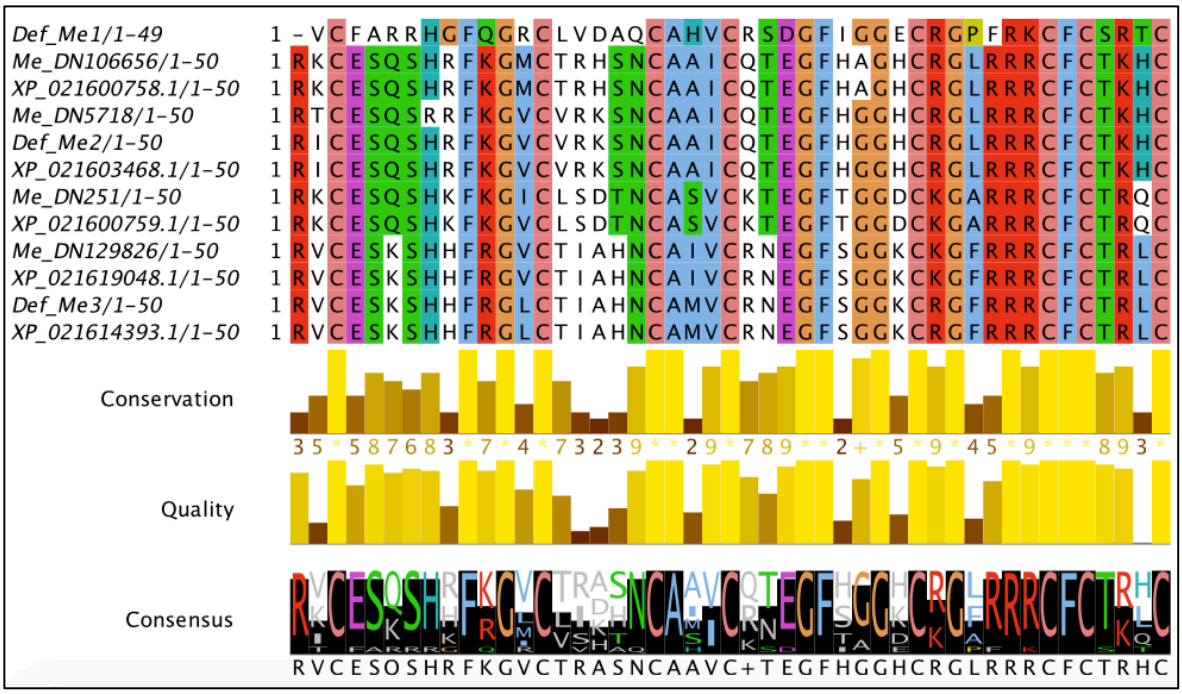

Figure 16 - Alignment of all the defensins found in the *M. esculenta* transcriptome (cultivar BRA337, CM6438-14 and Xinxuan), in the genome of *M. esculenta* V6 (cultivar AM560-2) and with the amplified and sequenced *M. esculenta* (cultivars Santo Estevão).

Discussion

Selection and characterization

The diversity of AMPs discovered in plants is so marked that it is difficult to categorize these peptides, except based on their structural features [11]. This diversity becomes more striking when it is observed that many AMP genes are specific to particular plant species or families [11,41–44]. Among these peptides, the defensins stand out as being small (45 to 54 amino acids) 5-7 kDa molecular weight proteins, generally cationic, rich in cysteine residues that generally form between four and five disulfide bridges with a highly conserved three-dimensional structure, usually having three antiparallel β-strands and one α-helix [11,12]. Indeed, all candidate defensin mature peptides) of *M. esculenta* ranged from 46 to 47 amino acids with a molecular weight ranging from 5.1 to 5.4 kDa. In addition, all sequences showed conserved γ-thionin domain, characteristic of plant defensins. With respect to the cysteine residues, all sequences showed a conserved pattern of eight cysteines forming four disulfide bonds. This binding specificity allows these AMPs to act more directly against particular groups of pathogens.

Defensins act as potent antifungal peptides interacting with specific sphingolipids present in the fungal cell membrane [11,45–48]. In addition, their small size and the high number of disulfide bonds found in plant AMPs allow defensins to fold into a compact size with important structural stability. Moreover, the secondary structure prediction for all *M. esculenta* candidate defensins predicted three β-sheets and one α-helix, consistent with the alignment of different plant defensins performed by Rogozhin et al. 2018 [49], showing that their tertiary structure is highly conserved even considering different plant species.

Amplification of genomic sequences

Selective pressure through plant-pathogen interactions throughout evolution provided plant AMPs with a vast repertoire of amino acid sequences through non-synonymous mutations, generating new interactions and different mechanisms of action [50]. In fact, the three amplified sequences (Def-Me1, 2 and 3) had a similarity

higher than 66%, although taking into account the physicochemical similarity of the amino acids, this similarity can reach more than 70%. In addition, differences between the amino acid sequences generated different values in the predicted antimicrobial characterization (**Table 7**). Therefore, the amplification of these genomic *M. esculenta* defensins was essential in order to check possible mutations that these sequences acquired since the material collected in this work (variety Santo Estevão) could present genomic differences when compared to the species with the genome available in Genbank (variety AM560-2).

Comparative modeling

Defensins have been studied extensively in the last decades due to their broad spectrum against pathogens and various mechanisms of action [12]. Due to their potential, many defensins had their tertiary structure resolved and are currently available in the Protein Data Bank (PDB), where some 177 structures are deposited from diverse organisms (2018-11-08). For plant defensins (γ-thionin) the number of resolved structures is much lower compared to the available number of models of mammal defensins (α and β). In the absence of experimentally determined structures, the models of protein structures available in the databases are often valuable in generating testable hypotheses, and are generally used for comparative modeling. This type of comparison relies on structural information of related proteins to guide the modeling procedure, useful to produce reliable models of structures with similar domains [51]. As there is no experimentally resolved *M. esculenta* defensin deposited in structural databases such as PDB, it was necessary to use similar structures to perform comparative modeling (*T. aestivum* and *M. truncatula)*. The three models generated for *M. esculenta* defensins presented structural similarity with an RMSD of 1.53 nm, as expected since the structure is three to ten times more conserved than the amino acid sequence [52]. In addition, in the alignment of the three structures (**Figure 15**) it is possible to observe that regions devoid of organized structure are regions of greaters alignments deviations, what reinforces the theory that β-sheets are much less likely to achieve this due to a higher free-energy barrier separating the folded and unfolded state [52]. In addition to its conserved tertiary structure, defensins exhibit a cationic surface charge that provides an electrostatic bond to the negative membrane

charge of the pathogens. Therefore, the amphipathic distribution of the amino acid residues directly interferes with their mechanism of action, giving these peptides an antimicrobial activity against a broad spectrum of pathogens [10]. Despite the structural similarity, the amino acid differences between them can increase or decrease based on Grand average of hydropathicity (GRAVY), as in the case of Def-Me1, having a GRAVY of -0.115, Def-Me2 with -0.555, and Def-Me3 with -0.340. The increase of GRAVY value indicates higher hydrophobicity.

Expression analysis in RT-qPCR

Plant defensins, in general, work at the level of innate non-specific immunity against varied pathogens and are not related to the specific effector triggered immunity involving R-Avr protein interactions. Despite the several literature reports on defensin antifungal activity, some appear to exhibit tissue-specific expression, as in the case of Def-Me1 and Def-Me4 that maintained the same expression levels in leaf, after inoculation with the pathogen. In contrast to these defensins, Def-Me2 and Def-Me3 were down-regulated, suggesting association to other pathogen or distinct tissue-specificity. This situation was reported in the literature, as in the case of the defensins of *Nicotiana alata* (NaD1) and *Petunia hybrida* (PhD1 and PhD2) that exhibited high expression levels in flowers (during early developmental stages), but lower expression in seeds [53]. Similarly, defensins identified in *Picea abies* (SPI1) [54] and *Picea glauca* (PgD5) [55] were reported to be expressed exclusively in roots. An *in silico* evaluation of the sugarcane transcriptome [56] uncovered that from 17 expressed defensins, 12 exhibited tissue or stage specificity, being mostly represented in reproductive tissues.

Tesfaye et al. (2013) [57] designed a microarray experiment containing a probe set for 317 and 684 defensins of *Arabidopsis thaliana* and *Medicago truncatula*, respectively, to catalog their expression in various plant organs at different stages of development in inflorescences (representing a pool of floral development stages, with the most mature at anthesis) and during symbiotic and pathogenic associations. The analysis provided evidence for the transcription of 71% and 90% of the defensins identified in *Arabidopsis* and *Medicago*, respectively, including many of the annotated genes that had no expression information. Some defensins were significantly up-regulated in *Arabidopsis* leaves inoculated with *Alternaria brassicicola* or

Pseudomonas syringae. Among these, the authors reported that some were dependent on the signaling of jasmonic acid or were associated with specific types of immune responses. The authors also noted that there were notable differences in the expression patterns of the defensin genes of *Arabidopsis* and *Medicago*, since most *Arabidopsis* defensins were expressed in inflorescences, while only a few exhibited increased expression in roots. In contrast, *Medicago* defensins were more prominently expressed in nitrogen-fixing root nodules. Thus, marked differences in the temporal and spatial expression of defensin suggest distinct signaling pathways and distinct roles for these proteins in different species and plant tissues.

In silico expression

Using the HMMER3 pattern recognition software package it was possible to find and characterize cassava defensin transcripts. When aligned and compared the four defensins found in the *M. esculenta* transcriptome (variety BRA337) showed similarity with the five defensins found in *M. esculenta* V6 genome (variety AM560-2), as well as with the three defensins amplified and sequenced (from the Brazilian variety Santo Estevão), as shown in **Figure 16**. The alignment exhibits a conservation concerning the eight cysteines characteristic of defensins [8], as well as the GXCX3-9C motif, a region considered responsible for antifungal activity [58]. Although similar, some defensins presented mutated regions (**Figure 16**), possibly due to the peptide-pathogen coevolution, considered a evolutionary force to the arising of different defensin isoforms (including different sizes and amino acids composition) [50].

Conclusions and perspectives

Three new complete defensins were identified and isolated from cassava genome sequences, all of them with predicted antimicrobial activity. All sequences exhibited eight conserved cysteines as reported in other studies, whereas Def-Me1 presented a differentiated primary structure. However, the secondary structure of Def-Me1 was not altered, presenting three ß-sheet and one α-helix. Despite their structural

uniformity, each of identified defensin had peculiarities, mainly in regions of turn, known to present greater standard deviation between the structures.

Regarding their expression, the defensins tested presented constitutive or down-regulated expression after fungal inoculation, reinforcing their role in the preformed plant defense system, but also indicating tissue or stage specificity, as reported for other defensins in the literature. We intend to test their possible association to bacterial infection, in order to confirm a possible pathogen specificity.

Indeed, the broad spectrum of defensin activities, dynamic ability to evolve and broad mechanisms of action are characteristics that turn these peptides into promising weapons for plant defense and also significant candidates for agricultural and pharmaceutical purposes.

REFERENCE

[1] K.M. Olsen, SNPs, SSRs and inferences on cassava's origin, Plant Mol. Biol. 56 (2004) 517–526. doi:10.1007/s11103-004-5043-9.

[2] N. Nassar, C.S. Vizzotto, C.A. Schwartz, O.R. Pires, Cassava diversity in Brazil: the case of carotenoid-rich landraces, Genet. Mol. Res. GMR. 6 (2007) 116–121.

[3] T. Sánchez, D. Dufour, J.L. Moreno, M. Pizarro, I.J. Aragón, M. Domínguez, H. Ceballos, Changes in extended shelf life of cassava roots during storage in ambient conditions, Postharvest Biol. Technol. 86 (2013) 520–528. doi:10.1016/j.postharvbio.2013.07.014.

[4] S. Liu, I.M. Zainuddin, H. Vanderschuren, J. Doughty, J.R. Beeching, RNAi inhibition of feruloyl CoA 6′-hydroxylase reduces scopoletin biosynthesis and post-harvest physiological deterioration in cassava (*Manihot esculenta* Crantz) storage roots, Plant Mol. Biol. 94 (2017) 185–195. doi:10.1007/s11103-017-0602-z.

[5] R. Sayre, J.R. Beeching, E.B. Cahoon, C. Egesi, C. Fauquet, J. Fellman, M. Fregene, W. Gruissem, S. Mallowa, M. Manary, B. Maziya-Dixon, A. Mbanaso, D.P. Schachtman, D. Siritunga, N. Taylor, H. Vanderschuren, P. Zhang, The BioCassava Plus Program: Biofortification of Cassava for Sub-Saharan Africa, Annu. Rev. Plant Biol. 62 (2011) 251–272. doi:10.1146/annurev-arplant-042110-103751.

[6] V. Lebot, J. Kaoh, Estimating sugars in 212 landraces and hybrids of sweet type cassava (*Manihot esculenta* Crantz, Euphorbiaceae), Genet. Resour. Crop Evol. 64 (2017) 2093–2103. doi:10.1007/s10722-017-0499-z.

[7] J.M. Ehiobu, G.I. Ogu, Phytochemical Content and *In Vitro* Antimycelial Efficacy of *Colocasia esculenta* (L), *Manihot esculenta* (Crantz) and *Dioscorea rotundata* (Poir) Leaf Extracts on *Aspergillus niger* and *Botryodiplodia theobromae*, J. Hortic. Plant Res. 1 (2018) 9–18. doi:10.18052/www.scipress.com/JHPR.1.9.

[8] A.M. Benko-Iseppon, S.L. Galdino, T. Calsa, E.A. Kido, A. Tossi, L.C. Belarmino, S. Crovella, Overview on plant antimicrobial peptides, Curr. Protein Pept. Sci. 11 (2010) 181–188.

[9] P. Tavormina, B. De Coninck, N. Nikonorova, I. De Smet, B.P.A. Cammue, The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions, Plant Cell. 27 (2015) 2095–2118. doi:10.1105/tpc.15.00440.

[10] R.K. Goyal, A.K. Mattoo, Plant antimicrobial peptides, in: Host Def. Pept. Their Potential Ther. Agents, Springer, 2016: pp. 111–136.

[11] M.L. Campos, C.M. de Souza, K.B.S. de Oliveira, S.C. Dias, O.L. Franco, The role of antimicrobial peptides in plant immunity, J. Exp. Bot. 69 (2018) 4997–5011. doi:10.1093/jxb/ery294.

[12] T.L. Cools, C. Struyfs, B.P. Cammue, K. Thevissen, Antifungal plant defensins: increased insight in their mode of action as a basis for their use to combat fungal infections, Future Microbiol. 12 (2017) 441–454. doi:10.2217/fmb-2016-0181.

[13] J. Tam, S. Wang, K. Wong, W. Tan, Antimicrobial Peptides from Plants, Pharmaceuticals. 8 (2015) 711–757. doi:10.3390/ph8040711.

[14] W.F. Porto, L. Irazazabal, E.S.F. Alves, S.M. Ribeiro, C.O. Matos, Á.S. Pires, I.C.M. Fensterseifer, V.J. Miranda, E.F. Haney, V. Humblot, M.D.T. Torres, R.E.W. Hancock, L.M. Liao, A. Ladram, T.K. Lu, C. de la Fuente-Nunez, O.L. Franco, *In silico* optimization of a guava antimicrobial peptide enables combinatorial exploration for peptide design, Nat. Commun. 9 (2018). doi:10.1038/s41467-018-03746-3.

[15] R. Hammami, J. Ben Hamida, G. Vergoten, I. Fliss, PhytAMP: a database dedicated to antimicrobial plant peptides, Nucleic Acids Res. 37 (2009) D963–D968. doi:10.1093/nar/gkn655.

[16] T.N. Petersen, S. Brunak, G. von Heijne, H. Nielsen, SignalP 4.0: discriminating signal peptides from transmembrane regions, Nat. Methods. 8 (2011) 785–786. doi:10.1038/nmeth.1701.

[17] A. Marchler-Bauer, S.H. Bryant, CD-Search: protein domain annotations on the fly, Nucleic Acids Res. 32 (2004) W327–W331. doi:10.1093/nar/gkh454.

[18] A. Ceroni, A. Passerini, A. Vullo, P. Frasconi, DISULFIND: a disulfide bonding state and cysteine connectivity prediction server, Nucleic Acids Res. 34 (2006) W177–W181. doi:10.1093/nar/gkl266.

[19] K. Weising, H. Nybom, M. Pfenninger, K. Wolff, G. Kahl, DNA fingerprinting in plants: principles, methods, and applications, CRC press, 2005.

[20] A.-M. Benko-Iseppon, P. Winter, B. Huettel, C. Staginnus, F.J. Muehlbauer, G. Kahl, Molecular markers closely linked to fusarium resistance genes in chickpea show significant alignments to pathogenesis-related genes located on *Arabidopsis* chromosomes 1 and 5, Theor. Appl. Genet. 107 (2003) 379–386. doi:10.1007/s00122-003-1260-x.

[21] A. Untergasser, H. Nijveen, X. Rao, T. Bisseling, R. Geurts, J.A.M. Leunissen, Primer3Plus, an enhanced web interface to Primer3, Nucleic Acids Res. 35 (2007) W71–W74. doi:10.1093/nar/gkm306.

[22] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, in: Nucleic Acids Symp. Ser., [London]: Information Retrieval Ltd., c1979-c2000., 1999: pp. 95–98.

[23] P. Stothard, The Sequence Manipulation Suite: JavaScript Programs for Analyzing and Formatting Protein and DNA Sequences, BioTechniques. 28 (2000) 1102–1104. doi:10.2144/00286ir01.

[24] S. Wu, Y. Zhang, MUSTER: Improving protein sequence profile-profile alignments by using multiple sources of structure information, Proteins Struct. Funct. Bioinforma. 72 (2008) 547–556. doi:10.1002/prot.21945.

[25] B. Webb, A. Sali, Comparative Protein Structure Modeling Using MODELLER, Curr. Protoc. Bioinforma. 47 (2014) 5.6.1-5.6.32. doi:10.1002/0471250953.bi0506s47.

[26] M. Wiederstein, M.J. Sippl, ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins, Nucleic Acids Res. 35 (2007) W407–W410. doi:10.1093/nar/gkm290.

[27] R.A. Laskowski, M.W. MacArthur, D.S. Moss, J.M. Thornton, PROCHECK: a program to check the stereochemical quality of protein structures, J. Appl. Crystallogr. 26 (1993) 283–291. doi:10.1107/S0021889892009944.

[28] V.S. Paes, P.L.M. Soares, O.O.O. Mari, J.M. Santos, A. Goes, Primeiro relato de uma doença complexa da seringueira (*Hevea brasiliensis*) causada por *Lasiodiplodia theobromae* e *Pratylenchus brachyurus* no Brasil, An. Uberlândia Nematol. Bras. (2012) 168–169.

[29] L. Zhao, Q. Ding, J. Zeng, F.-R. Wang, J. Zhang, S.-J. Fan, X.-Q. He, An Improved CTAB-Ammonium Acetate Method for Total RNA Isolation from Cotton: CTAB-Ammonium Acetate Method for RNA Isolation from Cotton, Phytochem. Anal. 23 (2012) 647–650. doi:10.1002/pca.2368.

[30] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Clin. Chem. 55 (2009) 611–622. doi:10.1373/clinchem.2008.112797.

[31] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method, Methods. 25 (2001) 402–408. doi:10.1006/meth.2001.1262.

[32] M.W. Pfaffl, Relative expression software tool (REST(C)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res. 30 (2002) 36e – 36. doi:10.1093/nar/30.9.e36.

[33] R. Leinonen, H. Sugawara, M. Shumway, on behalf of the International Nucleotide Sequence Database Collaboration, The Sequence Read Archive, Nucleic Acids Res. 39 (2011) D19–D21. doi:10.1093/nar/gkq1019.

[34] B.J. Haas, A. Papanicolaou, M. Yassour, M. Grabherr, P.D. Blood, J. Bowden, M.B. Couger, D. Eccles, B. Li, M. Lieber, M.D. MacManes, M. Ott, J. Orvis, N. Pochet, F. Strozzi, N. Weeks, R. Westerman, T. William, C.N. Dewey, R. Henschel, R.D. LeDuc, N. Friedman, A. Regev, *De novo* transcript sequence reconstruction from RNA-seq

using the Trinity platform for reference generation and analysis, Nat. Protoc. 8 (2013) 1494–1512. doi:10.1038/nprot.2013.084.

[35] S.R. Eddy, Accelerated Profile HMM Searches, PLoS Comput. Biol. 7 (2011) e1002195. doi:10.1371/journal.pcbi.1002195.

[36] S.R. Eddy, A new generation of homology search tools based on probabilistic inference, Genome Inform. Int. Conf. Genome Inform. 23 (2009) 205–211.

[37] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[38] Y. Lin, Y. Jeon, Random Forests and Adaptive Nearest Neighbors, J. Am. Stat. Assoc. 101 (2006) 578–590. doi:10.1198/016214505000001230.

[39] M. Karasuyama, I. Takeuchi, Multiple Incremental Decremental Learning of Support Vector Machines, IEEE Trans. Neural Netw. 21 (2010) 1048–1059. doi:10.1109/TNN.2010.2048039.

[40] S. Ding, H. Li, C. Su, J. Yu, F. Jin, Evolutionary artificial neural networks: a review, Artif. Intell. Rev. 39 (2013) 251–260. doi:10.1007/s10462-011-9270-6.

[41] K.A.T. Silverstein, Genome Organization of More Than 300 Defensin-Like Genes in *Arabidopsis*, Plant Physiol. 138 (2005) 600–610. doi:10.1104/pp.105.060079.

[42] K.A.T. Silverstein, W.A. Moskal, H.C. Wu, B.A. Underwood, M.A. Graham, C.D. Town, K.A. VandenBosch, Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants: *Under-predicted cysteine-rich peptides in plants*, Plant J. 51 (2007) 262–280. doi:10.1111/j.1365-313X.2007.03136.x.

[43] C.W. Gruber, A.G. Elliott, D.C. Ireland, P.G. Delprete, S. Dessein, U. Goransson, M. Trabi, C.K. Wang, A.B. Kinghorn, E. Robbrecht, D.J. Craik, Distribution and Evolution of Circular Miniproteins in Flowering Plants, Plant Cell online. 20 (2008) 2471–2483. doi:10.1105/tpc.108.062331.

[44] M. Oliveira-Lima, A. Benko-Iseppon, J. Neto, S. Rodriguez-Decuadro, E. Kido, S. Crovella, V. Pandolfi, Snakin: Structure, Roles and Applications of a Plant Antimicrobial Peptide, Curr. Protein Pept. Sci. 18 (2017) 368–374. doi:10.2174/1389203717666160619183140.

[45] K. Thevissen, R.W. Osborn, D.P. Acland, W.F. Broekaert, Specific Binding Sites for an Antifungal Plant Defensin from Dahlia (*Dahlia merckii*) on Fungal Cells Are Required for Antifungal Activity, Mol. Plant. Microbe Interact. 13 (2000) 54–61. doi:10.1094/MPMI.2000.13.1.54.

[46] M. Wilmes, B.P.A. Cammue, H.-G. Sahl, K. Thevissen, Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation, Nat. Prod. Rep. 28 (2011) 1350. doi:10.1039/c1np00022e.

[47] N. Hegedüs, F. Marx, Antifungal proteins: More than antimicrobials?, Fungal Biol. Rev. 26 (2013) 132–145. doi:10.1016/j.fbr.2012.07.002.

[48] A.F. Lacerda, Ã.A.R. Vasconcelos, P.B. Pelegrini, M.F. Grossi de Sa, Antifungal defensins and their role in plant defense, Front. Microbiol. 5 (2014). doi:10.3389/fmicb.2014.00116.

