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MARX OLIVEIRA DE LIMA

CARACTERIZAÇÃO E ANÁLISE ESTRUTURAL DE PEPTÍDEOS ANTIMICROBIANOS DE PLANTAS

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MARX OLIVEIRA DE LIMA

CARACTERIZAÇÃO E ANÁLISE ESTRUTURAL DE PEPTÍDEOS ANTIMICROBIANOS DE PLANTAS

Tese apresentada como parte dos requisitos para a obtenção do título de Doutor em Ciências Biológicas.
Área de concentração: Biotecnologia/Biologia Celular e Molecular.

Orientadora: **Profª Drª Ana Maria Benko-Iseppon**
Coorientadora: **Profª Drª Valesca Pandolfi**

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MARX OLIVEIRA DE LIMA

CARACTERIZAÇÃO E ANÁLISE ESTRUTURAL DE ESNAQUINAS EM PLANTAS DE INTERESSE ECONÔMICO

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos obrigatórios para a obtenção do título de Doutor em Ciências Biológicas.

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RESUMO

As esnaquinas são AMPs membros da família Snakin/GASA, devido ao seu domínio conservado que é caracterizado por um péptido sinal, uma região variável e um domínio C-terminal, o domínio GASA, que é composto por 12 cisteínas formando seis pontes dissulfeto. Este estudo objetivou a caracterização desta família de peptídeos no transcriptoma da soja, descrevendo sua estrutura, abundância e distribuição genômica. Para caracterizar os genes, foram utilizadas sequências *seed* de 10 famílias de plantas, assim, 33 sequências (não redundantes) de snakins foram identificadas no transcriptoma da soja, 20 das quais possuíam o domínio GASA completo, ponto isoelétrico variando de 5,53 a 9,32 para o peptídeo maduro e peso molecular de 6,88 a 18,04 KDa. Todas as sequências foram endereçadas ao meio extracelular e estão relacionadas tanto ao desenvolvimento natural da planta quanto aos estresses bióticos e abióticos, estes genes podem ser distribuídos em três subfamílias de acordo com os motivos internos. Uma nova esnaquina (GmSN2) tem aqui sua primeira descrição, isolamento gênico, sequenciamento genômico, validação da expressão RT-qPCR e análise de sua estrutura. O péptido mostra uma estrutura semelhante a outros AMPs como a tionina e é estabilizado por seis ligações dissulfeto com o padrão: Cis1-7, Cis2-5, Cis3-4, Cis6-12, Cis8-11 e Cis9-10. O isolado revelou expressão constitutiva, mesmo após inoculação com o fungo *Phakopsora pachyrhizi*. O gene possui três éxons e 40 homólogos distribuídos em 16 de 20 cromossomos de soja e em seis cromossomos de *P. vulgaris* e *M. truncatula*, com um padrão de distribuição diferente de outros genes de defesa, apresentando uma distribuição heterogênea. Aqui fornecemos uma fonte importante para mapear essa família de AMPs e também inferir sobre sua função.

Palavras-chave: Peptídeos antimicrobianos. Bioinformática. GENOSOJA. NordEST. Mineração de dados.

ABSTRACT

Snakins are members of the Snakin/GASA family, because of their conserved domain which is characterized by a signal peptide, a variable region and a C-terminal domain, the GASA domain, which is composed by 12 cysteines connecting six disulfide bonds. This study aimed the characterization of this peptide family in the soybean transcriptome describing structure, abundance and genomic distribution. To characterize the genes, we used seed sequences from 10 plant families, 33 (non-redundant) sequences of snakins were identified in the soybean transcriptome, 20 of which had the GASA domain complete, isoelectric point ranging from 5.53 to 9.32 for the mature peptide and molecular weight 6.88 to 18.04. All sequences were addressed to the extracellular environment and they are related to the natural development of the plant and to biotic and abiotic stresses, these genes can be distributed in three subfamilies according to internal motifs. A new snakin (GmSN2) has its first description, gene isolation, genomic sequencing, RT-qPCR expression validation and analysis of its structure. The peptide shows a similar structure to other AMPs like thionin and it is stabilized by six disulfide bonds with the pattern: Cys1-7, Cys2-5, Cys3-4, Cys6-12, Cys8-11, and Cys9-10. The isolated GmSN2 revealed constitutive expression, even after inoculation with the fungus *Phakopsora pachyrhizi*. The gene has three exons and 40 homologues distributed along 16 of 20 soybean chromosomes and in six chromosomes of *P. vulgaris* and *M. truncatula*, with a distribution pattern different from other defense genes, presenting a heterogenous distribution. Here we provide an important source to map this family of AMPs and also to infer about their function.

Keywords: Antimicrobial peptides. Bioinformatic. GENOSOJA. NordEST. Data mining.

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LISTA DE ABREVIAÇÕES

ABA - Ácido Abscísico (*Abscisic Acid*)

AMP - Peptídeos antimicrobianos (*Antimicrobial Peptide*)

Avr - Gene de Avirulência

BLAST - Ferramenta Básica de Busca e Alinhamento Local (*Basic Local Alignment Search Tool*)

CaMV - Vírus do Mosaico da Couve-Flor (*Cauliflower mosaic virus*)

CaSN - *Capsicum annuum snakin*

EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária

EST - Etiquetas de Sequencias Expressas (*Expressed Sequence Tags*)

FaGASA - *Fragaria ananassa* GASA

FBCBP - *French bean chitin-binding protein*

FsGASA - *Fagus sylvatica* GASA

GA - Ácido Giberélico (*Gibberellic acid*)

GASA - *Gibberellin acid-stimulated arabidopsis*

GAST - *Gibberellic acid-stimulated transcript*

GEG - *Gerbera homolog of GAST1* gene

Gip - *GA-induced proteins*

GmSN - *Glycine max Snakin*

GsGASA - *Glycine soja* GASA

GSL - Proteína estimulada por giberelina (*Gibberellin Stimulated Like*)

HR - Resposta Hipersensível (*Hypersensitive Response*)

LTP - Proteínas transferidoras de lipídeos (*Lipid Transfer Proteins*)

LRR - Repetições ricas em leucina (*Leucine rich repeat*)

NBS - Sítio de ligação de nucleotídeos (*Nucleotide binding site*)

NCBI - Centro Nacional de Informações sobre Biotecnologia (*National Center for Biotechnology Information*)

OsGASR - *Oryza sativa GA-stimulated transcript-related gene*

PDB - Banco de Dados de proteínas (*Protein Data Bank*)

PDC - Morte celular programada (Programmed cell death)

PR - Proteínas Relacionadas à Patogênese (*Pathogenesis Related Protein*)

R - Genes de Resistência

ROS - Espécies Reativas de Oxigênio (*Reactive Oxygen Species*)

RSI-1 - *Root system inducible*-1

AS - Ácido Salicílico (*Salicilic acid*)

SAGE - Análise Serial de Expressão Gênica (*Serial Analysis of Gene Expression*)

SAR - Resposta Sistêmica Adquirida (*Systemic Acquired Resistance*)

SNK - Snakin

StSN - *Solanum tuberosum* snakin

ZmGSL - *Zea mays gibberellin-stimulated like*

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1. INTRODUÇÃO

Ao longo dos processos evolutivos os vegetais desenvolveram diversos mecanismos de defesa que variam desde barreiras físicas, como a rigidez do caule e a cobertura serosa das folhas que ajudam a impedir a predação por insetos, a invasão por agentes microbianos e a perda de água por transpiração. Contudo, em se tratando de agentes antimicrobianos, muitas vezes tais moléculas são capazes de ir além destas barreiras, incitando a liberação de agentes químicos de defesa como as proteínas PR (*Pathogenesis-Related*) que incluem os peptídeos antimicrobianos - AMPs (*Antimicrobial peptides*) ricos em cisteínas (AMP) (Silverstein et al. 2007; Benko-Iseppon et al., 2010).

Encontrados em todas as famílias de mono e eudicotiledôneas, AMPs são codificados por famílias multigênicas, incluindo defensinas, thioninas, ciclotídeos, heveínas, esnaquinas, entre outros. Possuem em comum a carga positiva e a natureza anfipática, características que estão relacionadas à sua atividade de interação com membranas biológicas, além da presença de 4-12 resíduos cisteína, cujo motivo resulta na formação de 2-6 pontes dissulfeto – contribuindo para a compactação da estrutura, além de conferir estabilidade e resistência à degradação química e proteolítica. A expressão destes peptídeos pode ser constitutiva ou induzida por patógenos e predadores de diferentes naturezas, como insetos e nematoides, agindo como inibidores de proteases ou desestabilizadores de membranas (Tam et al. 2015).

Neste sentido, as esnaquinas – classificadas como membros da família snakin/GASA (Segura et al. 1999; Berrocal-lobo et al. 2002) – vêm recebendo crescente destaque por sua diversidade funcional, com ação contra fungos, bactérias e nematoides, além de atuarem como efetores do crescimento e de divisão celular. Tais peptídeos são expressos em vários tecidos (desde as raízes até as flores). Sua estrutura primária é composta por três motivos principais: (1) um peptídeo sinal na região N-Terminal, que pode conter de 18-29 resíduos, (2) uma região variável que possui entre 7-31 resíduos e o domínio C-Terminal GASA, composto por 60 aminoácidos, incluindo as 12 cisteínas. A estrutura terciária é formada por um domínio hélice-volta-hélice, uma hélice 3_{10} e uma terceira α-hélice. Usualmente, estes peptídeos são subcelularmente localizados na parede celular, mas podem também ser direcionados para o apoplasto ou para o núcleo (quando traduzidos sem o peptídeo sinal). Assim, devido à diversidade funcional e às suas características estruturais, as esnaquinas destacam-se como alvos biotecnológicos interessantes para uso tanto na transformação de plantas, como no desenvolvimento de novos fármacos.

Outra pequena família de AMPs ricos em cisteínas que apresenta interesse biotecnológico é a família das heveínas (*hevein-like*), tendo em vista sua capacidade de se ligar à quitina (componente presente em nematoides, na parede celular de fungos e no exoesqueleto de insetos). Além disso, estes peptídeos também demonstraram atividade contra bactérias gram-positivas e gram-negativas. Estruturalmente, heveínas são peptídeos básicos, pequenos (variando de 29-45 aminoácidos), ricos em cisteínas, glicinas, bem como alguns aminoácidos aromáticos, cujo domínio conservado (*hevein*, ligante de quitina) pode variar quanto à quantidade de cisteínas (de 6-10). A sua estrutura terciária é composta pelo motivo estrutural α- hélice–β1–β2–α-hélice–β3, onde as folhas-beta são antiparalelas. Estes peptídeos têm sido encontrados preferencialmente nos fluidos intracelulares de vegetais superiores, sendo explorado com sucesso na transformação de plantas, resultando em variedades resistentes a diversos fitopatógenos (Slavokhotova et al. 2017).

Tendo em vista o grande potencial destes peptídeos em diversas áreas biotecnológicas, um número cada vez maior de sequências tem sido depositado em bancos de dados biológicos. Entretanto, a identificação e caracterização destes peptídeos ainda é um grande desafio, em vista da carência de protocolos que recuperem, de forma rápida e apurada, todos os peptídeos antimicrobianos de interesse. Nesse contexto, a bioinformática tem sido indispensável na prospecção de alvos, tendo em vista o desenvolvimento de softwares e ferramentas direcionados a cada tipo de questão biológica, proporcionando as "pistas" necessárias para a montagem desse "quebra-cabeças". Contudo, frequentemente é preciso complementar os dados compilados e tratados com as metodologias de *wet lab* para que tenham uma maior robustez e confiabilidade.

O presente trabalho teve como objetivo identificar e caracterizar esnaquinas e heveínas em plantas de interesse econômico e selecionar os candidatos de maior potencial biotecnológico para uso no melhoramento genético vegetal e/ou uso farmacológico.

2. OBJETIVOS

2.1. Objetivo Geral:

- Identificar e caracterizar genes codificadores de peptídeos antimicrobianos de plantas, em especial esnaquinas, com dados gerados a partir de transcriptomas e genomas disponíveis em bancos de dados públicos e de acesso restrito, com ênfase em elementos desconhecidos do ponto de vista funcional, visando ao entendimento dos processos de defesa vegetal e à futura exploração do seu potencial biotecnológico.