[49] E. Rogozhin, D. Ryazantsev, A. Smirnov, S. Zavriev, Primary Structure Analysis of Antifungal Peptides from Cultivated and Wild Cereals, Plants. 7 (2018) 74. doi:10.3390/plants7030074.

[50] J.H. Nickling, T. Baumann, F.-J. Schmitt, M. Bartholomae, O.P. Kuipers, T. Friedrich, N. Budisa, Antimicrobial Peptides Produced by Selective Pressure Incorporation of Non-canonical Amino Acids, J. Vis. Exp. (2018). doi:10.3791/57551.

[51] B. Webb, A. Sali, Protein Structure Modeling with MODELLER, in: M. Kaufmann, C. Klinger, A. Savelsbergh (Eds.), Funct. Genomics, Springer New York, New York, NY, 2017: pp. 39–54. doi:10.1007/978-1-4939-7231-9_4.

[52] K. Illergård, D.H. Ardell, A. Elofsson, Structure is three to ten times more conserved than sequence-A study of structural response in protein cores, Proteins Struct. Funct. Bioinforma. 77 (2009) 499–508. doi:10.1002/prot.22458.

[53] F.T. Lay, Isolation and Properties of Floral Defensins from Ornamental Tobacco and Petunia, PLANT Physiol. 131 (2003) 1283–1293. doi:10.1104/pp.102.016626.

[54] P. Sharma, A. Lönneborg, Isolation and characterization of a cDNA encoding a plant defensin-like protein from roots of Norway spruce, Plant Mol. Biol. 31 (1996) 707–712.

[55] P. Picart, A. Pirttilä, D. Raventos, H.-H. Kristensen, H.-G. Sahl, Identification of defensin-encoding genes of *Picea glauca*: characterization of PgD5, a conserved spruce defensin with strong antifungal activity, BMC Plant Biol. 12 (2012) 180. doi:10.1186/1471-2229-12-180.

[56] L.C. Belarmino, P.V.S.Z. Capriles, S. Crovella, L.E. Dardenne, A.M. Benko-Iseppon, EST-Database Search of Plant Defensins – An Example Using Sugarcane, a Large and Complex Genome, Curr. Protein Pept. Sci. 11 (2010) 248–254. doi:10.2174/138920310791112048.

[57] M. Tesfaye, K.A. Silverstein, S. Nallu, L. Wang, C.J. Botanga, S.K. Gomez, L.M. Costa, M.J. Harrison, D.A. Samac, J. Glazebrook, F. Katagiri, J.F. Gutierrez-Marcos, K.A. VandenBosch, Spatio-Temporal Expression Patterns of *Arabidopsis thaliana* and *Medicago truncatula* Defensin-Like Genes, PLoS ONE. 8 (2013) e58992. doi:10.1371/journal.pone.0058992.

[58] U.S. Sagaram, R. Pandurangi, J. Kaur, T.J. Smith, D.M. Shah, Structure-Activity Determinants in Antifungal Plant Defensins MsDef1 and MtDef4 with Different Modes of Action against *Fusarium graminearum*, PLoS ONE. 6 (2011) e18550. doi:10.1371/journal.pone.0018550

5. Capítulo III

Manuscrito de artigo.
Em preparação.

NOVEL SYNTHETIC ANTIMICROBIAL PEPTIDE PDEF-ME1 MODIFIED FROM A *MANIHOT ESCULENTA* DEFENSIN

Carlos André dos Santos Silva[1]; Lívia Maria Batista Vilela[1]; Sérgio Crovella[1]; Isis Caroline da Silva Cirino[1]; Carlos Henrique Bezerra Cruz[3]; Marx Oliveira de Lima[1]; Tereza Cristina Leal-Balbino[2]; Roberto Dias Lins Neto[3]; Alessandro Tossi[4]; Ana Maria Benko-Iseppon[1]*

[1]Departamento de Genética, Centro de Biociências, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, 1235, CEP 50.670-423, Recife - PE, Brazil.

[2]Departamento de Microbiologia, Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, Av. Prof. Moraes Rego, s/n., Cidade Universitária, CEP 50740-465, Recife - PE, Brazil.

[3]Departamento de Virologia, Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, Av. Professor Moraes Rego, s/n., Cidade Universitária, CEP 50740-465, Recife - PE, Brazil.

[4]Department of Life Sciences, University of Trieste, Via Licio Giorgieri 1, 34127 Trieste, Italy.

*Corresponding Author's e-mail: ana.benko.iseppon@pq.cnpq.br

Abstract

Antimicrobial peptides (AMPs) are present in the innate immune system of multicellular organisms and often show broad-spectrum activity against several pathogens, including multiresistant strains. Therefore, AMPs are considered candidates for the development of new antibiotics either alone or in combination with traditional antibiotics. In this context, a new defensin was identified and selected in cassava (*M. esculenta*) genome, which was modified following a set of rules that favored the choice of PDef-Me1, being a twenty-amino acid amphipathic sequence with a β-sheets structure in hairpin. Its activity was tested against yeasts and bacteria, including resistant strains, with best results against *Staphylococcus aureus* (16 $\mu g.ml^{-1}$), *Acinetobacter baumanii* (64 $\mu g.ml^{-1}$) and *Candida parapsilosis* (64 $\mu g.ml^{-1}$). In addition, hemolytic activity assays as well as cell viability with rat macrophages were performed indicating low citotoxicity. While requiring further testing, PDef-Me1 showed potential to be used as a future broad-spectrum antimicrobial pharmaceutical compound.

Keywords: Antimicrobial peptides, *Manihot esculenta*, *Staphylococcus aureus, Acinetobacter baumanii* .

Introduction

The ability of bacteria to develop resistance to antibiotics has been reported after the discovery of penicillin in 1928. Initially, the arsenal of new antibiotics was sufficient to overcome the resistance observed, and antibiotics were often taken for granted. However, as the development of new antibiotics has declined, the frequency of bacterial resistance continues to increase throughout the world mainly due to the excessive consumption and indiscriminate use of antibiotics, in addition to the continuous evolution and dissemination of elements of mobile genetic resistance (Banin, Hughes, and Kuipers 2017; Collier and O'Neill 2018).

In recent years, several reports have highlighted the urgent and critical situation of antimicrobial resistance (AMR) and in 2014 the World Health Organization (WHO) acknowledged that AMR is a global public health challenge and if untreated, it is estimated that the annual number of deaths from antibiotic-resistant bacteria can reach 10 million by 2050, surpassing that of cancer by 1.2 million (O'Neill 2016; O'Brien-Simpson et al. 2018). In this scenario, antimicrobial peptides (AMPs) are considered as the next generation of molecules for the development of therapeutic strategies to combat AMR. These AMPs are small cationic molecules that exhibit antimicrobial activity against a wide range of bacteria, fungi, and viruses. For an AMP to be considered as a therapeutic option, it should have not only potent antibacterial properties but also low hemolytic and cytotoxic activities (Maturana et al. 2017).

The efficiency of AMPs is attributed to their ability to break down the cell membranes of microorganisms, in some cases causing pore formation, leading to microbial death (Cadwell et al. 2017). Binding of cationic AMPs to a bacterial membrane is driven by electrostatic contacts between the positively charged amino acids and the negatively charged cell surface, followed by hydrophobic interactions between the amphipathic domains of the peptide and the membrane phospholipids (Goering et al. 2018). These AMPs, present in several living beings, including plants, in this way a wide variety of sequences and combinations of amino acids can be found (Hancock and Sahl 2006; Benko-Iseppon et al. 2010).

These peptides have potent antimicrobial activity with a broad spectrum of roles and are also capable of mediating adaptive immune responses in a combined response to multiresistant bacteria (Yeaman and Yount 2003). The AMPs are generally small (<10 kDa), characterized by their cationic charge and structural rigidity. They are grouped according to their secondary structure, (α-helical, β-sheet or extended peptides) conferring distinct physicochemical properties and biological mechanisms of action. Approximately eukaryotic 3,014 AMPs have been reported (http://aps.unmc.edu/AP/main.php) with antibacterial activity (Wang, Li, and

Wang 2016). Approximately 90% of them were determined to adopt α-helix structure. This category has been extensively explored and reviewed elsewhere (Edwards et al. 2016; Rončević et al. 2018).

AMPs that adopt a β-sheet hairpin structure are less well known, representatives of this class of AMPs are among the most active antimicrobial molecules, due to their broad spectrum of action and resistance to internal environmental factors. Despite the relatively small number of known AMP-β-hairpin, their biological functions are very diverse. These peptides share a number of essential structural and functional characteristics, suitable to the development of new antibiotics based on their structure, specifically: small size (up to 25 amino acid residues), net positive charge and amphiphilic properties sufficient for the manifestation of membranotropic activity against a wide spectrum of bacterial targets, and a compact structure stabilized by disulfide bonds, providing greater proteolytic resistance (Panteleev et al. 2015; Panteleev et al. 2017). In this context, the natural compounds based on AMP β-hairpin may become the most promising drug candidates.

The present work identified a new defensin of cassava (*M. esculenta*), which was identified and modified to maintain the antimicrobial activity and to facilitate its synthesis. The results regarding its structure, antimicrobial activity and cytotoxicity are reported in the present work.

Materials and methods

Peptide design and synthesis

PDef-Me1 was designed based on the mature peptide of an *M. esculenta* defensin sequence (ID: Def_Me1, Santos-Silva et al., in prep). Some criteria were established for the design of PDef-Me1 as: (i) maximal size of 20 amino acids; (ii) cationic surface charge; (iv) cysteine residues to form disulfide bonds; and (v) values above the cut-off point for the four algorithms analyzed in the prediction of antimicrobial activity with the 'Predict Antimicrobial Peptides' tool (available in $CAMP_{R3}$) (Panteleev et al. 2015; Panteleev et al. 2017). These approaches reduced the number of candidates, and one of them (PDef-Me1) was chosen to be synthesized (Santos-Silva et al., in prep).

The peptide was obtained from *NovoPro Bioscience Inc*. (Shanghai, China). The purity of the peptide (>95 %) and was confirmed by RP-HPLC and mass spectrometry. Chromatographic separation was achieved on a reversed-phase Phenomenex Gemini-NX

column (C18, 5 μm, 110 Å, 4.6 mm × 250 mm) using a 25−50% acetonitrile/0.1% TFA gradient in 25 min at a 1 mL/min flow rate (see in supplementary material S1). Stock and test solutions were prepared by accurately dissolving aliquots of the peptide in distilled water.

Peptide modeling

The candidate models for PDef-Me1 were generated using the ROSETTA algorithm (Rohl et al. 2004). then the models were clustered according to their energy, and the final model was chosen from the models with the lowest energy (**Figure 17**). Mutations were induced with ROSETTA algorithm to form different combinations of disulfide bridges between the four cysteines present in the sequence (**Table 8**). Finally, PROCHECK (Laskowski et al. 1993) was used for the stereochemical analysis of the model, using the Ramachandran graph, observing the position of the amino acids in the most favorable regions.

Table 8 - Models for PDef-Me1 with different possibilities for the formation of disulfide bonds between the cysteine residues.

Model	Disulfide bonds*
1	CYS1-CYS2 and CYS3-CYS4
2	CYS1-CYS4 and CYS2-CYS3
3	CYS1-CYS3 and CYS2-CYS4

*CYS: Cysteine

Molecular dynamics

Simulations were conducted with the GROMACS 5.0.7 package. The proteins were placed in the center of a cubic box of dimensions 4 x 4 x 4 nm and solvated using SPC water model (Berendsen et al. 1981). The system included solvation in NaCl solution in physiological concentration (0.15M) replacing 46 solute molecules by 20 NA and 26 CL ions, with minimized energy. The temperature was maintained at 300 K in NpT ensemble with solute atoms constraint in the initial position. The LINCS method (Hess et al. 1997) was used to constrain bonds involving hydrogen atoms. Integration was carried out by the leapfrog algorithm (Hockney 1970) using a 2-fs integration time step. The systems were initially energy optimized using 50,000 steps of the steepest descent algorithm. All atomistic simulations were performed for 800 ns using the GROMOS 53A6 (Oostenbrink et al. 2004) force fields and periodic boundary conditions in the x, y and z directions using the NpT ensemble. Then, the molecular dynamics was performed unrestrained at constant pressure and temperature of 1 atm and 300 K.

Circular dichroism spectroscopy (CD)

CD spectra were obtained on a J-715 spectropolarimeter (Jasco, Tokyo, Japan) and regard the accumulation of three scans. They were measured using 2-mm path length quartz cells and 20 μM peptides in (a) water solution, (b) sodium dodecyl sulfate micelles (10 mM SDS in SPB) and (c) 50% TFE in SPB. The % helix content was determined as $[\theta]^{222}/[\theta]^{\alpha}$, where $[\theta]^{222}$ is the measured molar per residue ellipticity at 222 nm under any given condition and $[\theta]\alpha$ is the molar ellipticity for a perfectly formed α helix of the same length, estimated as described by Chen, Yang, and Chau (1974).

Antimicrobial activity

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against PDef-Me1 were determined by broth microdilution method following the recommendations of the Institute of Clinical and Laboratory Standards (CLSI, 2015) with modifications. For this, the PDef-Me1 in Cation-adjusted Müller-Hinton broth (CA-MHB) (Sigma) was added to the plate under serial dilution (50 μL). Briefly, bacterial cells were cultured in Müller Hinton (Himedia) with 1 mL of CA-MHB overnight at 37 °C and were determined by the dissolved oxygen density (OD_{625nn} of 0.08-0.13 AU) corresponding to 0.5 of the scale of McFarland (1-5 × 10^8 $CFU.mL^{-1}$). The solution was diluted (1:1000) in CA-MHB and inoculated into the wells of the microdilution plate (50 μL) so that the final concentration of bacterial cell density was approximately 1-5 × 10^5 $CFU.mL^{-1}$. The MIC was determined as the lowest peptide concentration that inhibited bacterial growth after incubation at 37 °C for 18 to 20 h. The uninoculated CA-MHB medium was used as the negative control and cultures without peptide addition served as positive control. For MBC, the plating of 10 μL of each well that showed no visible growth in Müller-Hinton plate and incubated for 24 h at 37 °C was performed, and MBC was considered the concentration in which it did not present bacterial growth. The bacterial strains used in the experiment were provided in the microbiology laboratory of the Aggeu Magalhães Institute (Fiocruz / Pernambuco). PDef-Me1 was tested against Gram-positive Strains *Staphylococcus aureus* ATCC 25923 and resistant *S. aureus* MRSA04673 and Gram-negative *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* resistant Pa_39, *Klebsiella pneumoniae* ATCC 13883 and *K. pneumoniae* resistant to Carbapenemase L016, *Acinetobacter baumannii* ATCC 19606 and *A. baumannii* resistant Acb

45, respectively. The experiments were performed in duplicates at different time intervals (CLSI, 2015).

The antifungal MIC was performed following the CLSI guidelines M27-A3 (2008). The PDef-Me1 was diluted in distilled water. The commercial antifungal Fluconazol® and the *Candida parapsilosis* strain ATCC 22019 as control standard yeast were used as a reference. Yeasts were grown on Sabouraud Agar Dextrose (SDA; Difco) and incubated for 48 h at 37 °C. For the yeasts, two serial dilutions from 1:100 and 1:20 were performed to obtain the final inoculum containing 1.0 to 5.0 x 10^6 CFU.mL^{-1}. This solution was inoculated throughout the 96 well plate containing standard culture medium RPMI 1640, buffered at pH 7.0 with 0.1 M morpholinopropanesulfonic acid (MOPS), to obtain the final concentration of 512 μg.mL^{-1} for each yeast tested. The yeasts were incubated at 37 °C and visualized 24 and 48h later. The fungi used in the experiment came from the microclinic located in the Medical Mycology Department of the Federal University of Pernambuco, including *Candida albicans* 4986, *Candida parapsilosis* 4970 and *Cryptococcus neoformans* 5980 resistant to multiple drugs, regarding strains of clinical origin. The MICs corresponded to the lower drug concentrations that showed growth inhibition compared to untreated fungi.

Hemolytic activity

The hemolytic activity of PDef-Me1 was measured by the amount of hemoglobin released by lysis of human erythrocytes. Human cells were suspended in 2% saline (NaCl 0.85 % + $CaCl_2$) and incubated in 96 well plates with different concentrations of PDef-Me1 (512 to 32 μg.mL^{-1}). Plates were incubated for a period of 3 hours at 37 °C under constant stirring and centrifuged at 3500 rpm for 4 min at 4 °C. The absorbance of the supernatant was measured at 540 nm to establish the percentage of hemolysis. As a positive control of cell lysis, 1 % Triton X-100 (Sigma-Aldrich, USA) and saline (0.85 % NaCl + 10mM $CaCl_2$) were used as the negative control. The experiments were performed in quadruplicate, with repetition at different time intervals.

Cytotoxic activity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – MTT]

The cytotoxic activity of PDef-Me1 was evaluated from the cells of the peritoneal exudate of mice (CEUA/IAM number 119/2017), provided by the microbiology laboratory of the Aggeu Magalhães Institute (Fiocruz-PE). For this, the cells were seeded in 96 well plates containing RPMI medium (Roswell Park Memorial Institute), without phenol red,

supplemented with 10% Fetal Bovine Serum (FBS) and incubated for 24 h at 37 °C in a 5 % atmosphere of CO_2. Five serial dilutions of PDef-Me1 were carried out starting from 1024 $\mu g.mL^{-1}$. The cultured cells were also used in the absence of the compounds, but maintained under the same conditions, as 100 % cell viability control. After 24 h the medium was removed, and the cells were treated in the presence of the different concentrations of PDef-Me1 and incubated at 37 °C with 5 % CO_2 for 24 h. After the incubation period, the effect of PDef-Me1 was determined by measuring the mitochondrial dehydrogenase activity, adding 10 µL per well of MTT (3- (5,4-dimethyl-tizol-2-I) -2-5-tretracil) and incubating at 37 °C for 3 hours. Then, the formazan crystals were solubilized in DMSO, and the absorbance was determined in a spectrophotometer at 540 nm. The concentration capable of killing 50 % of the cells (CC_{50}) was calculated by linear regression. Wells containing medium and MTT were used as reaction control. Each concentration of PDef-Me1, as well as the control without the compound, were evaluated in quadruplicate in two independent experiments.

Results

Peptide design

PDef-Me1 is a synthetic short peptide with twenty amino acids that was derived from a cassava defensin (*M. esculenta*) sequence called Def-Me1. However, PDef-Me1 maintained 60% identity with Def-Me1.

Sequence shortening and peptide synthesis

The *M. esculenta* defensin sequence available on GenBank (ID XP_021600759.1) was amplified, sequenced, characterized with the Gamma-thionin domain and modified from 46 to 20 amino acids, preserving about 60% identity to the original sequence (**Figure 18**). This new sequence possesses four cysteine residues that were intentionally maintained with the intention of favoring the formation of disulfide bonds (**Figure 18**). This modification in the original sequence followed a defined set of rules based on observed characteristics of a set of natural peptides, as well as on the *in silico* prediction of antimicrobial activity. Prediction values of antimicrobial activity before and after modification can be seen in **Table 8**.

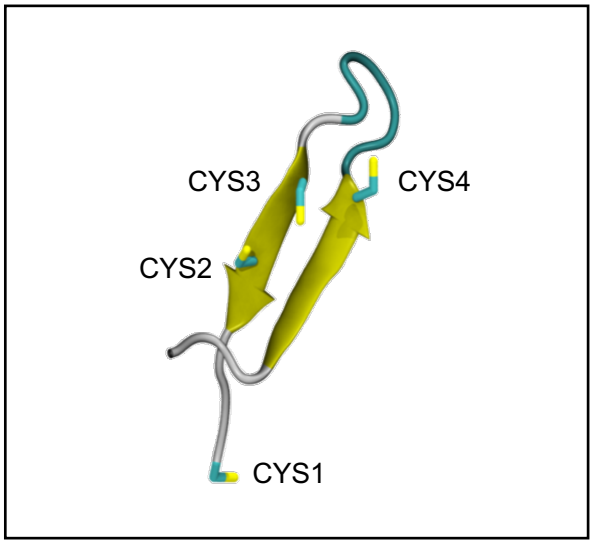

Figure 17. Three-dimensional structure of PDf-Me1 showing the four cysteine (CYS) residues. The antiparallel arrows in yellow represent both ß-sheets structure.

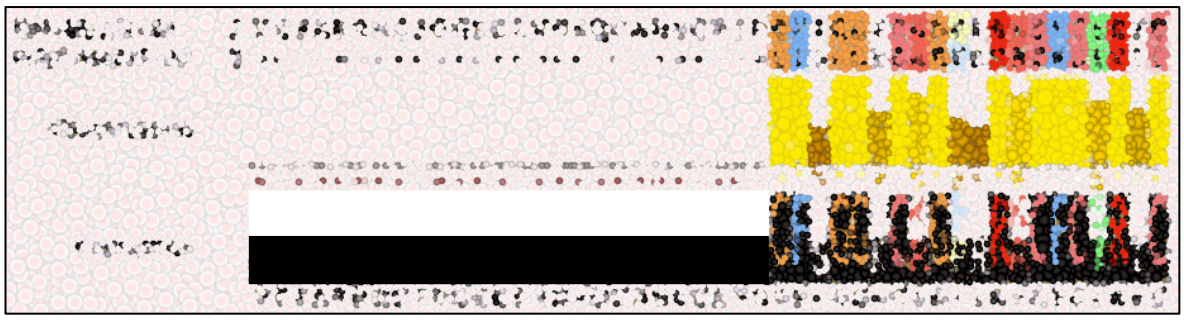

Figure 18 - Alignment of the Def-Me1 sequence with PDef-Me1, showing the selected region. The highlighted amino acids represent sequence conservation. In the bottom, the consensus amino acid sequence is presented.

Table 9 - *In silico* evaluation of antimicrobial activity using the four algorithms of the 'Predict Antimicrobial Peptides' tool available in CAMP$_{R3}$.

Sequences	Algorithm			
	*SVM	*RFC	*ANN	*DAC
Def-Me1	0.964	0.838	AMP	1.000
PDef-Me1	0.912	0.985	AMP	1.000

* Support Vector Machine (SVM), Random Forest Classifier (RFC), Artificial Neural Network (ANN), Discriminant Analysis Classifier (DAC).

Support Vector Machine is a specific type of supervised method of machine learning, aiming to classify data points by maximizing the margin between classes in a high-dimensional space. Randon Forest is a non-parametric tree-based approach that combines the ideas of adaptive neighbors with bagging for efficient adaptive data inference. Neural Network is an

information processing paradigm inspired by how a biological nerve system process information. It is composed of highly interconnected processing elements (neurons or nodes) working together to solve specific problems. Discriminant Analysis finds a set of prediction equations based on independent variables that are used to classify individuals into groups. (Lin and Jeon 2006; Karasuyama and Takeuchi 2010; Ding et al. 2013).

The synthesized PDef-Me1 had a molecular weight of 2.28 kDa and a purity level greater than 95%. Through mass spectrometry, it was possible to observe that the peptide formed two disulfide bonds. Chromatography showed the presence of a single peak in the 214 nm region.

Peptide modeling

After the synthesis, it was possible to observe that PDef-Me1 had formed two disulfide bonds, as predicted in its design. As there was no resolution data of the peptide structure, it was necessary to construct *ab initio* models with different combinations of disulfide bonds that could represent this peptide (**Table 9**). The models were generated by the ROSETTA algorithm and the consensus model found was a ß-sheets hairpin structure (**Figure 19**).