2.2. Objetivos Específicos:

- Identificar, quantificar e caracterizar domínios conservados de genes codificantes para esnaquinas nos bancos de dados públicos, comparativamente àqueles gerados por nossos projetos para soja e de feijão-caupi (GENOSOJA e NordEST), inferindo sua diversidade;
- Elucidar aspectos estruturais e funcionais de natureza gênica e proteica desses peptídeos com base em vias metabólicas, modelagem molecular e identificação de possíveis interações;
- Identificar regiões filogeneticamente informativas, inferindo sobre a evolução destes grupos;
- Estabelecer o perfil de expressão dos genes candidatos (*in silico* e experimentalmente), validando seus níveis de expressão em situações contrastantes (controle e sob estresse biótico e/ou abiótico, quando presentes), inferindo sobre sua função espacial e temporal;

3. REVISÃO BIBLIOGRÁFICA

3.1. Estresses ambientais e mecanismos de defesa das plantas

As plantas são constantemente submetidas a mudanças ambientais, as quais muitas vezes são estressantes. Estas condições incluem estresses bióticos, tais como infecção por patógenos e ataque de herbívoros, além dos estresses abióticos, como seca, temperaturas extremas, solos pobres em nutrientes, excesso de sais ou metais pesados no solo, entre outros. Como forma de defesa, os vegetais respondem através de mecanismos físicos e químicos, a fim de mitigar os danos e conservar recursos necessários ao seu crescimento e à reprodução (Rizhsky et al. 2004; Atkinson and Urwin 2012; Dorantes-Acosta et al. 2012).

Entre as barreiras desenvolvidas que servem tanto para a detecção quanto para a proteção contra estresses ambientais, destaca-se a cutícula, uma camada composta de cutina (um polímero de ácidos graxos) que ajuda tanto na defesa contra a penetração de microrganismos, quanto na minimização da perda de água (Serrano et al. 2014). Outro exemplo é a deposição de lignina na parede celular, uma barreira química que possui efeitos antimicrobianos (Vanetten et al. 1994). Apesar de eficientes, muitas vezes estas barreiras primárias não são suficientes para a defesa da planta e podem ser superadas, resultando em danos aos vegetais (Sels et al. 2008).

No caso de estresses bióticos, quando os agentes microbianos ultrapassam a primeira barreira (física), a planta aciona uma cascata de sinalização ativando moléculas de defesa (ex. metabólitos secundários e/ou proteínas), ou seja, uma forma de "contra-ataque" mais eficiente. Nesse mecanismo, hormônios – como o ácido salicílico, o ácido jasmônico e o etileno – agem como sinalizadores (Robert-Seilaniantz et al. 2011; Dorantes-Acosta et al. 2012), ao passo que as proteínas relacionadas à patogênese (PR, *Pathogenesis-Related*) atuam diretamente sobre o agente invasor (Saboki et al. 2011). Em conjunto, esses dois mecanismos constituem uma resposta de defesa mais complexa, eficiente e segura (Fu and Dong 2013; Pandey et al. 2016).

3.1.1 Resposta Hipersensível (HR) e Resistência Sistêmica Adquirida (SAR)

Quando um patógeno ou um herbívoro ataca a planta, superando suas barreiras primárias, esta responde ativando sinais nos locais de infecção, resultando em uma resposta

de hipersensibilidade (*Hypersensitive response*, HR), o que leva à morte celular programada (*Programmed cell death* - PCD) no entorno da região em contato com o patógeno (Dixon and Harrison, 1994; Morel and Dangl, 1997). O reconhecimento de padrões moleculares e/ou de efetores dos fitopatógenos (fungos, vírus e bactérias) pode levar à sinalização celular através da produção de espécies reativas de oxigênio (*Reactive Oxygen Species* - ROS) de uma série de hormônios vegetais (ex. giberelina e ácido salicílico), além de proteínas PR relacionadas à defesa contra patógenos, as quais fazem parte da segunda via das respostas químicas, levando a um tipo de resistência em plantas conhecida como Resistência Sistêmica Adquirida (*System Acquired Resistance* – SAR) (Liu and Ekramoddoullah 2006; van Verk et al. 2009; Zurbriggen et al. 2010; Kachroo and Robin 2013).

A HR pode ser desencadeada em resposta a vários patógenos (ocorrendo pouco tempo após a infecção), sendo condicionada pela interação dos produtos dos genes de resistência (*R*) do hospedeiro e de avirulência (*avr*) do patógeno (Morel and Dangl 1997; Mur et al. 2008; Wanderley-Nogueira et al. 2017). Os mecanismos de defesa são conservados no que diz respeito às modificações bioquímicas e metabólicas e, caso a infecção persista, vários genes de defesa serão induzidos, aumentando o nível de resistência e desencadeando a SAR (Dangl 1995; Bent 1996; Morel and Dangl 1997; Durrant and Dong 2004).

Inicialmente, a SAR envolve a geração de moléculas sinalizadoras nos tecidos infectados, com a finalidade de prover a percepção da invasão do patógeno pelos outros tecidos (Fu and Dong 2013; Kachroo and Robin 2013). Trata-se da forma de defesa mais duradoura (podendo chegar a alguns meses) e de largo espectro em plantas. Do ponto de vista molecular, ocorre aumento da expressão de uma vasta quantidade de genes *PR*, tanto no local da lesão, como em outros tecidos (Durrant and Dong 2004), um exemplo são as vias de sinalização por etileno que são ativadas pela liberação desse hormônio no local de infecção, fazendo com que ocorra um *feedback* positivo das proteínas PR (Kitajima and Sato 1999).

3.1.2. Genes R e PR

Dentre os mecanismos mais bem caracterizados de defesa de plantas e, portanto, amplamente utilizados em processos de transformação genética são aqueles mediados pelos genes *R* (Elvira et al. 2008; Pandolfi et al. 2017; Wanderley-Nogueira et al. 2017). Estes se apresentam com um alto grau de polimorfismo, um aparato de reconhecimento específico e

fazem parte de famílias gênicas que tendem a formar grupamentos (*clusters*) no genoma, os quais evoluíram por duplicação, recombinação e neofuncionalização (Ronald 1998; Kang et al. 2012). Só na cana-de-açúcar (*Saccharum officinarum*) e no eucalipto (*Eucalyptus* sp.), por exemplo, foram encontrados 280 e 210 genes *R*, respectivamente, com sítios de ligação de nucleotídeos (*Nucleotide Binding Site* - NBS) e repetições ricas em resíduos de leucina centrais (*Leucine Rich Repeat*, LRR) (Barbosa-da-Silva et al. 2005; Wanderley-Nogueira et al. 2007).

A resistência conferida pelos genes *R* parece seguir o padrão de interação *gene-a-gene* relatado por Flor (1971) e completado por Bourras et al. (2016), onde está relatado que a resistência seria mediada por interações envolvendo os genes *avr/svr* no patógeno e os genes *R* no hospedeiro. O mecanismo de resistência seria ativado quando o supressor (*svr*) estivesse inativo e os genes avr fossem produzidos em quantidade suficiente para serem detectados pelos genes *R*. Caso tais condições não ocorram, a planta pode sucumbir à infecção, o que geraria outra cascata de sinalização hormonal, dando início à SAR (Cameron et al. 1994; Vlot et al. 2008; Bourras et al. 2016).

A "*guard hypothesis"* proposta por Van Der Biezen e Jones (1998) por sua vez, sugere que as proteínas R (*guard*) estão constitutivamente associadas a proteínas celulares no hospedeiro (*guardee*), as quais são "exigidas" pelo patógeno para a infecção. Nesta situação, o patógeno causa modificações nas proteínas *guardee*, as quais são detectadas pela *guard.* Assim, qualquer modificação na estrutura quaternária das *guardee* pode resultar na detecção do patógeno e, portanto, induzir uma cascata de sinalização, resultando na resposta de resistência (Van Der Biezen and Jones 1998; Holt et al. 2003; Soosaar et al. 2005) (**Figura 1**).

Em ambas as hipóteses, as etapas subsequentes seguem um padrão, onde moléculas sinalizadoras (quinases, espécies reativas de oxigênio e alguns hormônios, por exemplo) atuam como indutores da expressão de proteínas PR (Soosaar et al. 2005; Sudisha et al. 2012).

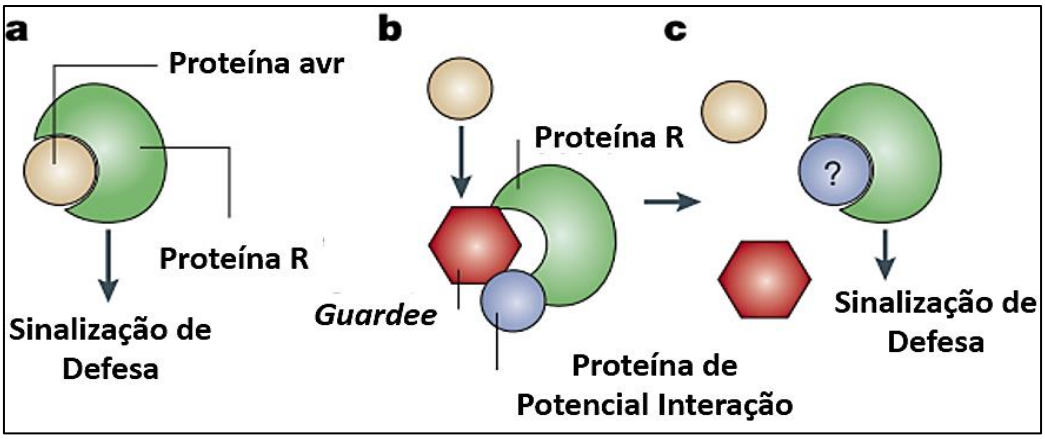

Figura 1. Hipótese do Receptor-Ligante *vs. guard hypothesis*. **(a)** Hipótese do Receptor-Ligante: proteínas de resistência (R) detectam o patógeno via interação direta com as proteínas de avirulência (avr), desencadeando a sinalização de defesa; **(b)** *Guard hypothesis:* os *guardees* são os alvos das proteínas avr, assim, as proteínas *guard* e *guardee* interagem dinamicamente podendo haver outras proteínas no complexo; **(c)** Situação onde ocorre a modificação das proteínas *guardee* (pelas proteínas avr), alterando sua interação e desencadeando os sinais de defesa (Soosaar et al. 2005).

Com base na sua estrutura e propriedades funcionais, as proteínas PR foram classificadas em 17 famílias (**Tabela 1**), tendo em vista suas homologias, similaridade, estrutura primária ou terciária e atividade biológica (Christensen et al. 2002; Benko-Iseppon et al. 2010; Sudisha et al. 2012). Outro parâmetro considerado nesta classificação foi o ponto isoelétrico (pI), prevalecendo a carga positiva para a maioria das PRs (van Loon et al. 1994; Christensen et al. 2002; Sudisha et al. 2012), uma vez que muitas delas tem a propriedade de aderir a membranas que possuem carga negativa (Zasloff 2002; Stec 2006). Dentre essas famílias, destacam-se as PRs 12, 13 e 14 (**Tabela 1**), que incluem as esnaquinas e as heveínas, bem como outros AMPs ricos em resíduos de cisteínas (Cys) (Silverstein et al. 2007).

Tabela 1. Classificação das proteínas relacionadas à Patogênese (proteínas PR)*.

Família	Membro tipo	Propriedades
PR1	PR-1a de tabaco	Antifúngica
PR2	PR-2 de tabaco	β-1,3-glucanase
PR3	Proteína P, Q de tabaco	Quitinase tipo: I, II, IV, V, VI e VII
PR4	Proteína R de tabaco	Quitinase tipo: I e II
PR5	Proteína S de tabaco	Taumatina, Antifúngica
PR6	Inibidor I de tomate	Inibidor de protease
PR7	P69 de tomate	Endoproteinase
PR8	Quitinase de pepino	Quitinase tipo III
PR9	Lignina peroxidase de tabaco	Peroxidase
PR10	*Petroselinum crispum* PR1	Ribonuclease
PR11	Quitinase classe V de tabaco	Quitinase tipo I
PR12	Rs-AFP3 de rabanete	Defensina
PR13	THI2.1 de Arabidopsis	Tionina
PR14	LTP4 de cevada	Proteína transferidora de lipídeos
PR15	OxOa de cevada	Oxalato oxidase
PR16	OxOLP de cevada	Oxalato oxidase-like
PR17	PRp27 de tabaco	Antifúngica e antiviral

*Fonte: Christensen et al. 2002; Sudisha et al. 2012.