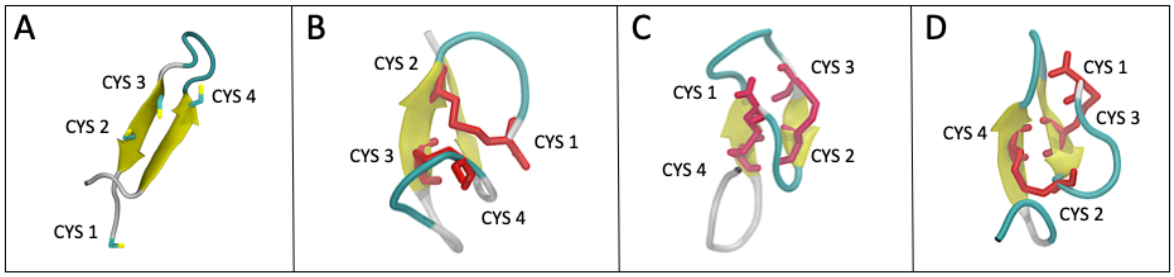

Figure 19 - Three-dimensional structures *ab initio* of PDf-Me1 showing the four cysteine residues (CYS) and the different combinations of disulfide bonds (B-D, in red). The antiparallel arrows in yellow represent the ß-sheets structure. In A- Model without bonds; B- Model-1; C- Model-2; D- Model-3.

Molecular dynamics

All three models with different combinations of disulfide bridges were submitted with the same conditions in molecular dynamics simulation in order to observe the possible modifications that the models could suffer and to infer which one of these models would be more likely to represent PDef-Me1. Therefore, some parameters were evaluated as root mean square deviation (RMSD), root mean square fluctuation (RMSF) and secondary peptide structure by DSSP graph (**Figure 20**).

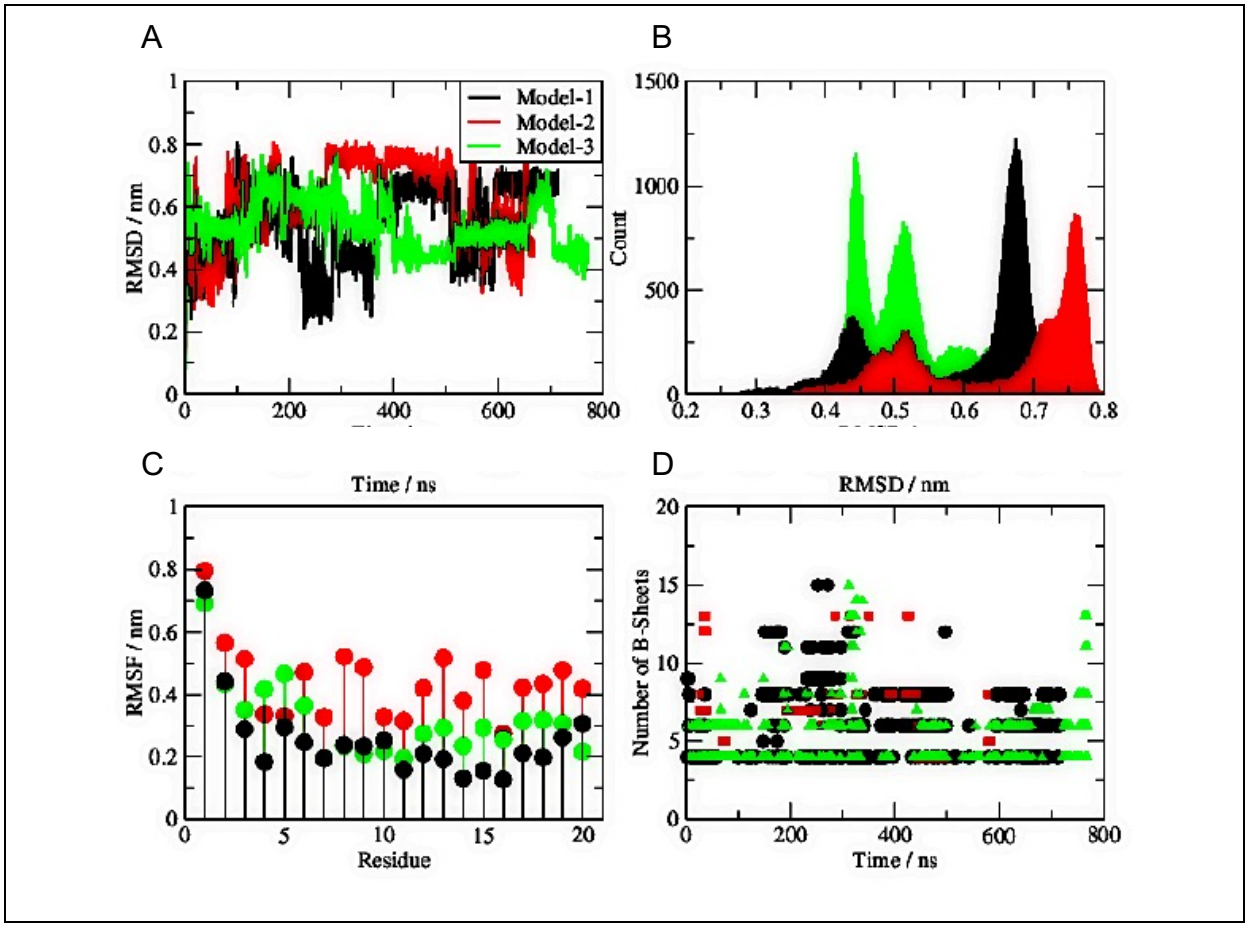

Figure 20 - Graphs associated to the molecular dynamics of the three models tested, with Model-1 represented in black, Model-2 in red and Model-3 in green (see Fig. 2.3). In A- the RMSD of the time models of 800 ns. In B- a clustering analysis of the consensus forms that models may have acquired during molecular dynamics; In C- the RMSF of the models represented by the fluctuation of amino acids by time. In D- a secondary structure analysis that each model maintained over time in molecular dynamics simulation.

Analyzing the graphs, it is possible to observe that the three models have left the original conformation. Model-1 exhibited the smallest variation in the clustering analysis (**Figure 21**). When observing the amino acids fluctuation, it was verified that the positions that suffered more changes were located in loop regions, being the Model-2 the one with more flexible amino acids. Regarding the secondary structure, it was observed that the Model-2 presented the least preserved ß-ribbons during the simulation.

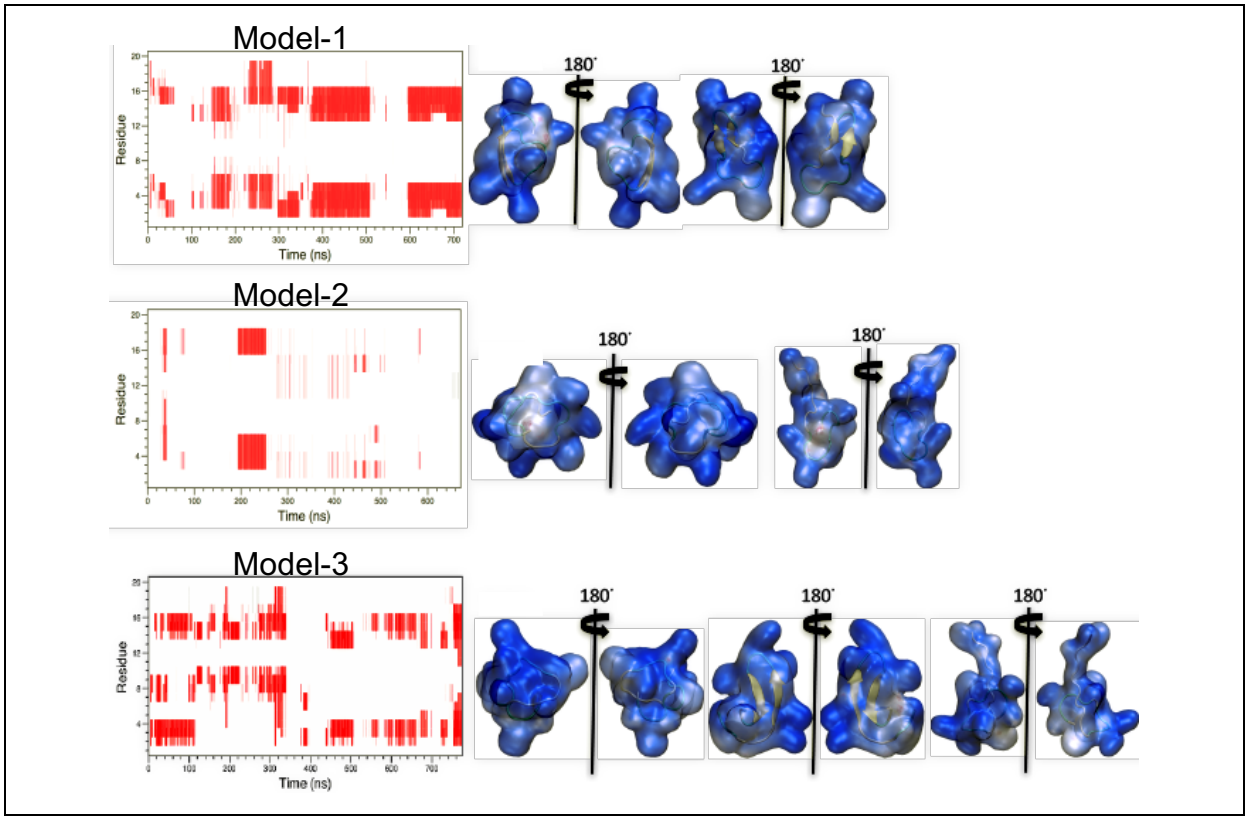

Figure 21 - On the right side, the three-dimensional structure of the models after cluster analysis, in blue the cationic surface charge of each model. On the left side, the graph of the secondary structure, in red the conservation of the β-sheets in relation to the simulation time in nanoseconds (ns).

Further to the observations on the structure, an analysis was made concerning the energies involved in the system (**Table 10**). Observing the energies of the models generated, the models 1 and 3 obtained very similar energies, being considered the potential representatives.

Table 10 - Energies regarding the simulations of the three models in molecular dynamics.

Model	Energy (kJ/mol)		
	Potential	Coulomb	VdW
1	-773 ± 26	-957 ± 20	-407 ± 5
2	-560 ± 21	-759 ± 18	-407 ± 8
3	-751 ± 14	-958 ± 10	-406 ± 6

Circular dichroism

The effect of environment on the structure of PDef-Me1 was determined by CD spectroscopy, as shown in **Figure 22**. As expected, spectra are consistent with a typical shape of a random coil in water, but in the presence of TFE (a solvent that enhances formation of H-bonds) or SDS micelles (an elementary model for lipid membranes), they show a partial transition to another conformation. The absence of peaks in the negative bands at 208 and 222 nm with the positive band at 192 or 198 nm, implies the absence of α-helix formation. However, based on the CD positive band at 195 nm and negative at approximately 230 nm (for TFE in water), this would correspond to a beta-sheet structure, suggesting that maybe the peptides fold to a beta-hairpin.

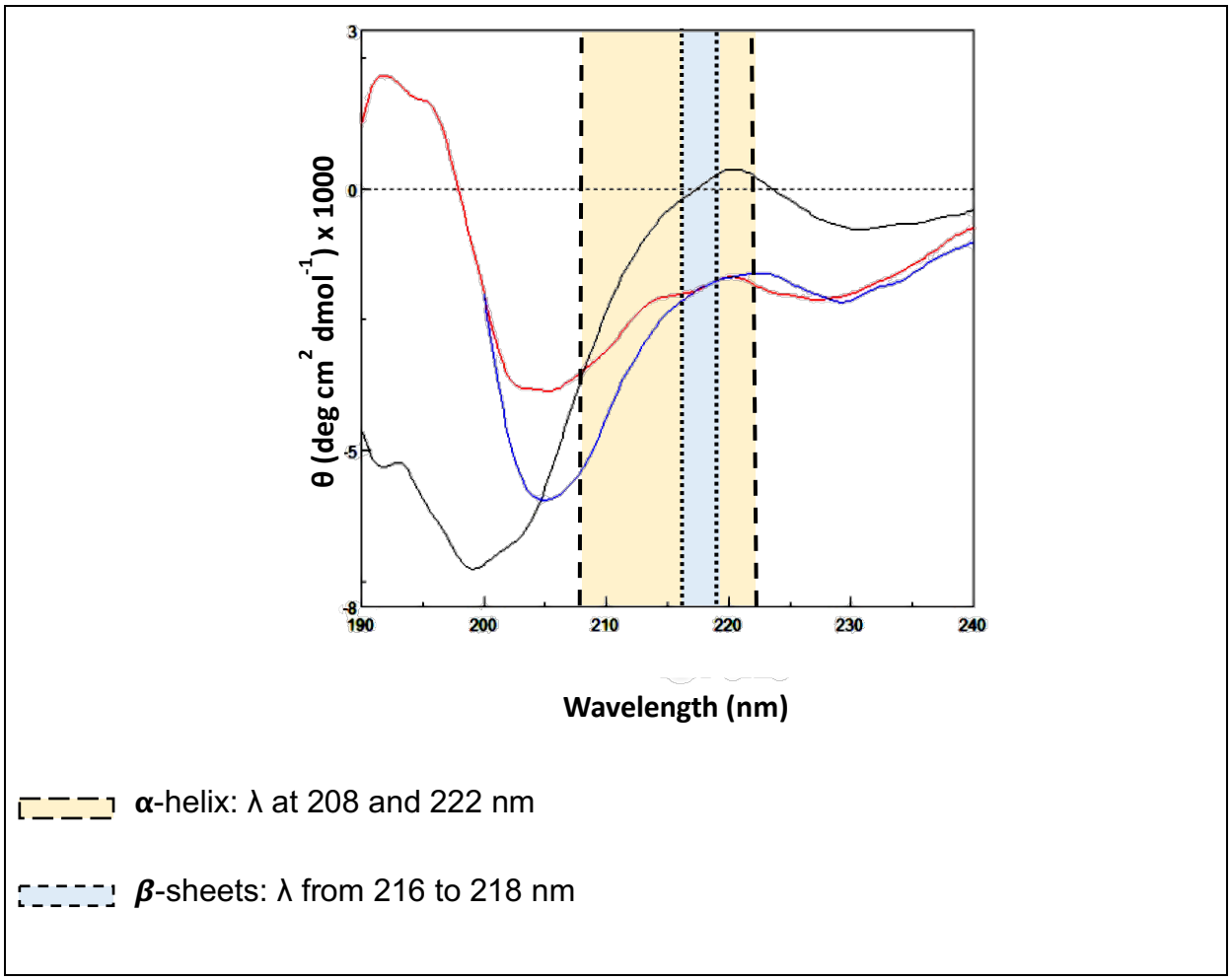

Figure 22 - CD spectra of PDef-Me1. The spectra were taken for a 20 μM solution in the following conditions: (—) black: water; (—) blue: 10 mM SDS (micelles); (—) red: 50% TFE in water. Spectra are the mean of three scans and subject to smoothing.

Antimicrobial activity

The results of the antimicrobial tests of PDef-Me1 against bacteria and fungi are presented in **Table 11**. In general, PDef-Me1 showed activity against all tested pathogens, presenting lower concentrations for *S. aureus* (single Gram-positive test) with 16 μg.mL^{-1} for the ATCC strain (25923) and 256 μg.mL^{-1} for methicillin-resistant *S. aureus* (MRSA04673). Considering Gram-negative strains, PDef-Me1 showed better results against *A. baumannii* with MIC of 64 μg.mL^{-1}, whereas for *K. pneumoniae* (ATCC 13883) and *P. aeruginosa* (Pa_39) the MIC was 128 μg.mL^{-1} for both.

However, at the minimum bactericidal concentration (MBC), PDef-Me1 presented 64 μg.mL^{-1} for *S. aureus* ATCC (25923), whereas for *S. aureus* resistant strain (MRSA04673) it had MBC of >1024 μg.mL^{-1} and similar results for *K. pneumoniae* resistant (L016) and *A. baumannii* ATCC (19606).

The antifungal activity of PDef-Me1 showed better results against *Candida parapsilosis* and *Cryptococcus neoformans* at the concentration of 64 μg.mL^{-1} for both yeasts, whereas for *Candida albicans* the active concentration was 128 μg.mL^{-1}.

Table 11 - Antimicrobial activity of PDef-Me1 against different pathogens tested, considering Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) for each tested strain.

Strains	**Microorganism**	**Result (μg.mL^{-1})**	
		MIC	**MBC**
ATCC 25923	*Staphylococcus aureus*	16	64
Resistant MRSA04673	*S. aureus*	256	>1024
ATCC 27853	*Pseudomonas aeruginosa*	256	256
Resistant Pa_39	*P. aeruginosa*	128	512
ATCC 13883	*Klebsiella pneumoniae*	128	512
Carbapenemase L016	*K. pneumoniae*	1024	>1024
ATCC 19606	*Acinetobacter baumannii*	64	1024
Resistant Acb 45	*A. baumannii*	128	512
4986	*Candida albicans*	128	-
4970	*C. parapsilosis*	64	-
5980	*Cryptococcus neoformans*	64	-

Cell viability assay (MTT) and hemolytic activity

By analyzing the cell viability of PDef-Me1 using rat macrophage cells, it was observed that concentrations lower than 256 μg.ml^{-1} presented similar results as the negative control. Thus, the cytotoxic activity of PDef-Me1 was CC_{50} 248.45, with cell viability below 50%, which decreases as the concentration of this peptide increases (**Figure 23**).

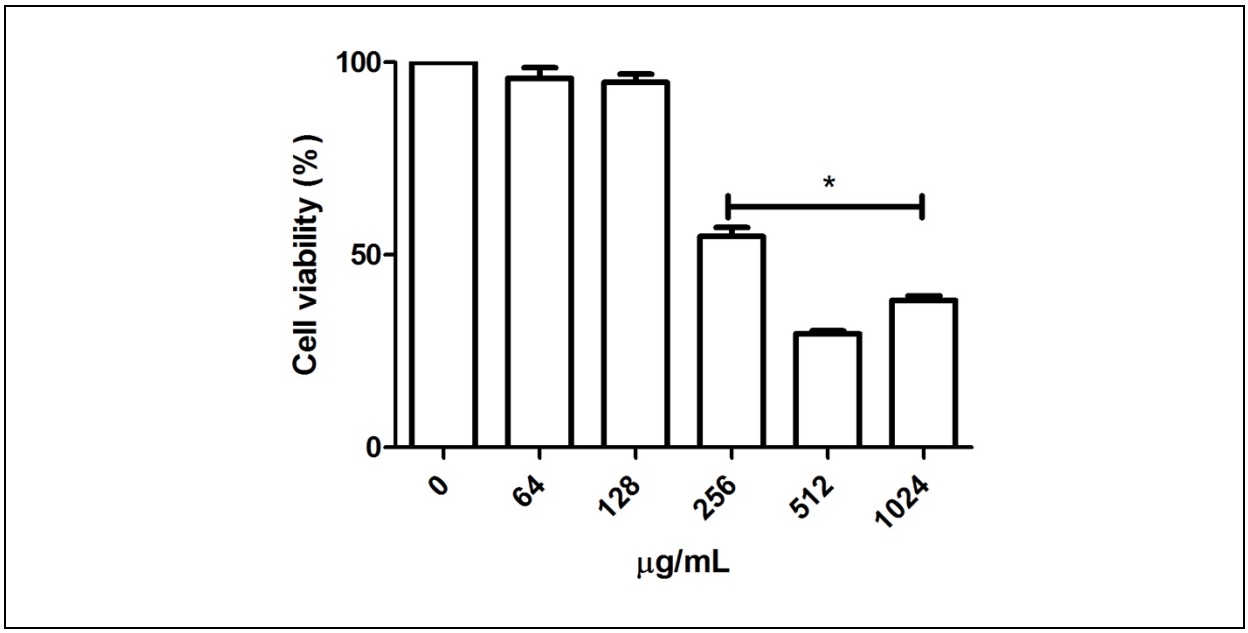

Figure 23 - Cell viability plot of rat macrophages in contact with PDef-Me1 using the MTT method.

Furthermore, no hemolytic activity was observed for PDef-Me1compared to the negative control using human erythrocyte cells (**Figure 24**).

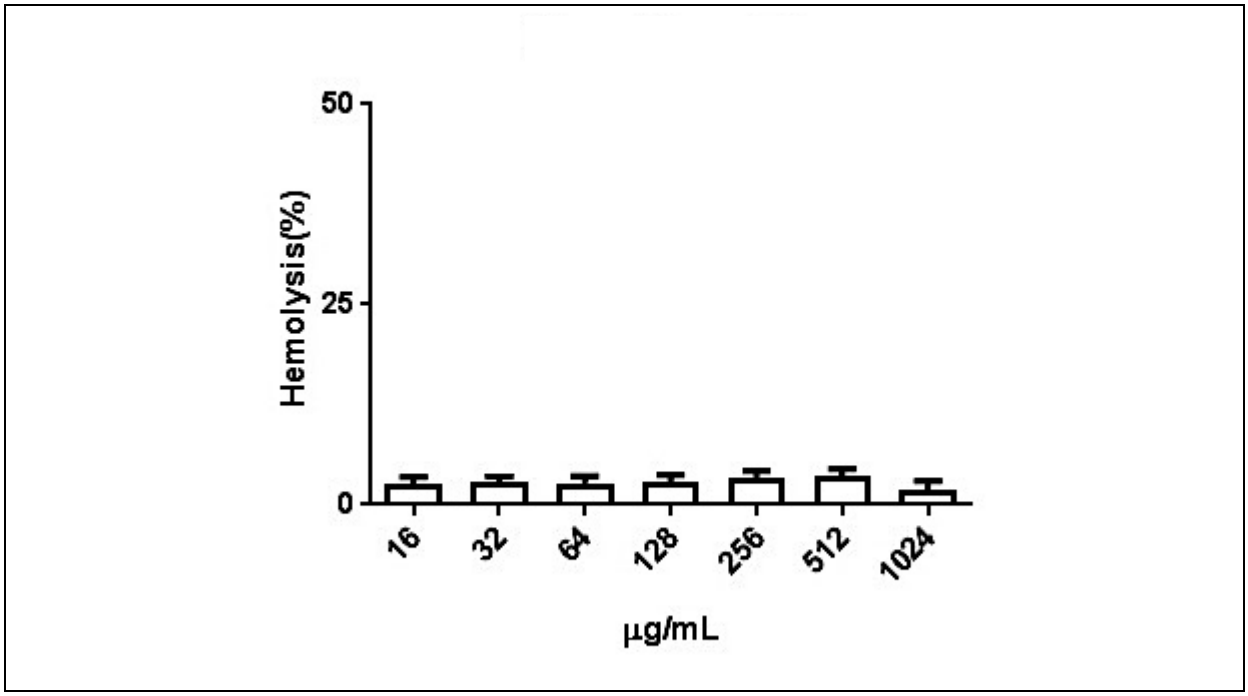

Figure 24 - Hemolytic activity plot of PDef-Me1 using human erythrocyte cells at different concentrations.

Discussion

Peptide design and structure

The rules imposed for the design of the peptide decreased the number of candidates for the *M. esculenta* defensin sequence. The amphipathic distribution of amino acids in the peptide seems to have been the criterion that more limited the possible conformations. This rule was applied with greater severity due to the knowledge already reported in the literature (Panteleev et al. 2015; Panteleev et al. 2017) that this type of conformation favors the biological activity of the peptide while maintaining its selectivity in its mechanism of action (Blondelle and Houghten 1992; Rončević et al. 2018).

When analyzing the secondary structure of the selected peptide (**Figure 25**), it was possible to observe the formation of two antiparallel ß-sheet in the hairpin. This conformation was probably favored by the interactions of the hydrophobic and hydrophilic amino acid alternations. This structure is possibly stabilized by the intramolecular hydrogen bonds between the ß-sheet of the peptide, as well as the turn structure that connects the two ß-sheet and favors the hydrophobic amino acid interactions.

Although the PDef-Me1 sequence had > 60% similarity to the β-sheet region of its derivative sequence, the modifications were essential to maintain their singularities without losing the conservation of their structure. Thus, the amino acid residue sequence of the peptide must be strategically positioned so that the side-chain scheduling of oppositely charged amino acids is achieved during packaging of the peptide (Rodriguez et al. 2016).

The CD result (50% TFE in water) for PDef-Me1 corroborates with the predicted *in silico* structure. This technique is widely reported in the literature as a rapid, non-destructive and quantitative technique. Moreover, thanks to the high sensitivity to the arrangement of the chromophores and to the eventual supramolecular organization, CD spectroscopy is used to monitor specific structural signatures in different complex arrangements, such as peptides (Betush, Urban, and Nilsson 2018). However, even if it is rich and very sensitive, the information incorporated in the CD spectra is, in some cases, difficult to relate directly to the three-dimensional model of the structure (Gattuso et al. 2017).

Molecular dynamics simulation analyzes indicated that out of the three models generated for PDef-Me1, Model-1 exhibited better overall results. Considering the results, the study of computational models for proteins still faces a series of challenges that make it difficult to obtain more concise and robust models. The choice of a model among the three ones here generated was based on parameters that presented similarities in some aspects, making difficult the unequivocal choice of the best candidate. However, such aspects are crucial for the understanding of each model limitations and for defining which interpretations and conclusions can be supported.

Antimicrobial activity

Cationic AMPs, including defensins, are peptides that have mostly the mechanism of action that targets the bacterial membrane through electrostatic interactions, thus facilitating pathogens death through the perturbation of their membrane, causing the formation of pores and leakage of the intracellular content (Schmidt et al. 2011; Cadwell et al. 2017). The major structural component of the cell membrane of most bacteria is peptidoglycan which, together with lipoteichoic acid and other polysaccharides, makes it highly polar, giving Gram-positive bacteria a thick, negatively charged hydrophilic surface. The Gram-negative bacteria in addition to the peptidoglycan membrane have the outer membrane which is also hydrophilic, corresponding to a second lipid bilayer located above the peptidoglycan, containing phospholipids, lipoproteins, proteins, and lipopolysaccharides, which also impart hydrophobic and negatively charged cell surface of these bacteria (Goering et al. 2018). These structural components in the bacteria cell membrane can help to explain the in vitro activity of PDef-Me1 that presented higher antimicrobial activity against *S. aureus*, corroborating with the works of Siebert et al. (2018), which tested sixteen peptides derivatives of mycophenolic acid against Gram-positive and Gram-negative bacteria with higher activity against *S. aureus*. Although three other compounds gave better results for *K. pneumoniae*, PDef-Me1 obtained better antimicrobial results for *A. baumannii*, compared to all sixteen compounds.

In recent years, a growing number of studies have taken the path of modifying the amino acid sequences of native/original peptides in order to develop a more potent antifungal activity. Several strategies designed to alter properties related to antifungal activity have been employed in the construction of these new peptides, such as modification of peptide length, net charge, and hydrophobicity (Fernandes and Carter 2017). In addition to its antimicrobial activity against bacteria, PDef-Me1 was tested against different yeast strains where its

antimicrobial capacity varied between 64 and 128 μg.ml^{-1}, a result in consonance with the work of Durnaś et al. (2016) and the review by Fernandes and Carter (2017).

Cell viability assay (MTT) and hemolytic activity

For an AMP to be considered as a therapeutic option, it should have not only potent antibacterial or antifungal properties but also low hemolytic and cytotoxic activities (Maturana et al. 2017). Generally, this is measured by the ability of AMPs to lyse red blood cells from mammals (Oddo and Hansen 2017). B-Hairpin antimicrobial peptides are generally amphipathic, binding to the membrane of several pathogens, but most members of this class also have a high and adverse cytotoxicity that prevents their pharmacological application as antimicrobial candidates (Edwards et al. 2016). In this context, the hemolysis assay has demonstrated that PDef-Me1 presents a low ability to lyse red blood cells. Even at the highest concentration tested (1024 μg.ml^{-1}), this peptide showed no significant hemolytic activity when compared to the negative control. These findings would allow them to be used as active molecules or substances in antimicrobial therapy as a possible medical treatment in humans.

The high hemolytic activity of some peptides prevents their therapeutic use, evidencing the need for improvements in the peptide design processes in order to minimize this effect. Although the results with erythrocyte cells were promising, future tests of the cellular viability of PDef-Me1 should be carried out, including more complex cells than rat macrophages, where this peptide showed CC_{50} = 248.45. Thus, it is necessary to test the peptide against several cells with different levels of structural complexities in order to evaluate the cytotoxic capacity, especially concerning the active concentration of the peptide.

Conclusions

The analysis of the relationship between the peptide structure and its antimicrobial activity confirmed the effect of the charge and the hydrophobicity on the studied biological activity. Other physicochemical descriptors used in this study, such as length and presence of cysteine residues forming disulfide bonds, have helped to form some secondary structures (e.g., ß-sheet in hairpin) showing good correlations for the design of this peptide. Therefore, taking into account the broad spectrum of antimicrobial activities and the low hemolytic activity of PDef-Me1, this peptide can be considered as a candidate molecule for new designs of pharmaceutical substances. Finally, this work opens a new possibility to study plant modified peptides with potential antimicrobial activity for biotechnological use.

REFERENCES

Banin, E., Hughes, D., and Kuipers, O. P. 2017. "Editorial: Bacterial Pathogens, Antibiotics and Antibiotic Resistance." *FEMS Microbiology Reviews* 41 (3): 450–52. https://doi.org/10.1093/femsre/fux016.

Benko-Iseppon, A. M., Lins Galdino, S., Calsa Jr, T., Akio Kido, E., Tossi, A., Carlos Belarmino, L., and Crovella, S. 2010. "Overview on Plant Antimicrobial Peptides." *Current Protein and Peptide Science* 11 (3): 181–88.

Berendsen, H. J., Postma, J. P., van Gunsteren, W. F., and Hermans, J. 1981. "Interaction Models for Water in Relation to Protein Hydration." In *Intermolecular Forces*, edited by Bernard Pullman, 14:331–42. Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-015-7658-1_21.

Betush, R. J., Urban, J. M., and Nilsson, B. L. 2018. "Balancing Hydrophobicity and Sequence Pattern to Influence Self-Assembly of Amphipathic Peptides." *Peptide Science* 110 (1): e23099. https://doi.org/10.1002/bip.23099.

Blondelle, S. E., and Houghten, R. A. 1992. "Design of Model Amphipathic Peptides Having Potent Antimicrobial Activities." *Biochemistry* 31 (50): 12688–94.

Cadwell, K., Niranji, S. S., Armstrong, V. L., Mowbray, C. A., Bailey, R., Watson, K. A., and Hall, J. 2017. "AvBD1 Nucleotide Polymorphisms, Peptide Antimicrobial Activities and Microbial Colonisation of the Broiler Chicken Gut." *BMC Genomics* 18 (1). https://doi.org/10.1186/s12864-017-4034-6.

Chen, Y. H., Yang, J. T., and Chau, K. H. 1974. "Determination of the Helix and Beta Form of Proteins in Aqueous Solution by Circular Dichroism." *Biochemistry* 13 (16): 3350–59.

Collier, P., and O'Neill, L. J. 2018. "Two Years on: An Update on Achievement towards the Recommendations of the Antimicrobial Resistance Report." *Journal of Applied Microbiology* 125 (2): 308–12. https://doi.org/10.1111/jam.13933.

Ding, S., Li, H., Su, C., Yu, J., and Jin, F. 2013. "Evolutionary Artificial Neural Networks: A Review." *Artificial Intelligence Review* 39 (3): 251–60. https://doi.org/10.1007/s10462-011-9270-6.

Durnaś, B., Wnorowska, U., Pogoda, K., Deptuła, P., Wątek, M., Piktel, E., ... and Bucki, R. 2016. "Candidacidal Activity of Selected Ceragenins and Human Cathelicidin LL-37 in Experimental Settings Mimicking Infection Sites." Edited by Joy Sturtevant. *Plos One* 11 (6): e0157242. https://doi.org/10.1371/journal.pone.0157242.

Edwards, I. A., Elliott, A. G., Kavanagh, A. M., Zuegg, J., Blaskovich, M. A., and Cooper, M. A. 2016. "Contribution of Amphipathicity and Hydrophobicity to the Antimicrobial Activity and Cytotoxicity of β-Hairpin Peptides." *ACS Infectious Diseases* 2 (6): 442–50. https://doi.org/10.1021/acsinfecdis.6b00045.

Fernandes, K. E., and Carter, D. A. 2017. "The Antifungal Activity of Lactoferrin and Its Derived Peptides: Mechanisms of Action and Synergy with Drugs against Fungal Pathogens." *Frontiers in Microbiology* 8 (January). https://doi.org/10.3389/fmicb.2017.00002.

Gattuso, H., García-Iriepa, C., Sampedro, D., Monari, A., and Marazzi, M. 2017. "Simulating the Electronic Circular Dichroism Spectra of Photoreversible Peptide Conformations." *Journal of Chemical Theory and Computation* 13 (7): 3290–96. https://doi.org/10.1021/acs.jctc.7b00163.

Goering, R., Dockrell, H., Zuckerman, M., and Chiodini, P. L. 2018. *Mims' Medical Microbiology E-Book*, 7-8. 5th ed. Elsevier Health Sciences.

Hancock, R. E., and Sahl, H. G. 2006. "Antimicrobial and Host-Defense Peptides as New Anti-Infective Therapeutic Strategies." *Nature Biotechnology* 24 (12): 1551–57. https://doi.org/10.1038/nbt1267.

Hess, B., Bekker, H., Berendsen, H. J., and Fraaije, J. G. 1997. "LINCS: A Linear Constraint Solver for Molecular Simulations." *Journal of Computational Chemistry* 18 (12): 1463–72. https://doi.org/10.1002/(SICI)1096-987X(199709)18:12<1463::AID-JCC4>3.0.CO;2-H.

Hockney, R. W. 1970. "Methods in Computational Physics." *Alder, B*, 136–211.

Karasuyama, M., and Takeuchi, I. 2010. "Multiple Incremental Decremental Learning of Support Vector Machines." IEEE *Transactions on Neural Networks* 21 (7): 1048–59. https://doi.org/10.1109/TNN.2010.2048039.

Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. 1993. "PROCHECK: A Program to Check the Stereochemical Quality of Protein Structures." *Journal of Applied Crystallography* 26 (2): 283–91. https://doi.org/10.1107/S0021889892009944.

Lin, Y., and Jeon, Y. 2006. "Random Forests and Adaptive Nearest Neighbors." *Journal of the American Statistical Association* 101 (474): 578–90. https://doi.org/10.1198/016214505000001230.

Maturana, P., Martinez, M., Noguera, M. E., Santos, N. C., Disalvo, E. A., Semorile, L., ... and Hollmann, A. 2017. "Lipid Selectivity in Novel Antimicrobial Peptides: Implication on Antimicrobial and Hemolytic Activity." *Colloids and Surfaces B: Biointerfaces* 153 (May): 152–59. https://doi.org/10.1016/j.colsurfb.2017.02.003.

O'Brien-Simpson, N. M., Hoffmann, R., Chia, C. S., and Wade, J. D. 2018. "Editorial: Antimicrobial and Anticancer Peptides." *Frontiers in Chemistry* 6 (February). https://doi.org/10.3389/fchem.2018.00013.

Oddo, A., and Paul R. H. 2017. "Hemolytic Activity of Antimicrobial Peptides." In *Antimicrobial Peptides*, 427–35. Springer.

O'Neill, J. 2016. "Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. Review of Antimicrobial Resistance. London: HM Government and Wellcome Trust; 2016." *There Is No Corresponding Record for This Reference*.

Oostenbrink, C., Villa, A., Mark, A. E., and Van Gunsteren, W. F. 2004. "A Biomolecular Force Field Based on the Free Enthalpy of Hydration and Solvation: The GROMOS Force-Field Parameter Sets 53A5 and 53A6." *Journal of Computational Chemistry* 25 (13): 1656–76. https://doi.org/10.1002/jcc.20090.

Panteleev, P. V., Bolosov, I. A., Balandin, S. V., and Ovchinnikova, T. V. 2015. "Structure and Biological Functions of β-Hairpin Antimicrobial Peptides." *Acta Naturae* 7 (1): 37–47.

Panteleev, P., V Balandin, S. V., T Ivanov, V., and V Ovchinnikova, T. V. 2017. "A Therapeutic Potential of Animal β-Hairpin Antimicrobial Peptides." *Current Medicinal Chemistry* 24 (17). https://doi.org/10.2174/0929867324666170424124416.

Rodriguez, L. M. D. L., Hemar, Y., Cornish, J., and Brimble, M. A. 2016. "Structure–Mechanical Property Correlations of Hydrogel Forming β-Sheet Peptides." *Chemical Society Reviews* 45 (17): 4797–4824. https://doi.org/10.1039/C5CS00941C.

Rohl, C. A., Strauss, C. E., Misura, K. M., and Baker, D. 2004. "Protein Structure Prediction Using Rosetta." In *Methods in Enzymology*, 383:66–93. Elsevier. https://doi.org/10.1016/S0076-6879(04)83004-0.

Rončević, T., Vukičević, D., Ilić, N., Krce, L., Gajski, G., Tonkić, M., ... and Juretić, D. 2018. "Antibacterial Activity Affected by the Conformational Flexibility in Glycine–Lysine Based α-Helical Antimicrobial Peptides." *Journal of Medicinal Chemistry* 61 (7): 2924–36. https://doi.org/10.1021/acs.jmedchem.7b01831.

Schmidt, N. W., Mishra, A., Lai, G. H., Davis, M., Sanders, L. K., Tran, D., ... and Selsted, M. E. 2011. "Criterion for Amino Acid Composition of Defensins and Antimicrobial Peptides Based on Geometry of Membrane Destabilization." *Journal of the American Chemical Society* 133 (17): 6720–27. https://doi.org/10.1021/ja200079a.

Siebert, A., Wysocka, M., Krawczyk, B., Cholewiński, G., and Rachoń, J. 2018. "Synthesis and Antimicrobial Activity of Amino Acid and Peptide Derivatives of Mycophenolic Acid." *European Journal of Medicinal Chemistry* 143 (January): 646–55. https://doi.org/10.1016/j.ejmech.2017.11.094.

Wang, G., Li, X., and Wang, Z. 2016. "APD3: The Antimicrobial Peptide Database as a Tool for Research and Education." *Nucleic Acids Research* 44 (D1): D1087–93. https://doi.org/10.1093/nar/gkv1278.

Yeaman, M. R., and Yount, N. Y. 2003. "Mechanisms of Antimicrobial Peptide Action and Resistance." *Pharmacological Reviews* 55 (1): 27–55.

6. DISCUSSÃO GERAL

O crescente problema da resistência dos patógenos aos medicamentos tradicionais e as dificuldades de produzir novos medicamentos geraram um desafio para a medicina moderna, demandando moléculas alternativas que possam driblar este problema. Por este motivo, pesquisas vêm sendo desenvolvidas na busca por novas moléculas antimicrobianas que tenham um amplo espectro de atividade. Neste cenário, compostos antimicrobianos derivados de plantas vêm sendo prospectados para combater diversas doenças provocadas por bactérias, fungos, protozoários e vírus, frequentemente com poucos efeitos colaterais, como revisado no Capítulo I. Com esta motivação, vem sendo feita uma vasta mineração nos bancos de dados públicos por sequências de peptídeos antimicrobianos de plantas, com ênfase para aqueles do tipo gama defensinas (pipeline descrito no Capítulo I). Defensinas previamente descritas e validadas foram utilizadas como sondas para a busca de homólogos em espécies da família Euphorbiaceae, a qual vem sendo estudada pela importância etnobotânica e capacidade de suas espécies de produzir moléculas bioativas.

Foram encontradas 12 sequências de defensinas candidatas entre os três genomas pesquisado (Mamona, Macaxeira e Pinhão manso).

Ainda que diferentes métodos de mineração tenham sido utilizados como o BLAST, HMM (*Hidden Markov Model*) e Regex (*Regular expression*, dados não mostrados) a diferença entre os resultados dos métodos utilizados não foram significativas (Capítulo II).

Dos 12 *primers* desenhados seis amplificaram, sendo que nenhum dos *primers* de *J. curcas* amplificou nas diferentes temperaturas testadas.

As sequências de *M. esculenta* (Def-Me1, Def-Me2 e Def-Me3) apresentaram baixa conservação dos aminoácidos, com exceção das cisteínas.

A modelgem das sequências de *M. esculenta* mostraram que apesar da pressão seletiva da interação peptídeo-patógeno resulte em mutações não sinônimas, estas não provocaram mudanças significativas nas estruturas analisadas. A análise do desvio da raiz quadra média (RMSD) indicou que todos os modelos eram similares, apesar de suas singularidades.

Def-Me1 e Def-Me2 foram modificados visando manter ou até melhorar a porção antimicrobiana da sequência, sendo Def-Me1 a melhor sequência para a síntese, sendo chamado de PDef-Me1.

PDef-Me1, um peptídeo curto sintético desenhado baseado com o auxílio de ferramentas de bioinformática, a partir de uma sequência de *M. esculenta* apresentou atividade antimicrobiana contra todos os patógenos testados (Capitulo III), incluindo leveduras do gênero *Candida* e contra bactérias Gram-positivas e Gram-negativas, incluindo linhagens resistentes a antibióticos tradicionais.

Desta forma PDef-Me1 surge como um potencial candidato para o desenvolvimento de um futuro composto farmacêutico com atividade antimicrobiana de amplo espectro. Sua atividade se dá por sua sequência anfipática com uma estrutura ß-fitas em grampo. Embora já relatado na literatura por possuir atividade antimicrobiana, este tipo de estrutura tem um grande problema com a seletividade de membrana, geralmente possuindo atividade hemolítica. Apesar disso, PDef-Me1 não exibiu atividade hemolítica detectável comparativamente ao controle negativo, mesmo na maior concentração testada, reforçando seu potencial terapêutico (Capítulo III).

Considerando-se os dados promissores, uma patente foi depositada (Pedido de Patente de Invenção, nº. INPI BR 1020190015187 em Janeiro/2019). Embora mais testes sejam necessários, com ênfase para estudos *in vivo*, PDef-Me1 apresenta-se como um agente microbiano com potencial para ser usado contra diversos tipos de patógenos.

7. CONCLUSÕES

- Até então não se sabia o número de defensinas no genoma de *Manihot esculenta*, neste estudo foram descobertas oito sequências no genoma, contudo apenas três dessas defensinas estavam completas, sendo amplificadas, sequenciadas e modeladas.
- Uma das defensinas sequenciadas (Def-Me1) mostrou-se diversos aminoácidos exclusivos em comparação à sequência mais similar identificada *in silico*.
- As três defensinas amplificadas possuíam domínio conservado gama-tionina e peptídeo maduro cujo tamanho variou entre 46 e 47 aminoácidos, apresentando o motivo conservado GXCX3-9C, formando quatro pontes dissulfeto.
- As modelagens tridimensionais das defensinas de *M. esculenta* indicaram semelhanças estruturais, com diferenças nas regiões de volta, além de se apresentarem com uma distribuição de resíduos anfipáticos diferentes ao longo da carga superficial.
- A análise da expressão de defensinas de *M. esculenta* indica expressão moderada em folhas a qual se manteve ou decresceu após inoculação com o fungo promírcuo *Lasiodiplodia theobromae*. Considera-se a possibilidade de que se tratem de defensinas tecido ou estágio-específicas, o que demanda futuras analyses de validação.
- Com base em uma das defensinas amplificadas de *M. esculenta* (Def-Me1) um peptídeo curto sintético (PDef-Me1) foi desenhado com o auxílio de técnicas e ferramentas de bioinformática. O peptídeo sintético apresentou atividade antimicrobiana contra leveduras do gênero *Candida*, bem como contra bactérias Gram-positivas e Gram-negativas, incluindo linhagens resistentes a antibióticos tradicionais.
- O peptídeo sintético PDef-Me1 apresenta uma sequência anfipática com uma estrutura ß-fitas em grampo observada tanto por técnica de modelagem *ab initio* como pelo dicroísmo circular.

- Apesar desse tipo de estrutura ser relatado na literatura como uma estrutura com pouca seletividade de membrana, PDef-Me1 não exibiu atividade hemolítica detectável comparativamente ao controle negativo, mesmo na maior concentração testada, reforçando seu potencial terapêutico.
- Embora mais testes in vitro e in vivo sejam necessários, PDef-Me1 apresenta-se como um agente microbiano com potencial para ser usado contra diversos tipos de patógenos.

REFERÊNCIAS

Acharya, C., Coop, A., E Polli, J., and D MacKerell, A. (2011) Recent advances in ligand-based drug design: relevance and utility of the conformationally sampled pharmacophore approach. Curr Comput Aided Drug Des 7:10–22.

Achten, W., Maes, W., Aerts, R., Verchot, L., Trabucco, A., Mathijs, E., ... and Muys, B. (2010) *Jatropha*: From global hype to local opportunity. J Arid Environ 74:164–165. doi: 10.1016/j.jaridenv.2009.08.010

Achtman, A. H., Pilat, S., Law, C. W., Lynn, D. J., Janot, L., Mayer, M. L., ... and Smyth, G. K. (2012) Effective Adjunctive Therapy by an Innate Defense Regulatory Peptide in a Preclinical Model of Severe Malaria. Sci Transl Med 4:135ra64-135ra64. doi: 10.1126/scitranslmed.3003515

Adeniyi, O. D., Kovo, A. S., Abdulkareem, A. S., and Chukwudozie, C. (2007) Ethanol Fuel Production from Cassava as a Substitute for Gasoline. J Dispers Sci Technol 28:501–504. doi: 10.1080/01932690701276940

Aerts, A. M., François, I. E. J. A., Cammue, B. P. A., and Thevissen, K. (2008) The mode of antifungal action of plant, insect and human defensins. Cell Mol Life Sci 65:2069–2079. doi: 10.1007/s00018-008-8035-0

Afolayan, A. J., Grierson, D. S., and Mbeng, W. O. (2014) Ethnobotanical survey of medicinal plants used in the management of skin disorders among the Xhosa communities of the Amathole District, Eastern Cape, South Africa. J Ethnopharmacol 153:220–232.

Ahmad, Z., and F Laughlin, T. (2010) Medicinal chemistry of ATP synthase: a potential drug target of dietary polyphenols and amphibian antimicrobial peptides. Curr Med Chem 17:2822–2836.

Ahmad, Z., Okafor, F., Azim, S., and F Laughlin, T. (2013) ATP synthase: a molecular therapeutic drug target for antimicrobial and antitumor peptides. Curr Med Chem 20:1956–1973.

Aidemark, M., Tjellström, H., Sandelius, A. S., Stålbrand, H., Andreasson, E., Rasmusson, A. G., and Widell, S. (2010) *Trichoderma viride* cellulase induces resistance to the antibiotic pore-forming peptide alamethicin associated with changes in the plasma membrane lipid composition of tobacco BY-2 cells. BMC Plant Biol 10:274. doi: 10.1186/1471-2229-10-274

Allem, A. C., Irgang, B. E., and Schultz, A. R. (1975) Flora ilustrada do Rio Grande do Sul: Euphorbiaceae tribo Euphorbieae. Impr. Univ.