Geralmente, as proteínas PR em plantas são expressas em decorrência do ataque de patógenos. No entanto, estes eventos não se referem apenas àqueles desencadeados pela HR ou SAR (onde as PRs são mais comumente observadas), mas em quase todos os tipos de situações de estresse causadas por patógenos, incluindo parasitismo e herbivoria (como nematoides e insetos, respectivamente). Em adição, proteínas PR podem estar presentes em situações de estresse abiótico, tanto em tecidos com sinais de clorose / necrose, como em tecidos intactos (van Loon 1999; Tuzun 2001; Sudisha et al. 2012). Embora frequentemente observada em tecidos foliares (van Loon et al. 2006), a expressão de PRs também tem sido relatada em outros tecidos/ órgãos, como tubérculos, flores, raízes e sementes (Belarmino et al. 2010; Sudisha et al. 2012) em uma grande variedades de espécies vegetais. Além disso, uma única espécie pode conter representantes de diferentes classes, como é o caso de *Arabidopsis thaliana*, *Medicago truncatula* e *Glycine max*, por exemplo, onde foram

identificados, respectivamente, 366, 585 e 310 transcritos de PRs das classes 1-9, 11, 12 e 14-16 (Wanderley-nogueira et al. 2012).

3.1.3 Peptídeos antimicrobianos de plantas ricos em cisteínas

De uma forma geral, peptídeos antimicrobianos são ubíquos e participam da defesa das plantas contra uma grande variedade de patógenos, incluindo microrganismos, fungos e animais (Egorov et al. 2005; Benko-Iseppon et al. 2010). Estruturalmente podem ser lineares (maioria) ou cíclicos (Tam et al. 2015; Lima et al. 2017), embora, em plantas se observe uma predominância da forma rica em resíduos de cisteínas (Cys; C) – fundamental para a formação das pontes de dissulfeto auxiliando na compactação da estrutura e servindo, portanto, de proteção contra eventual degradação proteolítica (Hammami et al. 2009b; Tam et al. 2015).

Em termos gerais, os AMPs de plantas compartilham algumas características com homólogos de organismos evolutivamente distantes (ex. mamíferos e insetos) como a carga positiva e natureza anfipática – propriedades associadas à atividade desestabilizadora da membrana dos microrganismos (Zasloff 2002; Padovan et al. 2010). Contudo, a característica que melhor separa as famílias de AMPs de plantas são os seus motivos ricos em cisteínas definidos, principalmente em relação aos demais resíduos de aminoácidos.

Baseado nessas propriedades, os AMPs de plantas foram classificados em diferentes famílias, tais como: defensinas, tioninas, LTPs, knotinas, heveínas e esnaquinas, entre outras, cuja quantidade de cisteínas pode variar de 4 a 12 resíduos (Manners 2007; Silverstein et al. 2007; Benko-Iseppon et al. 2010). Outras propriedades dos AMPs de plantas incluem: (i) tamanho pequeno (peso molecular de 2-6 kDa), básicos e com 2 - 6 pontes dissulfeto; (ii) similaridade de sequência e conservação estrutural secundária e terciária; (iii) múltiplas funções; (iv) todos são bioprocessados e, geralmente, possuem três domínios (peptídeo sinal, região variável além do peptídeo maduro) e (v) exibem alta estabilidade térmica, química e enzimática (Tam et al. 2015).

3.1.4. Esnaquinas (*Snakin*)

As esnaquina "*snakin'* (nomenclatura dada em função da similaridade de três aminoácidos com a "*desitegrin-like*" - uma peçonha da serpente *Agkistrodon rhodostoma*, embora também conhecidos por GSL - *gibberellin stimulated-like proteins*) são peptídeos

ricos em cisteínas, identificados pela primeira vez na batata (*Solanum tuberosum*) (Adler et al. 1991; Segura et al. 1999; Berrocal-lobo et al. 2002; Meiyalaghan et al. 2014).

Devido à sua similaridade com membros da família GASA (*Gibberellic Acid Stimulated,* de *Arabidopsis*), foram classificadas como membros integrantes da família "*snakin*/GASA" (Berrocal-lobo et al. 2002). Os genes que codificam esses peptídeos apresentam: (i) uma sequência sinal, de até 28 aminoácidos, servindo para seu direcionamento subcelular; (ii) uma região variável, e (iii) um peptídeo maduro, de ~60 aminoácidos, sendo 12 resíduos de cisteína (Cys) altamente conservados. Tais resíduos auxiliam na manutenção da estrutura tridimensional do peptídeo, através da formação de pontes dissulfeto, além de fornecer estabilidade à molécula – fato este de fundamental quando a planta está sob estresse (Segura et al. 1999; Berrocal-lobo et al. 2002; Mao et al. 2011; Yeung et al. 2016). A biossíntese desses peptídeos, embora ainda não tenha sido completamente eluncidada, relatos sugerem que, após sua síntese no núcleo, estes peptídeos são processados e transportados (atraves do retículo endoplasmático) para o ambiente extracelular, compondo a parede celular (Nahirñak et al. 2012).

São encontradas em diferentes tecidos vegetais: raíz, caule, folhas, flores e sementes (Zhang et al. 2009; Zimmermann et al. 2010; Almasia et al. 2010; Guzmán-rodríguez et al. 2013), com os mais variados níveis de expressão, desde constitutivo ao induzido por diferentes tipos de estresses (bióticos e/ou abióticos), especialmente agindo na desestabilização da membrana plasmática de fitopatógenos como fungos, bactérias e nematoides(Segura et al. 1999; Faccio et al. 2011; Mao et al. 2011; Herbel and Wink 2016). A expressão de homólogos de esnaquinas também tem sido relatada em eventos de divisão celular, elongação, crescimento, floração, embriogênese, além de várias vias de sinalização (Kotilainen et al. 1999; Furukawa et al. 2006; Roxrud et al. 2007; Lucau-Danila et al. 2010). Curiosamente, a atividade de um homólogo da família (*snakin*-Z - *Ziziphus jujuba* Mill.) foi associada ao tratamento de Alzheimer em humanos, tendo em vista sua ação antioxidante, aliada a uma significativa atividade como inibidor de colinesterase, fatores estes determinantes no tratamento dessa doença, embora tal propriedade ainda esteja em fase de estudo (Zare-zardini et al. 2013).