Almeida, M. S., Cabral, K. M., Kurtenbach, E., Almeida, F. C., and Valente, A. P. (2002) Solution structure of *Pisum sativum* defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action 1 1Edited by M. F. Summers. J Mol Biol 315:749–757. doi: 10.1006/jmbi.2001.5252

Almeida, P. M., Araújo, S. S., Marin-Morales, M. A., Benko-Iseppon, A. M., and Brasileiro-Vidal, A. C. (2015). Genotoxic potential of the latex from cotton-leaf physicnut (*Jatropha gossypiifolia* L.). Genetics and molecular biology, 38(1), 93-100.

Almeida, P. M., Araújo, S. S., Santos, I. R. M. R., Marin-Morales, M. A., Benko-Iseppon, A. M., Santos, A. V., ... and Brasileiro-Vidal, A. C. (2016). Genotoxic potential of leaf extracts of *Jatropha gossypiifolia* L. Genetics and molecular research: GMR, 15(1).

Aloys, N., and Hui Ming, Z. (2006) Traditional Cassava Foods in Burundi — A Review. Food Rev Int 22:1–27. doi: 10.1080/87559120500379761

Aluru, M., Curry, J., and O'Connell, M. A. (1999) Nucleotide sequence of a defensin or gamma-thionin-like gene from habanero Chile. Plant Physiol 120:633.

Alvarez-Ortega, C., Olivares, J., and Martínez, J. L. (2013) RND multidrug efflux pumps: what are they good for? Front Microbiol. doi: 10.3389/fmicb.2013.00007

Alves, M. V. (1998) Checklist of Euphorbiaceae Juss. of the semi-arid in Pernambuco State, Brazil. Acta Bot Bras 12:485–495. doi: 10.1590/S0102-33061998000400016

Alves, M., Araújo, M. F. L., Gusmão, G. L. S., Lira-Neto, A. C., Carvalho, R., and Benko-Iseppon, A. M. (2012). The genus *Croton* (Euphorbiaceae) in North-Eastern Brazil: diversity, uses and priorities for conservation. Medicinal Plants: Biodiversity and Drugs (Rai M, Cordell GA, Martinez JL, Marinoff M, et al., eds.). 1st edn. CRC Press, New York. Aminov, R. I. (2010) A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. Front Microbiol. doi: 10.3389/fmicb.2010.00134

Ananiadou, S., Kell, D. B., and Tsujii, J. I. (2006) Text mining and its potential applications in systems biology. Trends Biotechnol 24:571–579. doi: 10.1016/j.tibtech.2006.10.002

Antcheva, N., Zelezetsky, I., and Tossi, A. (2006) Cationic Antimicrobial Peptides — The Defensins. Handbook of Biologically Active Peptides. Elsevier, pp 55–66

APGII. (2003) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. Bot J Linn Soc 141:399–436. doi: 10.1046/j.1095-8339.2003.t01-1-00158.x

APGIII. (2009) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III: APG III. Bot J Linn Soc 161:105–121. doi: 10.1111/j.1095-8339.2009.00996.x

Apic, G., Ignjatovic, T., Boyer, S., and Russell, R. B. (2005) Illuminating drug discovery with biological pathways. FEBS Lett 579:1872–1877. doi: 10.1016/j.febslet.2005.02.023

Appiah-Opong, R., Nyarko, A. K., Dodoo, D., Gyang, F. N., Koram, K. A., and Ayisi, N. K. (2011) Antiplasmodial activity of extracts of *Tridax procumbens* and *Phyllanthus amarus* in *in vitro Plasmodium falciparum* culture systems. Ghana Med J 45:143–150.

Araújo, S. S., Fernandes, T. C. C., Cardona, Y. T., de Almeida, P. M., Marin-Morales, M. A., dos Santos, A. V., ... and Brasileiro-Vidal, A. C. (2015). Cytotoxic and genotoxic effects of ethanolic extract of *Euphorbia hyssopifolia* L. on HepG2 cells. Journal of ethnopharmacology, 170, 16-19.

Astafieva, A. A., Rogozhin, E. A., Andreev, Y. A., Odintsova, T. I., Kozlov, S. A., Grishin, E. V., and Egorov, T. A. (2013) A novel cysteine-rich antifungal peptide ToAMP4 from *Taraxacum officinale* Wigg. flowers. Plant Physiol Biochem 70:93–99. doi: 10.1016/j.plaphy.2013.05.022

Avison, M. B., von Heldreich, C. J., Higgins, C. S., Bennett, P. M., and Walsh, T. R. (2000) A TEM-2beta-lactamase encoded on an active Tn1-like transposon in the genome of a clinical isolate of *Stenotrophomonas maltophilia*. J Antimicrob Chemother 46:879–884.

Awanchiri, S. S., Trinh-Van-Dufat, H., Shirri, J. C., Dongfack, M. D. J., Nguenang, G. M., Boutefnouchet, S., ... and Wandji, J. (2009) Triterpenoids with antimicrobial activity from *Drypetes inaequalis*. Phytochemistry 70:419–423. doi: 10.1016/j.phytochem.2008.12.017

Baillon, H. (1858) Etude générale du groupe des Euphorbiacées. doi: 10.5962/bhl.title.50439

Batista, D. (1976) O complexo da Amazônia. Rio de Janeiro. Conquista 292.

Batista, D. (2007) O complexo da Amazônia: análise do processo de desenvolvimento. Editora Valer.

Batista, M. M. (2015) Avaliação da Ação do Peptídeo Antimicrobiano Dermadistinctina K em Membrana Modelo por Dinâmica Molecular.

Bechinger, B. (2004) Structure and Function of Membrane-Lytic Peptides. Crit Rev Plant Sci 23:271–292. doi: 10.1080/07352680490452825

Bechinger, B., and Lohner, K. (2006) Detergent-like actions of linear amphipathic cationic antimicrobial peptides. Biochim Biophys Acta BBA - Biomembr 1758:1529–1539. doi: 10.1016/j.bbamem.2006.07.001

Becker-Ritt, A. B, Portugal, C. S., and Carlini, C. R. (2017) Jaburetox: update on a urease-derived peptide. J Venom Anim Toxins Trop Dis. doi: 10.1186/s40409-017-0122-y

Bedran, T. B. L., Mayer, M. P. A., Spolidorio, D. P., and Grenier, D. (2014) Synergistic Anti-Inflammatory Activity of the Antimicrobial Peptides Human Beta-Defensin-3 (hBD-3) and Cathelicidin (LL-37) in a Three-Dimensional Co-Culture Model of Gingival Epithelial Cells and Fibroblasts. PLoS ONE 9:e106766. doi: 10.1371/journal.pone.0106766

Benavides, A., Benjumea, P., and Pashova, V. (2007) Castor oil biodiesel as an alternative fuel for diesel engines. 74:141–150.

Benko-Iseppon, A. M. and Crovella, S. (2010) Ethnobotanical bioprospection of candidates for potential antimicrobial drugs from Brazilian plants: state of art and perspectives. Current Protein and Peptide Science, 11(3), 189-194.

Benko-Iseppon, A. M., Lins Galdino, S., Calsa Jr, T., Akio Kido, E., Tossi, A., Carlos Belarmino, L., and Crovella, S. (2010) Overview on plant antimicrobial peptides. Curr Protein Pept Sci 11:181–188.

Bentham, G., and Hooker, J. D. (1880) Euphorbiaceae. Genera Plant 3:239–540.

Bergmann, M., and Zervas, L. (1932) A general procedure of the peptide synthesis. Ber Dtsch Chem Ges 65:1192–1201.

Bergmann, R., Kubeil, M., Zarschler, K., Chhabra, S., Tajhya, R. B., Beeton, C., ... and Stephan, H. (2017) Distribution and kinetics of the Kv1.3-blocking peptide HsTX1[R14A] in experimental rats. Sci Rep. doi: 10.1038/s41598-017-03998-x

Bernabé, K. J., Langendorf, C., Ford, N., Ronat, J. B., & Murphy, R. A. (2017). Antimicrobial resistance in West Africa: a systematic review and meta-analysis. International journal of antimicrobial agents.

Beutler, J. A., Alvarado, A. B., McCloud, T. G., and Cragg, G. M. (1989) Distribution of phorbol ester bioactivity in the euphorbiaceae. Phytother Res 3:188–192. doi: 10.1002/ptr.2650030507

Bhattacharya, A., Hopkins, S., Sallis, A., Budd, E. L., and Ashiru-Oredope, D. (2016) A process evaluation of the UK-wide Antibiotic Guardian campaign: developing engagement on antimicrobial resistance. J Public Health. doi: 10.1093/pubmed/fdw059

Blank, A. F., Almeida, C. S., Alves, R. P., Sampaio, T. S., et al. (2017). Formulação fungicida à base de óleo essencial de *Croton tetradenius* para controlar ou eliminar o fitopatógeno Fusarium solani. Patent BR-10-2017-000272-1. Instituto Nacional da Propriedade Industrial, Rio de Janeiro.

Bloch Jr, C., Patel, S. U., Baud, F., Zvelebil, M. J., Carr, M. D., Sadler, P. J., and Thornton, J. M. (1998) 1H NMR structure of an antifungal γ-thionin protein SIα1: Similarity to scorpion toxins. Proteins Struct Funct Genet 32:334–349. doi: 10.1002/(SICI)1097-0134(19980815)32:3<334::AID-PROT9>3.0.CO;2-H

Bocchinfuso, G., Palleschi, A., Orioni, B., Grande, G., Formaggio, F., Toniolo, C., ... and Stella, L. (2009) Different mechanisms of action of antimicrobial peptides: insights from fluorescence spectroscopy experiments and molecular dynamics simulations. J Pept Sci 15:550–558. doi: 10.1002/psc.1144

Bohlmann, H., and Broekaert, W. (1994) The Role of Thionins in Plant Protection. Crit Rev Plant Sci 13:1–16. doi: 10.1080/07352689409701905

Bolintineanu, D. S., and Kaznessis, Y. N. (2011) Computational studies of protegrin antimicrobial peptides: A review. Peptides 32:188–201. doi: 10.1016/j.peptides.2010.10.006

Bolton, M. D. (2009) Primary Metabolism and Plant Defense—Fuel for the Fire. Mol Plant Microbe Interact 22:487–497. doi: 10.1094/MPMI-22-5-0487

Borchardt, J. K. (2002) The Beginnings of Drug Therapy: Ancient Mesopotamian Medicine. Drug News Perspect 15:187–192.

Boucher, H. W., Talbot, G. H., Benjamin Jr, D. K., Bradley, J., Guidos, R. J., Jones, R. N., ... and Infectious Diseases Society of America. (2013) 10 x ’20 Progress - Development of New Drugs Active Against Gram-Negative Bacilli: An Update From the Infectious Diseases Society of America. Clin Infect Dis 56:1685–1694. doi: 10.1093/cid/cit152

Braga, R. (1976) Plantas do Nordeste, Especialmente do Ceará. Escola Superior de Agricultura de Mossoró, Fortaleza

Brandenburg, K., Andrä, J., Garidel, P., and Gutsmann, T. (2011) Peptide-based treatment of sepsis. Appl Microbiol Biotechnol 90:799–808. doi: 10.1007/s00253-011-3185-7

Brandstädter, J., Roßbach, C., and Theres, K. (1996) Expression of genes for a defensin and a proteinase inhibitor in specific areas of the shoot apex and the developing flower in tomato. Mol Gen Genet MGG 252:146–154.

Brasil. Ministério da Saúde. (2009) Plantas de Interesse ao SUS. http://portalarquivos.saude.gov.br/images/pdf/2014/maio/07/renisus.pdf. Accessed 5 May 2017

Broekaert, W. F., Cammue, B. P., De Bolle, M. F., Thevissen, K., De Samblanx, G. W., Osborn, R. W., and Nielson, K. (1997) Antimicrobial Peptides from Plants. Crit Rev Plant Sci 16:297–323. doi: 10.1080/713608148

Broekaert, W. F., Terras, F. R., Cammue, B. P., and Osborn, R. W. (1995) Plant Defensins: Novel Antimicrobial Peptides as Components of the Host Defense System. Plant Physiol 108:1353–1358. doi: 10.1104/pp.108.4.1353

Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250. doi: 10.1038/nrmicro1098

Brötz, H., Josten, M., Wiedemann, I., Schneider, U., Götz, F., Bierbaum, G., and Sahl, H. G. (1998) Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol Microbiol 30:317–327.

Brown, J. K. M. (2002) Yield penalties of disease resistance in crops. Curr Opin Plant Biol 5:339–344.

Bruix, M., Jimenez, M. A., Santoro, J., Gonzalez, C., Colilla, F. J., Mendez, E., and Rico, M. (1993) Solution structure of gamma 1-H and gamma 1-P thionins from barley and wheat endosperm determined by 1H-NMR: a structural motif common to toxic arthropod proteins. Biochemistry (Mosc) 32:715–724.

Burkill, H. M. (2004) The useful plants of West Tropical Africa. Vol. 6: General Index, 2. ed. Royal Botanic Gardens, Kew

Butler, M. S., and Cooper, M. A. (2011) Antibiotics in the clinical pipeline in 2011. J Antibiot (Tokyo) 64:413–425. doi: 10.1038/ja.2011.44

Butler, M. S., Blaskovich, M. A., and Cooper, M. A. (2013) Antibiotics in the clinical pipeline in 2013. J Antibiot (Tokyo) 66:571–591. doi: 10.1038/ja.2013.86

Butler, M. S., Blaskovich, M. A., and Cooper, M. A. (2017) Antibiotics in the clinical pipeline at the end of 2015. J Antibiot (Tokyo) 70:3–24. doi: 10.1038/ja.2016.72

Carrasco, L., Vázquez, D., Hernández-Lucas, C., Carbonero, P., and García-Olmedo, F. (1981) Thionins: plant peptides that modify membrane permeability in cultured mammalian cells. Eur J Biochem 116:185–189.

Carvalho, A., and Gomes, V. M. (2011) Plant defensins and defensin-like peptides - biological activities and biotechnological applications. Curr Pharm Des 17:4270–4293.

Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A. P., & Nautiyal, A. R. (2017). Antimicrobial Resistance and the Alternative Resources with Special Emphasis on Plant-Based Antimicrobials—A Review. Plants 16.

Chen, K. C., Lin, C. Y., Chung, M. C., Kuan, C. C., Sung, H. Y., Tsou, S. C., ... and Chen, C. S. (2002) Cloning and characterization of a cDNA encoding an antimicrobial protein from mung bean seeds. Bot. Bull. Acad. Sin. 43:

Chiang, C. C., and Hadwiger, L. A. (1991) The *Fusarium solani* induced expression of a pea gene family encoding high cysteine content proteins. Mol Plant-Microbe Interact MPMI 4:324–331.

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006) Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. Cell 124:803–814. doi: 10.1016/j.cell.2006.02.008

Colilla, F. J., Rocher, A., and Mendez, E. (1990) γ-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. FEBS Lett 270:191–194. doi: 10.1016/0014-5793(90)81265-P

Coradin, L., Siminski, A., and Reis, A. (2011) Espécies nativas da flora brasileira de valor econômico atual ou potencial: plantas para o futuro - Região Sul. Ministério do Meio Ambiente, Brasília, DF.

Cordeiro, I., Secco, R., Cardiel, J. M., Steinmann, V., Caruzo, M. B. R., Riina, R., Lima, L. R., Maya-L, C. A, Berry, P., Carneiro-Torres, D. S. ... and Silva, R. A. P. (2015) Euphorbiaceae in Lista de Espécies da Flora do Brasil.

Cragg, G. M. and Newman, D. J. (2013) Natural products: A continuing source of novel drug leads. Biochim Biophys Acta BBA - Gen Subj 1830:3670–3695. doi: 10.1016/j.bbagen.2013.02.008

da Rocha Pitta, M. G., da Rocha Pitta, M. G., and Galdino, S. L. (2010) Development of novel therapeutic drugs in humans from plant antimicrobial peptides. Curr Protein Pept Sci 11:236–247.

de Albuquerque, U. P., De Medeiros, P. M., De Almeida, A. L. S., Monteiro, J. M., Neto, E. M. D. F. L., de Melo, J. G., and Dos Santos, J. P. (2007) Medicinal plants of the caatinga (semi-arid) vegetation of NE Brazil: A quantitative approach. J Ethnopharmacol 114:325–354. doi: 10.1016/j.jep.2007.08.017

de la Fuente-Núñez, C., Cardoso, M. H., de Souza Cândido, E., Franco, O. L., and Hancock, R. E. W. (2016) Synthetic antibiofilm peptides. Biochim Biophys Acta BBA - Biomembr 1858:1061–1069. doi: 10.1016/j.bbamem.2015.12.015

De Samblanx, G. W., Goderis, I. J., Thevissen, K., Raemaekers, R., Fant, F., Borremans, F., ... and Broekaert, W. F. (1997) Mutational analysis of a plant defensin from radish (*Raphanus sativus* L.) reveals two adjacent sites important for antifungal activity. J Biol Chem 272:1171–1179.

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., ... and Ryals, J. (1994) A Central Role of Salicylic Acid in Plant Disease Resistance. Science 266:1247–1250. doi: 10.1126/science.266.5188.1247

Di Stasi, L. C., Oliveira, G. P., Carvalhaes, M. A., Queiroz-Junior, M., Tien, O. S., Kakinami, S. H., and Reis, M. S. (2002) Medicinal plants popularly used in the Brazilian Tropical Atlantic Forest. Fitoterapia 73:69–91.

Dodds, P. N., and Rathjen, J. P. (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. Nat Rev Genet 11:539–548. doi: 10.1038/nrg2812

Dongmo, A. B., Kamanyi, A., Anchang, M. S., Nkeh, B. C. A., Njamen, D., Nguelefack, T. B., ... and Wagner, H. (2001) Anti-inflammatory and analgesic properties of the stem

extrakt of *Drypetes molunduana* Pax and Hoffm. (Euphorbiaceae) in rats. Pharm Pharmacol Lett 11:61–63.

Dutta, C.S., A.V. Glyakina, A. Lahiri, and O.V. Galzitskaya. 2018. “Plant Antibacterial Peptides and Their Possible Oligomerization.” In . Pushchino, Moscow Region, Russia. https://doi.org/10.17537/icmbb18.73.

du Vigneaud, V., Ressler, C., Swan, J. M., Roberts, C. W., and Katsoyannis, P. G. (1954) The Synthesis of Oxytocin. J Am Chem Soc 76:3115–3121. doi: 10.1021/ja01641a004

Elya, B., Basah, K., Mun'im, A., Yuliastuti, W., Bangun, A., and Septiana, E. K. (2012) Screening of α-Glucosidase Inhibitory Activity from Some Plants of Apocynaceae, Clusiaceae, Euphorbiaceae, and Rubiaceae. J Biomed Biotechnol 2012:1–6. doi: 10.1155/2012/281078

Emmert-Streib, F., Dehmer, M., and Haibe-Kains, B. (2014) Gene regulatory networks and their applications: understanding biological and medical problems in terms of networks. Front Cell Dev Biol. doi: 10.3389/fcell.2014.00038

Emmert-Streib, F., Zhang, S. D., and Hamilton, P. (2015) Computational cancer biology: education is a natural key to many locks. BMC Cancer. doi: 10.1186/s12885-014-1002-2

Engel, T. (2006) Basic Overview of Chemoinformatics. J Chem Inf Model 46:2267–2277. doi: 10.1021/ci600234z

Ernst, M., Grace, O. M., Saslis-Lagoudakis, C. H., Nilsson, N., Simonsen, H. T., and Rønsted, N. (2015) Global medicinal uses of *Euphorbia* L. (Euphorbiaceae). J Ethnopharmacol 176:90–101. doi: 10.1016/j.jep.2015.10.025

Fannang, S. V., Kuete, V., Djama, C. M., Dongfack, M. D. J., Wansi, J. D., Tillequin, F., ... and Wandji, J. (2011) A new friedelane triterpenoid and saponin with moderate antimicrobial activity from the stems of *Drypetes laciniata*. Chin Chem Lett 22:171–174. doi: 10.1016/j.cclet.2010.09.022

Fant, F., Vranken, W., Broekaert, W., and Borremans, F. (1998) Determination of the three-dimensional solution structure of *Raphanus sativus* Antifungal Protein 1 by 1 H NMR 1 1Edited by P. E. Wright. J Mol Biol 279:257–270. doi: 10.1006/jmbi.1998.1767

Fatumo, S. A., Adoga, M. P., Ojo, O. O., Oluwagbemi, O., Adeoye, T., Ewejobi, I., ... and Nashiru, O. (2014) Computational Biology and Bioinformatics in Nigeria. PLoS Comput Biol 10:e1003516. doi: 10.1371/journal.pcbi.1003516

Félix-Silva, J., Giordani, R.B., Silva-Jr, A.A., Zucolotto, S.M., and Fernandes-Pedrosa, M. F. (2014a) *Jatropha gossypiifolia* L. (Euphorbiaceae): A Review of Traditional Uses, Phytochemistry, Pharmacology, and Toxicology of This Medicinal Plant. Evid Based Complement Alternat Med 2014:1–32. doi: 10.1155/2014/369204

Félix-Silva, J., Souza, T., Menezes, Y. A., Cabral, B., Câmara, R. B., Silva-Junior, A. A., ... and Fernandes-Pedrosa, M. F. (2014b) Aqueous Leaf Extract of *Jatropha gossypiifolia* L. (Euphorbiaceae) Inhibits Enzymatic and Biological Actions of Bothrops jararaca Snake Venom. PLoS ONE 9:e104952. doi: 10.1371/journal.pone.0104952

Ferri, M., Ranucci, E., Romagnoli, P., and Giaccone, V. (2017) Antimicrobial resistance: A global emerging threat to public health systems. Crit Rev Food Sci Nutr 57:2857–2876. doi: 10.1080/10408398.2015.1077192

Fischer, E., and Fourneau, E. (1901) Ueber einige Derivate des Glykocolls. Berichte Dtsch Chem Ges 34:2868–2877. doi: 10.1002/cber.190103402249

Fitzgerald-Hughes, D., Devocelle, M., and Humphreys, H. (2012) Beyond conventional antibiotics for the future treatment of methicillin-resistant *Staphylococcus aureus* infections: two novel alternatives. FEMS Immunol Med Microbiol 65:399–412. doi: 10.1111/j.1574-695X.2012.00954.x

Fjell, C.D., Hiss, J.A., Hancock, R. E. W., and Schneider, G. (2011) Designing antimicrobial peptides: form follows function. Nat Rev Drug Discov. doi: 10.1038/nrd3591

Florack, D. E., and Stiekema, W. J. (1994) Thionins: properties, possible biological roles and mechanisms of action. Plant Mol Biol 26:25–37.

Fogg, C. N., and Kovats, D. E. (2015) Computational Biology: Moving into the Future One Click at a Time. PLOS Comput Biol 11:e1004323. doi: 10.1371/journal.pcbi.1004323

Franco, O. L. (2011) Peptide promiscuity: An evolutionary concept for plant defense. FEBS Lett 585:995–1000. doi: 10.1016/j.febslet.2011.03.008

Friedrich, C. L., Rozek, A., Patrzykat, A., and Hancock, R. E. W. (2001) Structure and Mechanism of Action of an Indolicidin Peptide Derivative with Improved Activity against Gram-positive Bacteria. J Biol Chem 276:24015–24022. doi: 10.1074/jbc.M009691200

Friolani, M., Daleck, C. R., Repetti, C. S., and Alessi, A. C. (2017) Use of rubber tree (*Hevea brasiliensis*) latex biomembrane in diaphragmatic injuries in rabbits–an experimental study. Braz J Vet Pathol 4:41–43.

Fürstenberg-Hägg, J., Zagrobelny, M., and Bak, S. (2013) Plant Defense against Insect Herbivores. Int J Mol Sci 14:10242–10297. doi: 10.3390/ijms140510242

Gadamsetty, G., Maru, S., Tyagi, A., and Chakravarthula, S. N. (2013) Anti-inflammatory, cytotoxic and antioxidant effects of methanolic extracts of *Drypetes sepiaria* (Euphorbiaceae). Afr J Tradit Complement Altern Med AJTCAM 10:274–282.

Gallo, R. L., Murakami, M., Ohtake, T., and Zaiou, M. (2002) Biology and clinical relevance of naturally occurring antimicrobial peptides. J Allergy Clin Immunol 110:823–831.

Gangwar, M., Goel, R. K., and Nath, G. (2014) *Mallotus philippinensis* Muell. Arg (Euphorbiaceae): Ethnopharmacology and Phytochemistry Review. BioMed Res Int 2014:1–13. doi: 10.1155/2014/213973

Garrigues, S., Gandía, M., Borics, A., Marx, F., Manzanares, P., and Marcos, J. F. (2017) Mapping and Identification of Antifungal Peptides in the Putative Antifungal Protein AfpB from the Filamentous Fungus *Penicillium digitatum*. Front Microbiol. doi: 10.3389/fmicb.2017.00592

Garten, Y., and Altman, R. B. (2009) Pharmspresso: a text mining tool for extraction of pharmacogenomic concepts and relationships from full text. BMC Bioinformatics 10:S6. doi: 10.1186/1471-2105-10-S2-S6

Gazit, E., Boman, A., Boman, H. G., and Shai, Y. (1995) Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. Biochemistry (Mosc) 34:11479–11488.

Gennaro, R., and Zanetti, M. (2000) Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Biopolymers 55:31–49. doi: 10.1002/1097-0282(2000)55:1<31::AID-BIP40>3.0.CO;2-9

Gleadow, R., Pegg, A., and Blomstedt, C. K. (2016) Resilience of cassava (*Manihot esculenta* Crantz) to salinity: implications for food security in low-lying regions. J Exp Bot 67:5403–5413.

Gomes, J. A. D. S., Félix-Silva, J., Morais Fernandes, J., Geraldo Amaral, J., Lopes, N. P., Tabosa do Egito, E. S., ... and Fernandes-Pedrosa, M. D. F. (2016) Aqueous Leaf Extract of *Jatropha mollissima* (Pohl) Bail Decreases Local Effects Induced by Bothropic Venom. BioMed Res Int 2016:1–13. doi: 10.1155/2016/6101742

Goyal, R. K., and Mattoo, A. K. (2014) Multitasking antimicrobial peptides in plant development and host defense against biotic/abiotic stress. Plant Sci 228:135–149. doi: 10.1016/j.plantsci.2014.05.012

Goyal, R. K., and Mattoo, A. K. 2016. “Plant Antimicrobial Peptides.” In Host Defense Peptides and Their Potential as Therapeutic Agents, 111–36. Springer.

Gu, Q., Kawata, E. E., Morse, M. J., Wu, H. M., and Cheung, A. Y. (1992) A flower-specific cDNA encoding a novel thionin in tobacco. Mol Gen Genet MGG 234:89–96.

Guo, Y., Wang, J., Niu, G., Shui, W., Sun, Y., Zhou, H., ... and Rao, Z. (2011) A structural view of the antibiotic degradation enzyme NDM-1 from a superbug. Protein Cell 2:384–394. doi: 10.1007/s13238-011-1055-9

Hammami, R., Ben Hamida, J., Vergoten, G., and Fliss, I. (2009) PhytAMP: a database dedicated to antimicrobial plant peptides. Nucleic Acids Res 37:D963–D968. doi: 10.1093/nar/gkn655

Han, J., Jyoti, M. A., Song, H. Y., and Jang, W. S. (2016) Antifungal Activity and Action Mechanism of Histatin 5-Halocidin Hybrid Peptides against *Candida* ssp. Plos one 11:e0150196. doi: 10.1371/journal.pone.0150196

Hancock, R. E. W., and Sahl, H. G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol 24:1551–1557. doi: 10.1038/nbt1267

Haque, Z., Ahmed, T., Asma, K., Rumman, M., and Alam, A. (2015) Medicinal effect of Kameela and *Amraze jildiya* (skin diseases) described in Unani system of medicine and current research–an overview. Int J Pharmacogn 2:437–439.

He, Y., and Lazaridis, T. (2013) Activity Determinants of Helical Antimicrobial Peptides: A Large-Scale Computational Study. PLoS ONE 8:e66440. doi: 10.1371/journal.pone.0066440

Henriques, S. T., Melo, M. N., and Castanho, M. A. R. B. (2006) Cell-penetrating peptides and antimicrobial peptides: how different are they? Biochem J 399:1–7. doi: 10.1042/BJ20061100

Hu, L. F., Chang, X., Ye, Y., Wang, Z. X., Shao, Y. B., Shi, W., ... and Li, J. B. (2011) *Stenotrophomonas maltophilia* resistance to trimethoprim/sulfamethoxazole mediated by acquisition of sul and dfrA genes in a plasmid-mediated class 1 integron. Int J Antimicrob Agents 37:230–234. doi: 10.1016/j.ijantimicag.2010.10.025

Huang, Y. B., He, L. Y., Jiang, H. Y., and Chen, Y. X. (2012) Role of Helicity on the Anticancer Mechanism of Action of Cationic-Helical Peptides. Int J Mol Sci 13:6849–6862. doi: 10.3390/ijms13066849

Huttner, A., Harbarth, S., Carlet, J., Cosgrove, S., Goossens, H., Holmes, A., ... and Pittet, D. (2013) Antimicrobial resistance: a global view from the 2013 World Healthcare-Associated Infections Forum. Antimicrob Resist Infect Control 2:31. doi: 10.1186/2047-2994-2-31

Irvine, F. R. (1961) Woody plants of Ghana. Woody Plants Ghana

Jacob, J. T., Klein, G. E., Laxminarayan, R., Beldavs, Z., Lynfield, R., Kallen, A. J., Ricks, P., Edwards, J., Srinivasan, A., and Fridkin, S. (2013) Vital signs: carbapenem-resistant Enterobacteriaceae. Morb Mortal Wkly Rep 62:165–169.

Janeway Jr, C. A., and Medzhitov, R. (2002) Innate immune recognition. Annu Rev Immunol 20:197–216.

Janssen, B. J. C., Schirra, H. J., Lay, F. T., Anderson, M. A., and Craik, D. J. (2003) Structure of *Petunia hybrida* Defensin 1, a Novel Plant Defensin with Five Disulfide Bonds †. Biochemistry (Mosc) 42:8214–8222. doi: 10.1021/bi034379o

Jenssen, H., Hamill. P., and Hancock, R. E. W. (2006) Peptide Antimicrobial Agents. Clin Microbiol Rev 19:491–511. doi: 10.1128/CMR.00056-05

Jiang, Z., Vasil, A. I., Gera, L., Vasil, M. L., and Hodges, R. S. (2011) Rational Design of α-Helical Antimicrobial Peptides to Target Gram-negative Pathogens, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*: Utilization of Charge, 'Specificity Determinants,' Total Hydrophobicity, Hydrophobe Type and Location as Design Para: Antimicrobial Peptides to Target Gram-negative Pathogens. Chem Biol Drug Des 77:225–240. doi: 10.1111/j.1747-0285.2011.01086.x

Jones, J. D. G., and Dangl, J. L. (2006) The plant immune system. Nature 444:323–329. doi: 10.1038/nature05286

Jussieu, A. (1824) De Euphorbiacearum generibus medisque earumdem viribus tentamen ... doi: 10.5962/bhl.title.51511

Kaewklom, S., Euanorasetr, J., Intra, B., Panbangred, W., and Aunpad, R. (2016) Antimicrobial Activities of Novel Peptides Derived from Defensin Genes of *Brassica* hybrid cv Pule. Int J Pept Res Ther 22:93–100. doi: 10.1007/s10989-015-9488-2

Karunanandaa, B., Singh, A., and Kao, T. (1994) Characterization of a predominantly pistil-expressed gene encoding a γ-thionin-like protein of *Petunia inflata*. Plant Mol Biol 26:459–464. doi: 10.1007/BF00039555

Kaspar, A. A., and Reichert, J. M. (2013) Future directions for peptide therapeutics development. Drug Discov Today 18:807–817. doi: 10.1016/j.drudis.2013.05.011

Kim, D. H., Lee, D. G., Kim, K. L., and Lee, Y. (2001) Internalization of tenecin 3 by a fungal cellular process is essential for its fungicidal effect on *Candida albicans*. Eur J Biochem 268:4449–4458.

King, A. J., Brown, G. D., Gilday, A. D., Larson, T. R., and Graham, I. A. (2014) Production of Bioactive Diterpenoids in the Euphorbiaceae Depends on Evolutionarily Conserved Gene Clusters. Plant Cell 26:3286–3298. doi: 10.1105/tpc.114.129668

Komori, T., Yamada, S., and Imaseki, H. (1997) A cDNA clone for γ-thionin from *Nicotiana paniculata* (accession no. AB005250; PGR97–132). Plant Physiol 115:314.

Kozma, D., Simon, I., and Tusnády, G. E. (2012) PDBTM: Protein Data Bank of transmembrane proteins after 8 years. Nucleic Acids Res 41:D524–D529. doi: 10.1093/nar/gks1169

Kragh, K. M., Nielsen, J. E., Nielsen, K. K., Dreboldt, S., and Mikkelsen, J. D. (1995) Characterization and localization of new antifungal cysteine-rich proteins from Beta vulgaris. Mol Plant-Microbe Interact MPMI 8:424–434.

Kubinyi, H. (2003) Opinion: Drug research: myths, hype and reality. Nat Rev Drug Discov 2:665–668. doi: 10.1038/nrd1156

Kumar, N., Hendriks, B. S., Janes, K. A., de Graaf, D., and Lauffenburger, D. A. (2006) Applying computational modeling to drug discovery and development. Drug Discov Today 11:806–811. doi: 10.1016/j.drudis.2006.07.010

Kumari, I. 2018. “A Study of the Antimicrobial Effect of *Euphorbia Hirta* L. against *Escherichia Coli*.” *Vegetos*- An International Journal of Plant Research 31 (3): 123. https://doi.org/10.5958/2229-4473.2018.00083.6.

Labbé, C. M., Kuenemann, M. A., Zarzycka, B., Vriend, G., Nicolaes, G. A., Lagorce, D., ... and Sperandio, O. (2016) iPPI-DB: an online database of modulators of protein–protein interactions. Nucleic Acids Res 44:D542–D547. doi: 10.1093/nar/gkv982

Lacerda, A., Vasconcelos, É. A. R., Pelegrini, P. B., and Grossi-de-Sa, M. F. (2014) Antifungal defensins and their role in plant defense. Front Microbiol. doi: 10.3389/fmicb.2014.00116

Lafferty, M. K., Sun, L., Christensen-Quick, A., Lu, W., and Garzino-Demo, A. (2017) Human Beta Defensin 2 Selectively Inhibits HIV-1 in Highly Permissive CCR6+CD4+ T Cells. Viruses 9:111. doi: 10.3390/v9050111

Lambert, P. (2005) Bacterial resistance to antibiotics: Modified target sites. Adv Drug Deliv Rev 57:1471–1485. doi: 10.1016/j.addr.2005.04.003

Laughlin, T. F., and Ahmad, Z. (2010) Inhibition of Escherichia coli ATP synthase by amphibian antimicrobial peptides. Int J Biol Macromol 46:367–374. doi: 10.1016/j.ijbiomac.2010.01.015

Lay, F. T. (2003) Isolation and Properties of Floral Defensins from Ornamental Tobacco and Petunia. PLANT Physiol 131:1283–1293. doi: 10.1104/pp.102.016626

Lay, F. T., and Anderson, M. A. (2005) Defensins--components of the innate immune system in plants. Curr Protein Pept Sci 6:85–101.

Lehrer, R. I., Lichtenstein, A. K., and Ganz, T. (1993) Defensins: Antimicrobial and Cytotoxic Peptides of Mammalian Cells. Annu Rev Immunol 11:105–128. doi: 10.1146/annurev.iy.11.040193.000541

Li, H. Y., and Gray, J. E. (1999) Molecular characterization of a cDNA, NTS13, encoding a defensin-like protein in tobacco styles. Plant Physiol 120:633–633.

Li, X. Z., Plésiat, P., and Nikaido, H. (2015) The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria. Clin Microbiol Rev 28:337–418. doi: 10.1128/CMR.00117-14

Li. T., Wang, N., Chen, S., Lu, R., Li, H., and Zhang, Z. (2017) Antibacterial activity and cytocompatibility of an implant coating consisting of TiO2 nanotubes combined with a GL13K antimicrobial peptide. Int J Nanomedicine 12:2995.

Liang, Y., and Kelemen, A. (2017) Dynamic modeling and network approaches for omics time course data: overview of computational approaches and applications. Brief Bioinform. doi: 10.1093/bib/bbx036

Liaw, S. J., Lee, Y. L., and Hsueh, P. R. (2010) Multidrug resistance in clinical isolates of *Stenotrophomonas maltophilia*: roles of integrons, efflux pumps, phosphoglucomutase (SpgM), and melanin and biofilm formation. Int J Antimicrob Agents 35:126–130. doi: 10.1016/j.ijantimicag.2009.09.015

Lima, L. R., and Pirani, J. R. (2008) Revisão taxonômica de *Croton* sect. Lamprocroton (Müll. Arg.) Pax (Euphorbiaceae s.s.). Biota Neotropica 8:177–231. doi: 10.1590/S1676-06032008000200018

Liu, B., Peng, H., Yao, Q., Li, J., Van Damme, E., Balzarini, J., and Bao, J. K. (2009) Bioinformatics analyses of the mannose-binding lectins from *Polygonatum cyrtonema*, Ophiopogon *japonicus* and *Liparis noversa* with antiproliferative and apoptosis-inducing activities. Phytomedicine 16:601–608. doi: 10.1016/j.phymed.2008.12.010

Liu, X., Li, Z., Wang, X., Chen, Y., Wu, F., Men, K., ... and Yang, L. (2016) Novel antimicrobial peptide–modified azithromycin-loaded liposomes against methicillin-resistant *Staphylococcus aureus*. Int J Nanomedicine Volume 11:6781–6794. doi: 10.2147/IJN.S107107

Lobanov, V. (2004) Using artificial neural networks to drive virtual screening of combinatorial libraries. Drug Discov Today BIOSILICO 2:149–156. doi: 10.1016/S1741-8364(04)02402-3

Loutet, S. A., and Valvano, M. A. (2011) Extreme Antimicrobial Peptide and Polymyxin B Resistance in the Genus *Burkholderia*. Front Microbiol. doi: 10.3389/fmicb.2011.00159

Manko, A., Motta, J. P., Cotton, J. A., Feener, T., Oyeyemi, A., Vallance, B. A., ... and Buret, A. G. (2017) Giardia co-infection promotes the secretion of antimicrobial peptides beta-defensin 2 and trefoil factor 3 and attenuates attaching and effacing bacteria-induced intestinal disease. PLOS ONE 12:e0178647. doi: 10.1371/journal.pone.0178647

Marqus, S., Pirogova, E., and Piva, T. J. (2017) Evaluation of the use of therapeutic peptides for cancer treatment. J Biomed Sci. doi: 10.1186/s12929-017-0328-x

Marrer, E., and Dieterle, F. (2007) Promises of Biomarkers in Drug Development ? A Reality Check. Chem Biol Drug Des 69:381–394. doi: 10.1111/j.1747-0285.2007.00522.x

Marrer, E., and Dieterle, F. (2008) Biomarkers in oncology drug development: rescuers or troublemakers? Expert Opin Drug Metab Toxicol 4:1391–1402. doi: 10.1517/17425255.4.11.1391

Martinez, J. L. (2009a) The role of natural environments in the evolution of resistance traits in pathogenic bacteria. Proc R Soc B Biol Sci 276:2521–2530. doi: 10.1098/rspb.2009.0320

Martinez, J. L. (2009b) Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ Pollut 157:2893–2902. doi: 10.1016/j.envpol.2009.05.051

Mathieu, C., Augusto, M. T., Niewiesk, S., Horvat, B., Palermo, L. M., Sanna, G., ... and Santos, N. C. (2017) Broad spectrum antiviral activity for paramyxoviruses is modulated by biophysical properties of fusion inhibitory peptides. Sci Rep 7:43610. doi: 10.1038/srep43610

Mátyus, E., Kandt, C., and Tieleman, D. P. (2007) Computer simulation of antimicrobial peptides. Curr Med Chem 14:2789–2798.

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., ... and Kalan, L. (2013) The Comprehensive Antibiotic Resistance Database. Antimicrob Agents Chemother 57:3348–3357. doi: 10.1128/AAC.00419-13

Méité, S., Bahi, C., Yéo, D., Datté, J. Y., Djaman, J. A., and N'guessan, D. J. (2010) Laxative activities of *Mareya micrantha* (Benth.) Müll. Arg. (Euphorbiaceae) leaf aqueous extract in rats. BMC Complement Altern Med. doi: 10.1186/1472-6882-10-7

Mendez, E., Moreno, A., Colilla, F., Pelaez, F., Limas, G. G., Mendez, R., ... and de Haro, C. (1990) Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, gamma-hordothionin, from barley endosperm. Eur J Biochem 194:533–539.

Merrifield, R. B. (1963) Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. J Am Chem Soc 85:2149–2154. doi: 10.1021/ja00897a025

Metraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990) Increase in Salicylic Acid at the Onset of Systemic Acquired Resistance in Cucumber. Science 250:1004–1006. doi: 10.1126/science.250.4983.1004

Meyer, B., Houlne, G., Pozueta-Romero, J., Schantz, M. L., and Schantz, R. (1996) Fruit-specific expression of a defensin-type gene family in bell pepper. Upregulation during ripening and upon wounding. Plant Physiol 112:615–622.

Michaelson, D., Rayner, J., Couto, M., and Ganz, T. (1992) Cationic defensins arise from charge-neutralized propeptides: a mechanism for avoiding leukocyte autocytotoxicity? J Leukoc Biol 51:634–639.

Milligan, S. B., and Gasser, C. S. (1995) Nature and regulation of pistil-expressed genes in tomato. Plant Mol Biol 28:691–711. doi: 10.1007/BF00021194

Moreno, M., Segura, A., and García-Olmedo, F. (1994) Pseudothionin-St1, a potato peptide active against potato pathogens. Eur J Biochem 223:135–139.

Moses, H., Dorsey, E. R., Matheson, D. H. M., and Thier, S. O. (2005) Financial Anatomy of Biomedical Research. JAMA 294:1333. doi: 10.1001/jama.294.11.1333

Mueller. (1873) Euphorbiaceae. In Flora brasiliensis (C.F.P. Martius and A.G. Eichler, eds.).

Muñoz, A., Gandía, M., Harries, E., Carmona, L., Read, N. D., and Marcos, J. F. (2013) Understanding the mechanism of action of cell-penetrating antifungal peptides using the rationally designed hexapeptide PAF26 as a model. Fungal Biol Rev 26:146–155. doi: 10.1016/j.fbr.2012.10.003

Muttenthaler, M., Albericio, F., and Dawson, P. E. (2015) Methods, setup and safe handling for anhydrous hydrogen fluoride cleavage in Boc solid-phase peptide synthesis. Nat Protoc 10:1067–1083. doi: 10.1038/nprot.2015.061

Mwine, J. T., and Van Damme, P. (2011) Why do Euphorbiaceae tick as medicinal plants? A review of Euphorbiaceae family and its medicinal features. J Med Plants Res 5:652–662.

Myers, S., and Baker, A. (2001) Drug discovery an operating model for a new era. Nat Biotechnol 19:727–730. doi: 10.1038/90765

Nawrot, R., Barylski, J., Nowicki, G., Broniarczyk, J., Buchwald, W., and Goździcka-Józefiak, A. (2014) Plant antimicrobial peptides. Folia Microbiol (Praha) 59:181–196. doi: 10.1007/s12223-013-0280-4

Nenkep, V. N., Shirri, J. C., Van-Dufat, H. T., Sipepnou, F., Vérité, P., Seguin, E., ... and Wandji, J. (2008) New flavan and unusual chalcone glycosides from *Drypetes parvifolia*. Chin Chem Lett 19:943–946. doi: 10.1016/j.cclet.2008.04.048

Nguyen, L. T., Haney, E. F., and Vogel, H. J. (2011) The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol 29:464–472. doi: 10.1016/j.tibtech.2011.05.001

Nikaido, H., and Pagès, J. M. (2012) Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol Rev 36:340–363. doi: 10.1111/j.1574-6976.2011.00290.x

O'Neill. (2016) Tackling drug-resistant infections globally: final report and recommendations. Rev Antimicrob Resist 15:526–526. doi: 10.1038/nrd.2016.160

Oberholzer-Gee, F., and Inamdar, S. N. (2004) Merck's Recall of Rofecoxib — A Strategic Perspective. N Engl J Med 351:2147–2149. doi: 10.1056/NEJMp048284

Okamura, N., Mori, M., Furumoto, S., Yoshikawa, T., Harada, R., Ito, S., ... and Kudo, Y. (2011) *In vivo* detection of amyloid plaques in the mouse brain using the near-infrared fluorescence probe THK-265. J Alzheimers Dis JAD 23:37–48. doi: 10.3233/JAD-2010-100270

Ou-Yang, S., Lu, J., Kong, X., Liang, Z., Luo, C., and Jiang, H. (2012) Computational drug discovery. Acta Pharmacol Sin 33:1131–1140. doi: 10.1038/aps.2012.109

Outterson, K., Rex, J. H., Jinks, T., Jackson, P., Hallinan, J., Karp, S., ... and Dixon, D. M. (2016) Accelerating global innovation to address antibacterial resistance: introducing CARB-X. Nat Rev Drug Discov 15:589–590. doi: 10.1038/nrd.2016.155

Pachón-Ibáñez, M. E., Smani, Y., Pachón, J., and Sánchez-Céspedes, J. (2017) Perspectives for clinical use of engineered human host defense antimicrobial peptides. FEMS Microbiol Rev 41:323–342. doi: 10.1093/femsre/fux012

Paiva, A. D., and Breukink, E. (2013) Antimicrobial peptides produced by microorganisms. Antimicrobial Peptides and Innate Immunity. Springer, pp 53–95

Pande, J., Szewczyk, M. M., and Grover, A. K. (2010) Phage display: concept, innovations, applications and future. Biotechnol Adv 28:849–858. doi: 10.1016/j.biotechadv.2010.07.004

Park, H. C., Kang, Y. H., Chun, H. J., Koo, J. C., Cheong, Y. H., Kim, C. Y., ... and Koo, Y. D. (2002) Characterization of a stamen-specific cDNA encoding a novel plant defensin in Chinese cabbage. Plant Mol Biol 50:59–69.

Pax, F. (1910) Aleurites in Euphorbiaceae—*Jatropheae* in Engler.

Pax, F. (1924) Die phylogenie der Euphorbiaceae. Bot Jahrb Für Syst Pflanzengesch Pflanzengeogr 59:129–182.