3.2 Bioinformática – Do armazenamento ao processamento de dados biológicos

Os recentes avanços nas tecnologias de sequenciamento têm favorecido, em grande escala, a disponibilidade de informações genômicas de interesse, principalmente na geração de marcadores moleculares, bem como na identificação de genes de interesse agronômico e para fins biotecnologicos. Assim, para que houvesse uma maior eficiência na caracterização dos genomas, fez-se necessário organizar a informação de forma "navegável" e desenvolver ferramentas que pudessem processá-la de forma eficiente. Neste sentido, bancos de dados inteiros vêm sendo dedicados ao armazenamento e tratamento deste tipo de informação (Edwards and Batley 2010; Batley 2015). Esses conjuntos de dados (*datasets*) representam uma fonte valiosa de informações derivadas das diferentes "ômicas" que têm proporcionando um melhor conhecimento/entendimento da genômica funcional às relações filogenéticas entre genes, proteínas e organismos (Soares-Cavalcanti et al. 2012; Porto et al. 2017).

A bioinformática, por sua vez, tem possibilitado a junção e interpretação do conhecimento biológico (Mochida and Shinozaki 2010), uma vez que todas as análises nessa área, necessariamente remetem à etapas *in silico*. Neste contexto, a bioinformática tem contribuido muito, principalmente pela constante evolução no desenvolvimento de ferramentas cada vez mais eficientes e aplicadas na área das ômicas.Dentre as principais contribuições da bioinformática no campo biológico destacam-se: (i) a obtenção da informação em bancos de dados biológicos (dedicados ou não a um objeto de estudo específico, organismo ou família gênica), (ii) alinhamento comparativo de sequências (seja ele local ou global), (iii) a análise funcional de domínios conservados e/ou motivos de sequências, (iv) a análise estrutural (gênica ou proteica), (v) a análise de elementos regulatórios, (vi) a identificação de polimorfismo de nucleotídeo único (*Single Nucleotide Polymorphism - SNP*) e/ou inserções e deleções (*Indels*), (vii) as análises fenéticas ou filogenéticas, (viii) a simulação de dinâmica molecular e (ix) a análise de transcritos, dentre outros (Rahman et al. 2016).

Assim, integrar essas informações e associá-las a um contexto biológico tem sido o grande desafio, uma vez que as interações entre os organismos e o meio em que estão inseridos são extremamente complexas (Sheth and Thaker 2014).

3.2.1. Bancos de dados biológicos

Bancos de dados biológicos (*databank* – DB) são coleções organizadas de dados de natureza diversa, que podem ser recuperados utilizando diferentes entradas (*inputs*). O gerenciamento dessa informação é feito através de vários *softwares* e *hardwares*, cuja recuperação e organização das informações são realizadas de forma mais rápida e eficiente quanto possível (Eltabakh et al. 2006). Em se tratando de dados biológicos, as informações podem ser classificadas em: (i) primária (sequências), (ii) secundária (de estrutura, de expressão, de vias metabólicas, tipos de drogas, etc..) e (iii) especializada (contendo informações sobre uma espécie ou classe de proteína) (Rahman et al. 2016).

Tendo em vista que a geração de dados biológicos tem ocorrido em uma escala exponencial, os repositórios dessas informações precisam acompanhar tal processo. Comparativamente, o desenvolvimento do primeiro banco de dados dedicado a sequências de proteínas, na década de 60, por Margaret Dayhoff (Strasser 2010), contava com apenas algumas dezenas de sequências. Nos últimos anos, somente no UniProt (*Universal Protein Resource*) foram depositados dezenas de milhões de sequências (Bairoch et al. 2004; Wasmuth and Lima 2016). Após uma busca simples por palavras-chave no NCBI (*National Center for Biotechnology Information*), por exemplo, é possível recuperar informações acerca de publicações (PubMed), sequências de proteínas e nucleotídeos, genomas completos, informações taxonômicas, entre muitas outras opções. Adicionalmente, uma ferramenta simples, com o uso de filtros, permite refinar a busca, auxiliando na identificação de novos genes e proteínas, bem como na elucidação de funções biológicas (Rahman et al. 2016; Porto et al. 2017).

Todas estas funcionalidades juntas possibilitaram a construção de outros tipos de bancos que tratam as informações de forma mais específica e dirigida. Neste sentido a construção de bases de dados destinados à AMPs foi um passo importante para organizar e disponibilizar dados provenientes deste tipo de peptídeo.

Ao longo da última década, várias bases de dados foram geradas com intuito de comportar a grande diversidade, deposição, consulta e mineração de AMPs (Tabela 2) (Zhou and Huang 2015). De forma geral, essas bases de dados são classificadas em dois grupos: gerais e específicas. Segundo Porto et al. (2017), as bases de dados específicas podem ser divididas em dois subgrupos - aquelas contendo apenas um grupo específico de AMPs (ex. defensinas ou ciclotídeos) e aquelas contendo dados de grandes grupos de peptídeos, incluindo os peptídeos vegetais, animais ou peptídeos cíclicos) (Tabela 2) (Mulvenna 2006;

Seebah et al. 2007; Hammami et al. 2009a). Ambos os bancos de dados compartilham de algumas características, como por exemplo, a forma como os dados estão disponíveis e/ou a forma de acesso às diferentes ferramentas de análise.

Tabela 2. Exemplos de bancos de dados de AMPs gerais e específicos

Banco de dados	**Tipo**	**Url**
CAMP	Geral	http://www.camp.bicnirrh.res.in/
APD	Geral	http://aps.unmc.edu/AP/main.php
LAMP	Geral	http://biotechlab.fudan.edu.cn/database/lamp
DBAASP	Geral	https://dbaasp.org/
Defensin Knowledgebase	Específico	http://defensins.bii.a-star.edu.sg/
Cybase	Específico	http://www.cybase.org.au/
Phytamp	Específico	http://phytamp.hammamilab.org/

Em linhas gerais, as bases de dados de AMPs podem reunir dados (peptídeos) advindos de diferentes fontes, de naturais a sintéticos, validados experimentalmente ou não, podendo possuir, também, sequências patenteadas (Wang 2004; Waghu et al. 2016). Com relação ao acesso às informações, essas podem ser obtidas por por palavras-chave ou através de alinhamentos locais. Algoritmos baseados em aprendizado de máquina que permitem a predição de AMPs, a partir de dados de sequências, tambem podem ser disponibilizados por alguns bancos de dados, a exemplo da *Collection of Antimicrobial Peptides* - CAMP (Waghu et al. 2016).