Pax, F., and Hoffmann, K. (1931) Euphorbiaceae *Macaranga*. Engler-Prantl, ed. Nat. Pflanzenfam

Peschel, A., and Sahl, H. G. (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol 4:529–536. doi: 10.1038/nrmicro1441

Pimentel, L. A., Riet-Correa, B., Dantas, A. F., Medeiros, R. M. T., and Riet-Correa, F. (2012) Poisoning by *Jatropha ribifolia* in goats. Toxicon 59:587–591. doi: 10.1016/j.toxicon.2012.01.002

Pino-Angeles, A., Leveritt, J. M., and Lazaridis, T. (2016) Pore Structure and Synergy in Antimicrobial Peptides of the Magainin Family. PLOS Comput Biol 12:e1004570. doi: 10.1371/journal.pcbi.1004570

Policy, I. P. (2010) The 10 ‘20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. Clin Infect Dis 50:1081–1083.

Prithiviraj, R. M., Paschke, M. W., and Vivanco. J. M. (2007) Root communication: the role of root exudates. Encyclopedia Plant and Crop Sci. doi: 10.1081/E-EPCS

Punt, W. (1962) Pollen morphology of the euphorbiaceae with special reference to taxonomy. Wentia 7:1–116. doi: 10.1111/j.1438-8677.1962.tb00010.x

Raja, A., Vipin, C., and Aiyappan, A. (2013) Biological importance of marine algae-an overview. Int J Curr Microbiol Appl Sci 2:222–227.

Ramsden, J. (2015) Bioinformatics: an introduction, 3rd ed. Springer, London

Román-Figueroa, C., Olivares-Carrillo, P., Paneque, M., Palacios-Nereo, F. J., and Quesada-Medina, J. (2016) High-yield production of biodiesel by non-catalytic

supercritical methanol transesterification of crude castor oil (*Ricinus communis*). Energy 107:165–171.

Ruangnoo, S., Kuropakornpong, P., and Itharat, A. (2017) Cytotoxic activity of medicinal plants selected from Euphorbiaceae family against Hepatocellular carcinoma cell. Planta Medica Int Open 4:Tu-PO-83.

Ryan, R. P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M. B., ... and Dow, J. M. (2009) The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat Rev Microbiol 7:514–525. doi: 10.1038/nrmicro2163

Sabandar, C. W., Ahmat, N., Jaafar, F. M., and Sahidin, I. (2013) Medicinal property, phytochemistry and pharmacology of several *Jatropha* species (Euphorbiaceae): A review. Phytochemistry 85:7–29. doi: 10.1016/j.phytochem.2012.10.009

Sagaram, U. S., Pandurangi, R., Kaur, J., Smith, T. J., and Shah, D. M. (2011) Structure-Activity Determinants in Antifungal Plant Defensins MsDef1 and MtDef4 with Different Modes of Action against *Fusarium graminearum*. PLoS ONE 6:e18550. doi: 10.1371/journal.pone.0018550

Sakata, T., and Winzeler, E. A. (2007) Genomics, systems biology and drug development for infectious diseases. Mol Biosyst 3:841. doi: 10.1039/b703924g

Sánchez, T., Salcedo, E., Ceballos, H., Dufour, D., Mafla, G., Morante, N., ... and Moreno, I. X. (2009) Screening of Starch Quality Traits in Cassava (*Manihot esculenta* Crantz). Starch - Stärke 61:12–19. doi: 10.1002/star.200800058

Santana, K. C. B. (2012) Isolamento de gene de defensina em *Euphorbia hyssopifolia* L., caracterização *in silico*, propriedades químicas e função putativa da proteína codificada.

Santussi, W. M., Bordon, K. C., Rodrigues Alves, A. P., Cologna, C. T., Said, S., and Arantes, E. C. (2017) Antifungal Activity against Filamentous Fungi of Ts1, a Multifunctional Toxin from *Tityus serrulatus* Scorpion Venom. Front Microbiol. doi: 10.3389/fmicb.2017.00984

Schaaper, W. M. M., Posthuma, G. A., Meloen, R. H., Plasman, H. H., Sijtsma, L., Van Amerongen, A., ... and Broekaert, W. F. (2001) Synthetic peptides derived from the beta2-beta3 loop of *Raphanus sativus* antifungal protein 2 that mimic the active site. J Pept Res 57:409–418. doi: 10.1034/j.1399-3011.2001.00842.x

Schwessinger, B., and Zipfel, C. (2008) News from the frontline: recent insights into PAMP-triggered immunity in plants. Curr Opin Plant Biol 11:389–395. doi: 10.1016/j.pbi.2008.06.001

Secco, R. D. S., Cordeiro, I., Senna-Vale, L. D., Sales, M. F. D., Lima, L. R. D., Medeiros, D., ... and Bigio, N. C. (2012) An overview of recent taxonomic studies on Euphorbiaceae s.l. in Brazil. Rodriguésia 63:227–242. doi: 10.1590/S2175-78602012000100014

Segura, A., Moreno, M., Molina, A., and García-Olmedo, F. (1998) Novel defensin subfamily from spinach (*Spinacia oleracea*). FEBS Lett 435:159–162.

Shai, Y. (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1462:55–70.

Shai, Y. (2002) Mode of action of membrane active antimicrobial peptides. Biopolymers 66:236–248. doi: 10.1002/bip.10260

Sharma, P., and Lönneborg, A. (1996) Isolation and characterization of a cDNA encoding a plant defensin-like protein from roots of Norway spruce. Plant Mol Biol 31:707–712.

Shekhar, C. (2008) *In Silico* Pharmacology: Computer-Aided Methods Could Transform Drug Development. Chem Biol 15:413–414. doi: 10.1016/j.chembiol.2008.05.001

Shewry, P. R., and Lucas, J. A. (1997) Plant Proteins that Confer Resistance to Pests and Pathogens. Advances in Botanical Research. Elsevier, pp 135–192

Silva, O. N., Fensterseifer, I. C., Rodrigues, E. A., Holanda, H. H., Novaes, N. R., Cunha, J. P., ... and Bocca, A. L. (2015) Clavanin A Improves Outcome of Complications from Different Bacterial Infections. Antimicrob Agents Chemother 59:1620–1626. doi: 10.1128/AAC.03732-14

Stotz, H. U., Waller, F., and Wang, K. (2013) Innate Immunity in Plants: The Role of Antimicrobial Peptides. In: Hiemstra PS and Zaat SAJ (eds) Antimicrobial Peptides and Innate Immunity. Springer Basel, Basel, pp 29–51

Strahilevitz, J., Mor, A., Nicolas, P., and Shai, Y. (1994) Spectrum of Antimicrobial Activity and Assembly of Dermaseptin-b and Its Precursor Form in Phospholipid Membranes. Biochemistry (Mosc) 33:10951–10960. doi: 10.1021/bi00202a014

Subramani, R., Narayanasamy, M., and Feussner, K. D. (2017) Plant-derived antimicrobials to fight against multi-drug-resistant human pathogens. 3 Biotech 7:172.

Sudharshan, S. J., Chinmaya, A., Valleesha, N. C., Kekuda, T. R., Rajeshwara, A. N., and Syed, M. (2009) Central Nervous System (CNS) depressant and analgesic activity of methanolic extract of Drypetes roxburghii wall in experimental animal model. Res J Pharm Technol 2:854–857.

Sun, L., Zheng, C., and Webster, T. (2016) Self-assembled peptide nanomaterials for biomedical applications: promises and pitfalls. Int J Nanomedicine Volume 12:73–86. doi: 10.2147/IJN.S117501

Tan, J. J., Cong, X. J., Hu, L. M., Wang, C. X., Jia, L., and Liang, X. J. (2010) Therapeutic strategies underpinning the development of novel techniques for the treatment of HIV infection. Drug Discov Today 15:186–197. doi: 10.1016/j.drudis.2010.01.004

Tan, T., Wu, D., Li, W., Zheng, X., Li, W., and Shan, A. (2017) High Specific Selectivity and Membrane-Active Mechanism of Synthetic Cationic Hybrid Antimicrobial Peptides Based on the Peptide FV7. Int J Mol Sci 18:339. doi: 10.3390/ijms18020339

Tang, Y., Zhu, W., Chen, K., and Jiang, H. (2006) New technologies in computer-aided drug design: Toward target identification and new chemical entity discovery. Drug Discov Today Technol 3:307–313. doi: 10.1016/j.ddtec.2006.09.004

Tavormina, P., De Coninck, B., Nikonorova, N., De Smet, I., and Cammue, B. P. A., (2015) The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions. Plant Cell 27:2095–2118. doi: 10.1105/tpc.15.00440

Terras, F. (1995) Small Cysteine-Rich Antifungal Proteins from Radish: Their Role in Host Defense. Plant Cell online 7:573–588. doi: 10.1105/tpc.7.5.573

Thevissen, K., Cammue, B. P., Lemaire, K., Winderickx, J., Dickson, R. C., Lester, R. L., ... and Broekaert, W. F. (2000) A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of *Saccharomyces cerevisiae* to an antifungal plant defensin from dahlia (*Dahlia merckii*). Proc Natl Acad Sci 97:9531–9536. doi: 10.1073/pnas.160077797

Thevissen, K., Ferket, K. K. A., François, I. E. J. A., and Cammue, B. P. A. (2003) Interactions of antifungal plant defensins with fungal membrane components. Peptides 24:1705–1712. doi: 10.1016/j.peptides.2003.09.014

Thomma, B. P., Eggermont, K., Penninckx, I. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P., and Broekaert, W. F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci U S A 95:15107–15111.

Thomma, B., Cammue, B., and Thevissen, K. (2002) Plant defensins. Planta 216:193–202. doi: 10.1007/s00425-002-0902-6

Tomlinson, P. B. (2016) The botany of mangroves, 1st pbk. ed. Cambridge University Press, Cambridge ; New York

Tortora, G. J., Funke, B. R., and Case, C. L. (2010) Microbiology: an introduction, 10th ed. Pearson Benjamin Cummings, San Francisco, CA

Tripathi, A. K., Kumari, T., Harioudh, M. K., Yadav, P. K., Kathuria, M., Shukla, P. K., ... and Ghosh, J. K. (2017) Identification of GXXXXG motif in Chrysophsin-1 and its implication in the design of analogs with cell-selective antimicrobial and anti-endotoxin activities. Sci Rep. doi: 10.1038/s41598-017-03576-1

Vaara, M. (2009) New approaches in peptide antibiotics. Curr Opin Pharmacol 9:571–576. doi: 10.1016/j.coph.2009.08.002

Van Damme, P. L. J. (2001) *Euphorbia tirucalli* for High Biomass Production. In: Pasternak D and Schlissel A (eds) Combating Desertification with Plants. Springer US, Boston, MA, pp 169–187

van der Weerden, N. L., and Anderson, M. A. (2013) Plant defensins: Common fold, multiple functions. Fungal Biol Rev 26:121–131. doi: 10.1016/j.fbr.2012.08.004

Villanueva, J., Quirós, L. M., and Castañón, S. (2015) Purification and partial characterization of a ribosome-inactivating protein from the latex of *Euphorbia trigona* Miller with cytotoxic activity toward human cancer cell lines. Phytomedicine 22:689–695. doi: 10.1016/j.phymed.2015.04.006

Vlieghe, P., Lisowski, V., Martinez, J., and Khrestchatisky, M. (2010) Synthetic therapeutic peptides: science and market. Drug Discov Today 15:40–56. doi: 10.1016/j.drudis.2009.10.009

Voukeng, I. K., Nganou, B. K., Sandjo, L. P., Celik, I., Beng, V. P., Tane, P., and Kuete, V. (2017) Antibacterial activities of the methanol extract, fractions and compounds from *Elaeophorbia drupifera* (Thonn.) *Stapf*. (Euphorbiaceae). BMC Complement Altern Med. doi: 10.1186/s12906-016-1509-y

Vriens, K., Cammue, B., and Thevissen, K. (2014) Antifungal Plant Defensins: Mechanisms of Action and Production. Molecules 19:12280–12303. doi: 10.3390/molecules190812280

Wandji, J., Tillequin, F., Mulholland, D. A., Temgoua, A. D., Wansi, J. D., Seguin, E., and Fomum, Z. T. (2003) Phenolic constituents from Drypetes armoracia. Phytochemistry 63:453–456.

Wandji, J., Wansi, J. D., Fuendjiep, V., Dagne, E., Mulholland, D. A., Tillequin, F., ... and Njamen, D. (2000) Sesquiterpene lactone and friedelane derivative from drypetes molunduana. Phytochemistry 54:811–815.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., ... and Ryals, J. A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085–1094.

Watson, L., and Dallwitz, M. J. (1992) The grass genera of the world. C.A.B.International, Wallingford, Oxon, UK

Webster, G. L. (1967) The genera of euphorbiaceae in the southeastern united states. J Arnold Arbor 48:363–430.

Webster, G. L. (1975) Conspectus of a New Classification of the Euphorbiaceae. Taxon 24:593. doi: 10.2307/1220725

Webster, G. L. (1994) Synopsis of the Genera and Suprageneric Taxa of Euphorbiaceae. Ann Mo Bot Gard 81:33. doi: 10.2307/2399909

Webster, G. L. (2014) Euphorbiaceae. In: Kubitzki K (ed) Flowering Plants. Eudicots. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 51–216

Webster, G. L., and Carpenter, K. J. (2002) Pollen morphology and phylogenetic relationships in neotropical *Phyllanthus* (Euphorbiaceae). Bot J Linn Soc 138:325–338. doi: 10.1046/j.1095-8339.2002.00009.x

Whiles, J. A., Brasseur, R., Glover, K. J., Melacini, G., Komives, E. A., and Vold, R. R. (2001) Orientation and Effects of Mastoparan X on Phospholipid Bicelles. Biophys J 80:280–293. doi: 10.1016/S0006-3495(01)76013-4

Wilmes, M., Cammue, B. P. A., Sahl, H. G., and Thevissen, K. (2011) Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. Nat Prod Rep 28:1350–1358. doi: 10.1039/c1np00022e

Wimley, W. C. (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol 5:905–917. doi: 10.1021/cb1001558

Wisniewski, M. E., Bassett, C. L., Artlip, T. S., Webb, R. P., Janisiewicz, W. J., Norelli, J. L., ... and Droby, S. (2003) Characterization of a defensin in bark and fruit tissues of peach and antimicrobial activity of a recombinant defensin in the yeast, *Pichia pastoris*. Physiol Plant 119:563–572. doi: 10.1046/j.1399-3054.2003.00204.x

Wong, H., Bowie, J. H., and Carver, J. A. (1997) The solution structure and activity of caerin 1.1, an antimicrobial peptide from the Australian green tree frog, *Litoria splendida*. Eur J Biochem 247:545–557.

World Health Organization. (2015) Global antimicrobial resistance surveillance system: manual for early implementation.

Wright, G. (2005) Bacterial resistance to antibiotics: Enzymatic degradation and modification. Adv Drug Deliv Rev 57:1451–1470. doi: 10.1016/j.addr.2005.04.002

Wu, Q., Jiri P., Eugenie N., and Kamil K. 2019. "*Jatropha gossypiifolia* L. and Its Biologically Active Metabolites: A Mini Review." *Journal of Ethnopharmacology*, January. https://doi.org/10.1016/j.jep.2019.01.022.

Wurdack, K. J., Hoffmann, P., Samuel, R., de Bruijn, A., van der Bank, M., and Chase, M. W. (2004) Molecular phylogenetic analysis of Phyllanthaceae (Phyllanthoideae pro parte, Euphorbiaceae sensu lato) using plastid RBCL DNA sequences. Am J Bot 91:1882–1900. doi: 10.3732/ajb.91.11.1882

Xu, W., Zhu, X., Tan, T., Li, W., and Shan, A. (2014) Design of embedded-hybrid antimicrobial peptides with enhanced cell selectivity and anti-biofilm activity. PLoS One 9:e98935.

Yang, L., Harroun, T. A., Weiss, T. M., Ding, L., and Huang, H. W. (2001) Barrel-Stave Model or Toroidal Model? A Case Study on Melittin Pores. Biophys J 81:1475–1485. doi: 10.1016/S0006-3495(01)75802-X

Yang, N., Liu, X., Teng, D., Li, Z., Wang, X., Mao, R., ... and Wang, J. (2017) Antibacterial and detoxifying activity of NZ17074 analogues with multi-layers of selective antimicrobial actions against *Escherichia coli* and *Salmonella enteritidis*. Sci Rep. doi: 10.1038/s41598-017-03664-2

Yao, L., Evans, J. A., and Rzhetsky, A. (2010) Novel opportunities for computational biology and sociology in drug discovery. Trends Biotechnol 28:161–170. doi: 10.1016/j.tibtech.2010.01.004

Yeaman, M. R. (2003) Mechanisms of Antimicrobial Peptide Action and Resistance. Pharmacol Rev 55:27–55. doi: 10.1124/pr.55.1.2

Yeung, A. T. Y., Gellatly, S. L., and Hancock, R. E. W. (2011) Multifunctional cationic host defence peptides and their clinical applications. Cell Mol Life Sci 68:2161–2176. doi: 10.1007/s00018-011-0710-x

Youenou, B., Favre-Bonte, S., Bodilis, J., Brothier, E., Dubost, A., Muller, D., and Nazaret, S. (2015) Comparative Genomics of Environmental and Clinical *Stenotrophomonas maltophilia* Strains with Different Antibiotic Resistance Profiles. Genome Biol Evol 7:2484–2505. doi: 10.1093/gbe/evv161

Yount, N. Y., and Yeaman, M. R. (2013) Peptide antimicrobials: cell wall as a bacterial target: Peptide antimicrobials. Ann N Y Acad Sci 1277:127–138. doi: 10.1111/nyas.12005

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395. doi: 10.1038/415389a

Zerbe, P., Hamberger, B., Yuen, M. M., Chiang, A., Sandhu, H. K., Madilao, L. M., ... and Bohlmann, J. (2013) Gene Discovery of Modular Diterpene Metabolism in Nonmodel Systems. Plant Physiol 162:1073–1091. doi: 10.1104/pp.113.218347

Zhang, S. (2011) Computer-Aided Drug Discovery and Development. In: Satyanarayanajois SD (ed) Drug Design and Discovery. Humana Press, Totowa, NJ, pp 23–38

ANEXO A
CURRÍCULO LATTES

Carlos André dos Santos Silva

Formação acadêmica/titulação

2015 Doutorado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
com período sanduíche em Universita degli Studi di Trieste (Orientador : Sergio Crovella)
Título: GENES CODIFICANTES PARA PEPTÍDEOS ANTIMICROBIANOS (AMPs) A PARTIR DE PLANTAS DA FAMÍLIA EUPHORBIACEAE
Orientador: Ana Maria Benko-Iseppon
Co-orientador: Cláudia Sampaio de Andrade Lima
Bolsista do (a): Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco
Áreas do conhecimento: Genética Vegetal

2013 - 2015 Mestrado em Engenharia Biomédica.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: PREPARAÇÃO E CARACTERIZAÇÃO DE FILMES POLIMÉRICOS A BASE DE KEFIRANA PARA APLICAÇÃO COMO SUPORTE PARA CULTIVO CELULAR., Ano de obtenção: 2015
Orientador: Ricardo Yara
Co-orientador: Cláudia Sampaio de Andrade Lima
Bolsista do (a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Palavras-chave: Kefirana, Filmes Poliméricos, Cultivo Celular
Áreas do conhecimento: Engenharia Biomédica

2008 - 2009 Especialização em Hematologia Laboratorial.
Centro Universitário CESMAC, FEJAL, Maceio, Brasil
Título: Leucemia de Células Plasmáticas (Estudo de Caso)
Orientador: Luiz Arthur Calheiros Leite

2004 - 2007 Graduação em Biomedicina.
Fundação Educacional Jayme de Altavila, FEJAL, Maceió, Brasil
Título: Determinação do perfil imunológico dos renais crônicos cadastrados no laboratório de imunogénetica do Hemocentro de Alagoas (HEMOAL).
Orientador: Maíza de Brito Ramos Bezerra

Atuação profissional

1. Laboratório DILAB - DILAB

Vínculo institucional

2011 - 2013 Vínculo: Celetista formal, Enquadramento funcional: Biomédico, Carga horária: 20, Regime: Parcial

Outras informações:

Atuação como biomédico responsável pelo setor da Hematologia, Imunologia e Uranálise.

2. Escola Técnica de Saúde Santa Bárbara - ETSSB

Vínculo institucional

2011 - 2012 Vínculo: Professor Visitante, Enquadramento funcional: Professor de Hematologia, Carga horária: 8, Regime: Parcial

Outras informações:

Professor do Curso Técnico em Análises Clínicas, na disciplina Hematologia. 120 horas.

Projetos

Projetos de pesquisa

2015 - Atual Rede InterSys: Biologia Sistêmica no Estudo de Função Gênica em Interações Bióticas

Descrição: O projeto envolve nove instituições e 17 subprojetos. Pretende estabelecer a rede INTERSYS, voltada para a formação de pessoal e geração de conhecimento científico de alto nível envolvendo interações bióticas a partir de abordagens multidisciplinares de biologia sistêmica (ômicas, biologia celular e bioinformática) através da integração de grupos nacionais e internacionais experientes.
Situação: Em andamento Natureza: Projetos de pesquisa
Integrantes: Carlos André dos Santos Silva (Responsável); ; Lívia Maria Batista Vilela; Ana Maria Benko Iseppon
.

2015 - Atual Bioprospecção de Defensinas de Plantas da Caatinga visando ao Desenvolvimento de Agentes Antimicrobianos

Descrição: O projeto pretende isolar, caracterizar estruturalmente, sintetizar e avaliar a atividade de defensinas isoladas de modelos vegetais, incluindo duas espécies da flora nativa da caatinga nordestina, comparativamente a outras plantas nativas ou cultivadas, testando sua atividade antimicrobiana com vistas ao desenvolvimento de novos fármacos para produção em larga escala. Pretende também desenvolver competências técnicas e infra-estrutura para a geração de um grupo de excelência na avaliação, isolamento e síntese de compostos antimicrobianos a partir de plantas da flora nativa.
Situação: Em andamento Natureza: Projetos de pesquisa
Integrantes: Carlos André dos Santos Silva (Responsável); ; Lívia Maria Batista Vilela; Ana Maria Benko Iseppon
.

2013 - Atual PREPARAÇÃO E CARACTERIZAÇÃO DE FILMES POLIMÉRICOS A BASE DE KEFIRANA PARA APLICAÇÃO COMO SUPORTE PARA CULTIVO CELULAR

Descrição: Desenvolver filmes poliméricos baseados em kefirana para ser utilizados como

suporte para cultivo celular. Espera-se obter um biopolímero na forma de filme que possua potencialidade para ser utilizado como suporte para crescimento celular. Devido as características intrínsecas da kefirana quanto a sua ação anti-inflamatória espera-se gerar um produto que além de biocompatível este se apresente como bioativo, devendo assim facilitar a resolução de processos inflamatórios fator primordial nos processos de implantação de biomateriais.
Situação: Em andamento Natureza: Projetos de pesquisa
Alunos envolvidos: Graduação (3); Mestrado acadêmico (1);
Integrantes: Carlos André dos Santos Silva (Responsável); ; Ricardo Yara; Cládia Sampaio de Andrade Lima
.

2006 - 2007 Obtenção e Caracterização de Extratos de Algas Para Uso na Indústria de Cosméticos

Descrição: Integrantes: Ivanilde Miciele da Silva Santos - Integrante / Erickson Marcos Santos Feitosa - Integrante / Cristhiano Sibaldo de Almeida - Integrante / Carlos André Santos Silva - Integrante / Laura Marina Pinotti - Coordenador.
Situação: Concluído Natureza: Projetos de pesquisa
Alunos envolvidos: Graduação (3);
Integrantes: Carlos André dos Santos Silva (Responsável); ; ALMEIDA, C. S.; FEITOSA, E. M. S.; SANTOS, I. M. S.; PINOTTI, L. M.
Financiador(es): Centro Universitário CESMAC-FEJAL
.