Consequentemente, os bancos de dadospassaram, ao longo dos anos, de repositórios de informação de sequências biológicas para servirem, tambem, como ferramentas na análise de dados em diversas instâncias, permitindo aos usuários, uma pesquisa bem direcionada e eficiente em termos de custo financeiro e/ou de tempo (Bry and Kruger 2003; Rahman et al. 2016).

3.2.2. Recuperação e anotação de sequências

O primeiro passo para a inferência da função de uma determinada sequência (anotação) é a sua recuperação nos bancos de dados. Para tal, duas metodologias apresentam grande utilidade: o alinhamento local (*Basic Local Alignment Search Tool* BLAST; Altschul

et al. 1990; FASTA – Pearson 1990) e a busca por padrões, a qual envolve duas estratégias principais: expressão regular (*Regular Expression* – REGEX) ou busca por padrões usando modelos de Markov (*Hidden Markov Model*- HMM) (Porto et al. 2017).

Alinhar sequências é a principal forma de compará-las, uma vez que a maior parte da informação disponível nos bancos encontra-se sob esta forma. O modo mais simples de conduzir esta abordagem é o alinhamento local (Polyanovsky et al. 2011), sendo o BLAST (*Basic Local Alignment Search Tool*) a ferramenta mais utilizada para este fim. O princípio desta ferramenta é dividir a sequência em tamanhos menores (*words*), comparando-os com o banco de dados de interesse. Tal abordagem, no entanto, apresenta uma limitação: pequenos motivos podem não ser encontrados, uma vez que muitos destes compreendem porções menores que 20 % do tamanho total para determinadas sequências (Tam et al. 2015; Porto et al. 2017). Visando diminuir os efeitos das limitações do alinhamento local, estratégias alternativas baseadas na busca por padrões são também utilizadas, como REGEX (Thompson 1968) ou HMM (Eddy 1998).

A ferramenta REGEX é uma forma precisa e sucinta de descrever um determinado padrão em uma sequência de caracteres (*string*) onde cada posição da REGEX deve ser fixada; embora caracteres ambíguos ou "curingas" também possam ser usados. Por exemplo, se quisermos encontrar uma sequência que tenha *match* com os fonemas da palavra "VALESCA", podemos utilizar a seguinte expressão: [VW]AL{1,2}ES[CK]A, ou seja, esta expressão encontraria uma palavra que iniciasse com "V" ou "W", seguida por um "A", um ou dois "L", "ES", "C" ou "K" e terminando com um "A". No caso dos perfis de HMM, há um perfil probabilístico inserido no modelo, o qual é calculado a partir de um alinhamento de sequências e uma pontuação é determinada sítio a sítio. Por não haver perfis estatísticos associados à REGEX, esta é menos restritiva que o HMM (Porto et al. 2017).

3.2.3. Alinhamento Múltiplo

O alinhamento múltiplo envolve a comparação entre três ou mais sequências, sejam elas de natureza nucleotídica ou peptídica. Estes alinhamentos podem também ser construídos comparando todas as sequências par a par, chamado de alinhamento pareado. Qualquer que seja a abordagem utilizada pelo algoritmo, a comparação é construída através da combinação e pontuação aplicada a *gaps*, *mismatches* e *matches*. Os programas costumam incluir ainda uma matriz de distância que funciona como medida qualitativa do conjunto de sequências a ser comparado e uma etapa de otimização, onde, dependendo da metodologia,

pode ser incluída a construção de uma árvore filogenética (árvore guia) que agrupa os entes mais similares e rodadas de iteração que refazem as comparações, assim possibilitando que os alinhamentos obtidos sejam aqueles com maior *score* (Figura 2) (Do and Katoh 2008; Pirovani and Heringa 2008; Pais et al. 2014).

Esta abordagem pode ser utilizada para se observar diferenças entre motivos proteicos, inserções e deleções em sequências nucleotídicas bem como auxiliar na construção de análises fenéticas e filogenéticas.

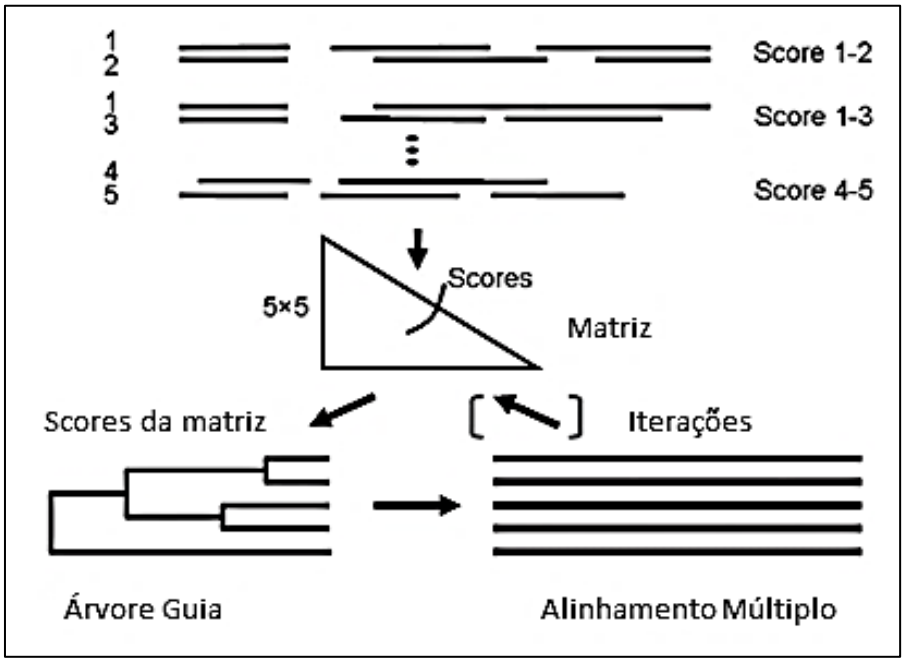

Figura 2. Representação de um alinhamento progressivo. Os alinhamentos construídos par a par passam por etapas qualitativas e de refinamento até o alinhamento final (Pirovani and Heringa 2008).