Áreas de atuação

1. Genética Vegetal
2. Biologia Molecular
3. Bioinformática
4. Molecular Biofísica Química

Idiomas

Inglês Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

Prêmios e títulos

2017 Segundo lugar na apresentação oral do trabalho intitulado Seleção e caracterização de genes codificantes para AMPs a partir de Manihot esculenta, Universidade Federal de Pernambuco

2006 Primeiro lugar na categoria pesquisa científica do projeto semente FEJAL/CESMAC, FEJAL

Producão

Produção bibliográfica
Artigos completos publicados em periódicos

1. BEZERRA, Maiza de Brito Ramos; GALDINO, Márcio Lins; **SILVA, C. A. S.**
DETERMINAÇÃO DO PERFIL IMUNOLÓGICO DOS PACIENTES RENAIS CRÔNICOS CADASTRADOS NO LABORATÓRIO DE IMUNOGENÉTICA DO HEMOCENTRO DE ALAGOAS (HEMOAL). Saúde em evidência (FEJAL). , v.II, p.99-102 - 102, 2008.
2. ALMEIDA, C. S.; FEITOSA, E. M. S.; **SILVA, C. A. S.**; SANTOS, I. M. S.; GUEDES, E. A. C.; COLIN, F. N.; CAETANO, L. C.; SILVA, B. L. R.; PINOTTI, L. M.

Obtenção e Caracterização de Extratos de Algas para Uso na Indústria de Cosméticos. Infarma (Brasília)., v.19, p.154-159 - 159, 2007.

Trabalhos publicados em anais de eventos (resumo)

1. ALMEIDA, C. S.; FEITOSA, E. M. S.; SANTOS, I. M. S.; **SILVA, C. A. S.**; PINOTTI, L. M.
A obtenção e caracterização de extratos de algas para uso na indústria de cosméticos In: V Seminário de Iniciação Científica, 2006, Maceió.
V Seminário de Iniciação Científica., 2006.

Apresentação de trabalho e palestra

1. **SILVA, C. A. S.**
Análises in silico, modelagem e engenharia de peptídeos antimicrobianos a partir de plantas da família Euphorbiaceae, 2018. (Simpósio,Apresentação de Trabalho)

2. **SILVA, C. A. S.**
Genes codificantes para peptídeos antimicrobianos a partir de plantas da família Euphorbiaceae, 2017. (Seminário,Apresentação de Trabalho)

3. **SILVA, C. A. S.**
PREPARAÇÃO E CARACTERIZAÇÃO DE FILMES POLIMÉRICOS A BASE DE KEFIRANA PARA APLICAÇÃO COMO SUPORTE PARA CULTIVO CELULAR., 2014. (Conferência ou palestra,Apresentação de Trabalho)

4. BEZERRA, Maiza de Brito Ramos; GALDINO, Márcio Lins; **SILVA, C. A. S.**
DETERMINAÇÃO DO PERFIL IMUNOLÓGICO DOS PACIENTES RENAIS CRÔNICOS CADASTRADOS NO LABORATÓRIO DE IMUNOGENÉTICA DO HEMOCENTRO DE ALAGOAS (HEMOAL), 2007. (Simpósio,Apresentação de Trabalho)

Produção técnica

Demais produções técnicas

1. **SILVA, C. A. S.**; Lima, M. O.
Modelagem de Proteína: uma abordagem teórico-prática para pequenas proteínas, 2018. (Outro, Curso de curta duração ministrado)

2. BENKO-ISEPPON, A. M.; SILVA, R. L. O.; **SILVA, C. A. S.**; MOURA, M. T.; VILELA, L. M. B.; SILVA, J. B.; SANTOS, R. F.
Desenho de primers: Uma abordagem teórico-prática, 2017. (Aperfeiçoamento, Curso de curta duração ministrado)

3. **SILVA, C. A. S.**; VILELA, L. M. B.
Extração, purificação e amplificação de DNA, 2017. (Aperfeiçoamento, Curso de curta duração ministrado)

4. **SILVA, C. A. S.**; VILELA, L. M. B.
Extração, purificação e amplificação de DNA, 2017. (Outro, Curso de curta duração ministrado)

5. **SILVA, C. A. S.**; SILVA, R. L. O.
Extração, purificação e amplificação de DNA genômico, 2017. (Aperfeiçoamento, Curso de curta duração ministrado)

6. **SILVA, C. A. S.**; SILVA, R. L. O.
Extração, purificação e amplificação de DNA genômico, 2017. (Outro, Curso de curta duração ministrado)

7. BENKO-ISEPPON, A. M.; **SILVA, C. A. S.**; VILELA, L. M. B.; MOURA, M. T.; SILVA, J. B.; SANTOS, R. F.
PCR Quantitativa em Tempo Real: Bases e Aplicações na Análise da Expressão Gênica, 2017. (Aperfeiçoamento, Curso de curta duração ministrado)
Palavras-chave: PCR, Primers, Amplificação

Patentes e registros

Patente

A Confirmação do status de um pedido de patentes poderá ser solicitada à Diretoria de Patentes (DIRPA) por meio de uma Certidão de atos relativos aos processos

1. BENKO-ISEPPON, A. M. B.; VILELA, L. M. B.; **SILVA, C. A. S.**; CROVELLA, S.; Lima, M. O.
PEPTÍDEO SINTÉTICO, PROCESSO DE OBTENÇÃO E USO DO PEPTÍDEO SINTÉTICO NA PREPARAÇÃO DE UM AGENTE ANTIMICROBIANO, 2019. Categoria: Produto. Instituição onde foi depositada: INPI - Instituto Nacional da Propriedade Industrial. País: Brasil. Natureza: Patente de Invenção. Número do registro: BR10201900152. Número do depósito PCT: 870190008102. Data de depósito: 24/01/2019. Depositante/Titular: Ana Maria Benko Iseppon. Depositante/Titular: Universidade Federal de Pernambuco.

2. BENKO-ISEPPON, A. M. B.; **SILVA, C. A. S.**; VILELA, L. M. B.; CROVELLA, S.; Lima, M. O.
PEPTÍDEO SINTÉTICO, PROCESSO DE OBTENÇÃO E USO DO PEPTÍDEO SINTÉTICO NA PREPARAÇÃO DE UM AGENTE CONTRA BACTÉRIAS E FUNGOS, 2019. Categoria: Produto. Instituição onde foi depositada: INPI - Instituto Nacional da Propriedade Industrial. País: Brasil. Natureza: Patente de Invenção. Número do registro: BR10201900151. Número do depósito PCT: 870190008096. Data de depósito: 24/01/2019. Depositante/Titular: Ana Maria Benko Iseppon. Depositante/Titular: Universidade Federal de Pernambuco.

3. CORDEIRO, N. A. O.; PADILHA, R. J. S. A.; PICASSO, R. S.; LIMA, C. S. A.; YARA, R.; KIDO, E. A.; NASCIMENTO, S. C.; **SILVA, C. A. S.**; SILVA, Y. E. O.; VIEIRA, J. R. C.; MELO, C. M. L.; SOARES, P. A. G.; MOURAO, P. A. S.; NASCIMENTO, A. L.
PROCESSO DE OBTENÇÃO DE EXOPOLISSACARÍDEO TIPO BETA-GLUCANA, EXTRAÍDO DE CONSÓRCIO MICROBIANO DO KEFIR, COM CARACTERÍSTICAS DE HIDROGEL SUPERABSORVENTE, AÇÃO CITOTÓXICA PARA LINHAGENS NEOPLÁSICAS, AÇÃO IMUNOMODULADORA E MATÉRIA-PRIMA PARA FILME POLIMÉRICO BIOATIVO, 2017. Categoria: Produto e Processo. Instituição onde foi depositada: INPI - Instituto Nacional da Propriedade Industrial. País: Brasil. Natureza: Patente de Invenção. Número do registro: BR1020170256278. Data de depósito: 29/11/2017. Data da concessão: 02/01/2018. Depositante/Titular: INSTITUTO TECNOLÓGICO DAS CADEIAS BIOSSUSTENTÁVEIS. Depositante/Titular: Universidade Federal de Pernambuco.

Demais produções técnicas

1. **SILVA, C. A. S.**; Lima, M. O.
Modelagem de Proteína: uma abordagem teórico-prática para pequenas proteínas, 2018. (Outro, Curso de curta duração ministrado)

2. **SILVA, C. A. S.**; VILELA, L. M. B.
Extração, purificação e amplificação de DNA, 2017. (Outro, Curso de curta duração ministrado)

3. **SILVA, C. A. S.**; SILVA, R. L. O.
Extração, purificação e amplificação de DNA genômico, 2017. (Outro, Curso de curta duração ministrado)

Eventos

Participação em eventos

1. Apresentação de Poster / Painel no(a) **III Simpósio Norte e Nordeste de Bioinformática**, 2018. (Simpósio)
Análises *in silico*, modelagem e engenharia de peptídeos antimicrobianos a partir de plantas da família Euphorbiaceae.

2. Apresentação de Poster / Painel no(a) **IX Escola de Modelagem Molecular em Sistemas**

Biológicos, 2018. (Encontro)
Avaliação da estrutura tridimensional de um novo peptídeo antimicrobiano sintético de *Manihot esculenta*.

3. Apresentação de Poster / Painel no(a) **XXII Encontro de Genética do Nordeste**, 2018. (Encontro)
Análises *in silico*, modelagem e engenharia de peptídeos antimicrobianos a partir da espécie *Manihot esculenta*.

4. **VII Jornada de Genética**, 2017. (Outra)
Seleção e caracterização de genes codificantes para AMPs a partir de *Manihot esculenta*", durante a VII Jornada de Pós-Graduação em Genética.

5. **Encontro Científico tecnológico Extensionista e didático do Departamento de Biofísica e Radiologia.De**, 2014. (Encontro)
PREPARAÇÃO E CARACTERIZAÇÃO DE FILMES POLIMÉRICOS A BASE DE KEFIRANA PARA APLICAÇÃO COMO SUPORTE PARA CULTIVO CELULAR.

Bancas

Participação em banca de trabalhos de conclusão

Graduação

1. **SILVA, C. A. S.**
Participação em banca de Ádamo Yésus Brito da Silva. **Características genotípicas do HIV associadas a falhas na predição computacional da resposta ao tratamento antirretroviral em pessoas vivendo com HIV/AIDS**, 2017
(Biomedicina) Universidade Federal de Pernambuco

2. **SILVA, C. A. S.**
Participação em banca de Luisa de Medeiros Conserva e Stephanie Kelly Moreira Chagas. **Fatores de risco associado ao Diabetes Mellitus tipo 2 em pacientes pós-transplantados em uso de tacrolimus**, 2011
(Biomedicina) Centro Universitário CESMAC

3. **SILVA, C. A. S.**
Participação em banca de Alice Ricelle Tavares Ramos. **Hemofilia B e seu inibidor específico: relato de caso**, 2011
(Biomedicina) Centro Universitário CESMAC

4. **SILVA, C. A. S.**
Participação em banca de Joelma Carvalho Santos e Larissa Rodrigues Nolasco de Araújo. **Prevenção à rejeição de órgãos em pacientes tlansplantados através da monitoração terapêutica do tacrolimus**, 2011
(Biomedicina) Centro Universitário CESMAC

5. **SILVA, C. A. S.**
Participação em banca de Edlaine Maria de Souza Barros e Sarah Montenegro Villanova B. **Síndrome do anticorpo antifosfolípide: relato de caso**, 2011
(Biomedicina) Centro Universitário CESMAC

ANEXO B

SUPLEMENTARES

Supplementary Figures

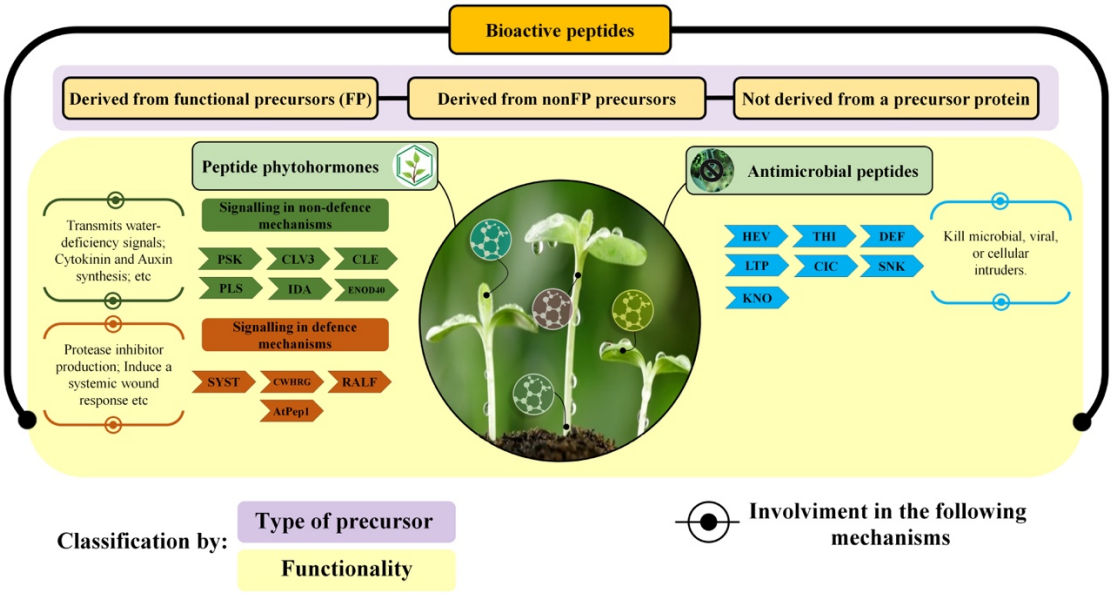

Figure S1-1. Classification system and some biological processes of Plant bioactive peptides (BP). According to Tavormina et al. (2015) [4], considering the BP precursors, classified them into: (i) derived from functional precursors or (ii) nonfunctional precursors, and (iii) not derived from a precursor protein (top of the figure) or according to. Farrokhi et al.[5] (center of the figure) based on functional groups, being classified into antimicrobial peptides or peptide phytohormones (with signaling in defense or non-defense mechanisms).

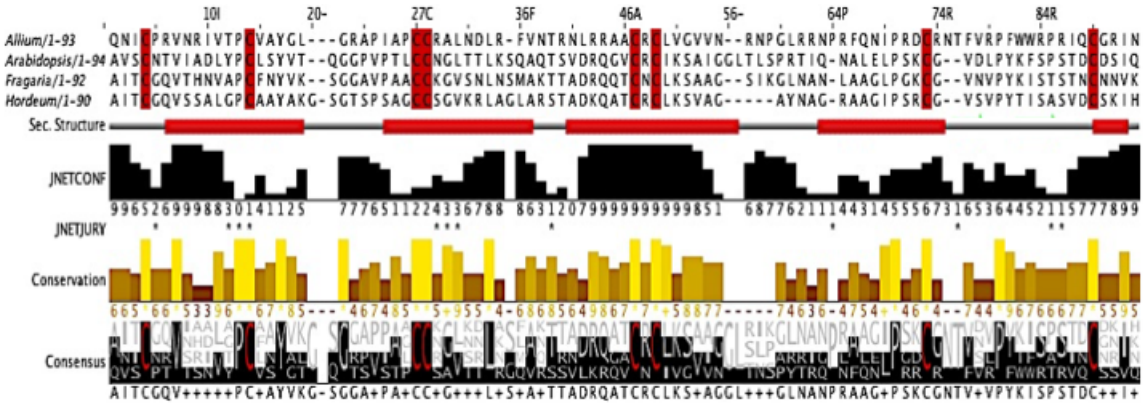

Figure S1-2. Alignment of plant LTP1 sequences isolated from onion, arabidopsis, strawberry and barley. Cysteine residues are highlighted in red in the alignment. Below the alignment, the secondary structure prediction generated by JalView. Bars in yellow/brown show sequence conservation. Amino acid consensus sequence is shown at the bottom of the figure.

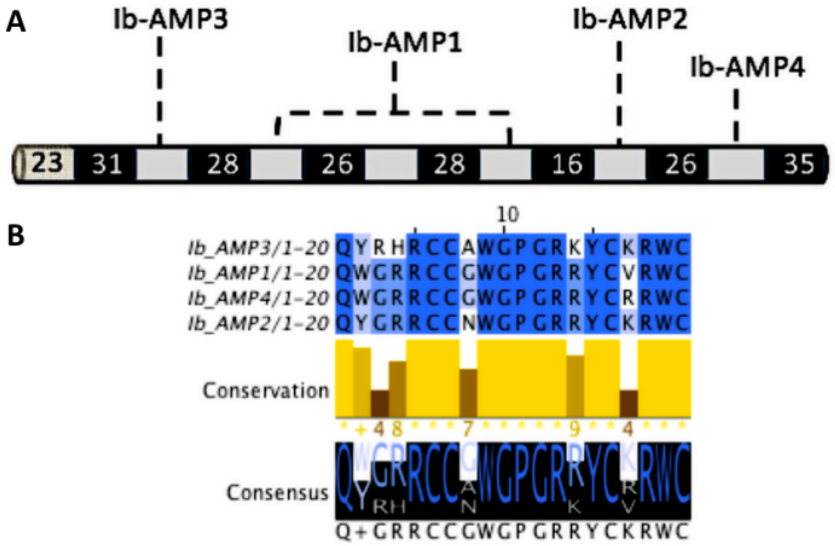

Figure S1-3. General gene structure and alignment of Ib (*Impatiens balsamina*) peptide sequences based on Tailor et al.[[81]]. (A) Structure of the predicted translation product of 333 amino acids with the individual 20 amino acid domains representing the mature Ib-AMP peptides (in gray), with the Ib-AMPs indicated above the dashed line. The regions of the propeptide are in black, the predetermined region of the pre-peptide (signal peptide) is textured at the beginning of the sequence, and the number of amino acids comprising each of those regions is indicated. (B) Alignment of the predicted translation amino acid sequence of each mature domain. In blue the conserved amino acid residues among Ib-AMPs. The yellow bars show conservation generally between the sequences and below the consensus sequence.

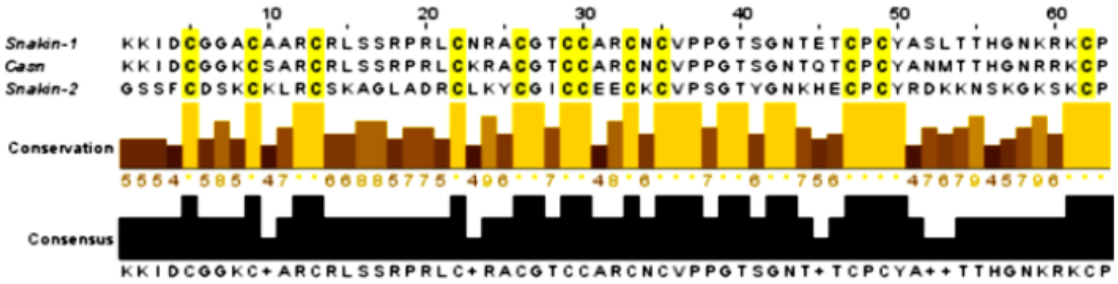

Figure S1-4. Comparative alignment of three Snakins of *Solanum tuberosum* (Snakin-1 and -2) and *Capsicum annuum* (Casn), evidencing the GASA domain with the conserved motif of twelve cysteines (in yellow). The yellow bars in the show conservation between the sequences and below the consensus sequence is presented.

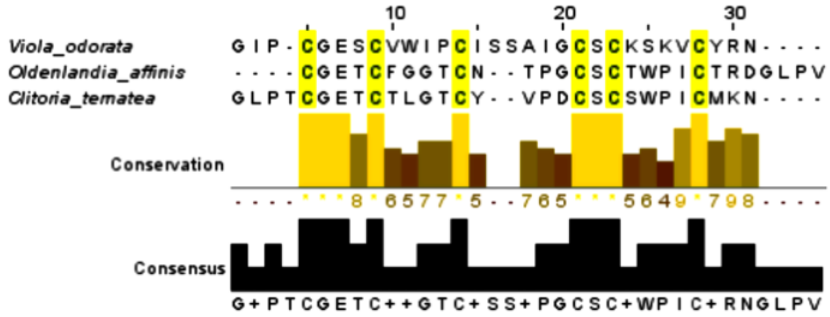

Figure S1-5. Alignment of cyclotides of *Viola odorata* (Violaceae), *Oldenlandia affinis* (Rubiaceae) and *Clitoria ternatea* (Fabaceae) evidencing the conserved motif of eight cysteines (in yellow). The yellow bars in the bottom show conservation between the sequences and below the consensus sequence is presented.

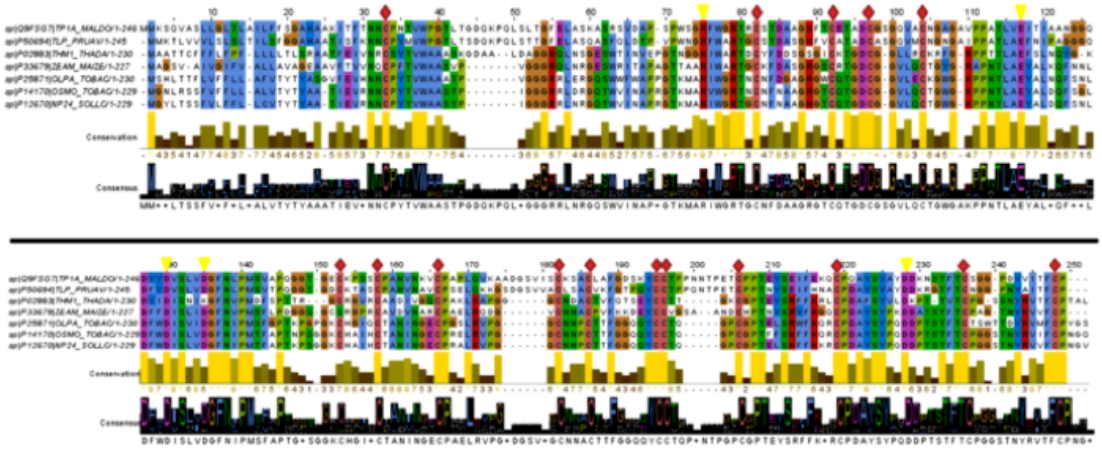

Figure S1-6. Alignment of TLP (thaumatin-like protein) amino-acid sequences of six different species (*Malus domestica*, *Prunus avium*, *Thaumatococcus daniellii*, *Zea mays*, *Nicotiana tabacum*, and *Solanum lycopersicum*). Sequence comparison was carried out with Clustal Omega software with standard parameters. Colored bars show conserved residues and motifs. Pink diamonds indicate cysteine residues that form eight disulfide bonds; Yellow triangles indicate the position of the REDDD motif and yellow bars in the base show the level of conservation for each position.

Table Supplementary

Table 1. General and specific antimicrobial peptide databases with their corresponding Uniform Resource Locator (URL).

Database	Type	Url
CAMP	General	http://www.camp.bicnirrh.res.in/
APD	General	http://aps.unmc.edu/AP/main.php
LAMP	General	http://biotechlab.fudan.edu.cn/database/lamp
DBAASP	General	https://dbaasp.org/
DEFENSIN KNOWLEDGEBASE	Specific	http://defensins.bii.a-star.edu.sg/
CYBASE	Specific	http://www.cybase.org.au/
PHYTAMP	Specific	http://phytamp.hammamilab.org/