3.2.4. Domínios e motivos conservados

O "domínio conservado" (*conserved domain*) compreende uma região da estrutura proteica frequentemente (porém não sempre) composta por uma sequência contínua de aminoácidos e com uma função distinta do esqueleto proteico (Buljan and Bateman 2009). Já o termo "motivo" (*motif*) é utilizado em dois sentidos na biologia estrutural: primeiro, uma sequência de aminoácidos característica de uma função bioquímica específica. A sequência: "CXX(XX)CXXXXXXXXXXXXHXXXH", chamada de *zinc finger*, encontrada em muitas famílias proteicas com função de ligação ao DNA (Aitken 1999) é um

exemplo de motivo conservado; O segundo sentido envolve segmentos contínuos da estrutura secundária com significante importância funcional, por exemplo, o motivo hélice-volta-hélice presente em diversos peptídeos, como nas esnaquinas (Yeung et al. 2016), de proteínas com grampos (*hairpins*) (Nolde et al. 2011) e das tioninas (Romagnoli et al. 2000).

Como nem todos os domínios consistem de cadeias polipeptídicas contínuas, em alguns casos os domínios podem ser interrompidos por uma cadeia que se dobra em um domínio distinto, depois do qual a sequência original continua (como ocorre, por exemplo, na enzima alanina racemase) (Petsko and Ringe 2004a). Os domínios também podem variar em tamanho, embora não sejam maiores que 250 aminoácidos (a maioria varia entre 51-150 aminoácidos) (Petsko and Ringe 2004b). O conhecimento desses aspectos é fundamental quando a proposta inclui o estudo e a caracterização estrutural de um gene.

Existem várias ferramentas atualmente disponíveis em servidores *online* para identificação de domínios proteicos. Dentre as ferramentas mais utilizadas destacam-se oSMART (Letunic et al. 2002), o InterProScan (Jones et al. 2014) e o CD-Search (Marchler-Bauer and Bryant 2004). Essas ferramentas são baseadas em algoritmos que alinham as sequências em questão com bancos de domínios conhecidos (similar ao BLAST) ou, ainda, por comparação de posições específicas, sendo o domínio inferido. A busca por possíveis assinaturas contidas na sequência submetida à análise (*query*) também pode ser uma estratégia na busca de domínios e, neste caso, um dos programas mais utilizados é o InterProScan (Jones et al. 2014).

3.2.5. Predição *in silico* de estruturas proteicas

Um dos maiores desafios para a bioinformática estrutural consiste na predição da estrutura tridimensional das proteínas a partir de sua sequência unidimensional (sequência de aminoácidos). O objetivo é determinar que conformação determinados resíduos irão assumir em conjunto, gerando o desafio de determinar se a estrutura que a sequência vai adotar é nova ou possui semelhança com outras já conhecidas e depositadas em bancos de dados especializados. Desta forma, três principais abordagens são geralmente aplicadas para resolução do modelo: *ab initio* (ou *de novo*), *threading* e modelagem comparativa (Huzefa and Karypis 2010; Pantazes et al. 2011).

Quando os *templates* para a construção de um modelo não estão disponíveis, sua construção é guiada a partir dos princípios das leis físico-quimicas, baseadas na hipótese termodinâmica a qual tem como princípio que "*o estado nativo de uma proteína corresponde*

ao seu nível mínimo de energia livre" (Anfinsen 1973). Os métodos que se baseiam em tais ideias são: *ab initio* (Wu et al. 2007), também conhecidos como modelagem *de novo* (Bradley et al. 2005) ou modelagem livre (*free modeling*) (Jauch et al. 2008), cuja abordagem pode auxiliar a elucidar questões sobre como e por que as proteínas adotam certos padrões de dobragem.

Geralmente as técnicas de modelagem *ab initio* fazem uma busca conformacional orientada por uma dada função de energia. Este procedimento produz um número de possíveis conformações a partir das quais os modelos finais são selecionados. Assim, uma modelagem *ab initio* depende de três fatores: (1) uma função energética precisa com a qual a estrutura nativa de uma proteína corresponda ao estado mais termodinamicamente estável, em comparação com todas as estruturas possíveis; (2) um método de busca eficiente que possa identificar os estados de mínima energia através da busca conformacional; (3) seleção de modelos nativos a partir de um conjunto estruturas (Hardin et al. 2002; Lee et al. 2009).

Em alguns casos, uma *query* poderá ter um padrão de dobragem nativo semelhante a outro já conhecido e depositado em um banco de dados. Contudo, suas sequências podem não compartilhar muitas semelhanças. Uma estrutura que pode servir de exemplo é o barril-TIM, encontrado em diversas proteínas não relacionadas diretamente (Brändén 1991; Dorn et al. 2014).

À medida que os métodos de alinhamento ganham mais refinamento para encontrar similaridade entre sequências, como consequência do aumento na demanda causada em vista da quantidade crescente de sequências disponíveis em bancos de dados, o número de estruturas proteicas em questão não segue o mesmo ritmo (Figura 3). As técnicas para resolver as estruturas destas sequências residem em montar a *query* comparativamente a uma série de estruturas modelares (*templates*). A esta técnica dá-se o nome de *threading* (Eisenberg et al. 1991; Jones et al. 1992; Jones 1999).

A modelagem comparativa (ou modelagem por homologia) é utilizada quando existe uma relação clara entre uma *query* e a sequência de uma estrutura conhecida, geralmente quando a identidade está em torno de 30 % ou mais (Xiang 2006). A abordagem mais simples para a predição da estrutura de uma determinada proteína é realizada com um alinhamento de sequências pareado comparando a *query* contra as sequências das estruturas conhecidas, gerando-se, então, o alinhamento a partir do qual pode-se construir uma estrutura com base no melhor *template* reportado. Alguns algoritmos (como por exemplo, o MODELLER; Webb and Sali 2016) realizam ainda uma etapa de refinamento dos modelos

gerados, fornecendo modelos com o mínimo de energia livre (Schwede et al. 2008; Huzefa and Karypis 2010).

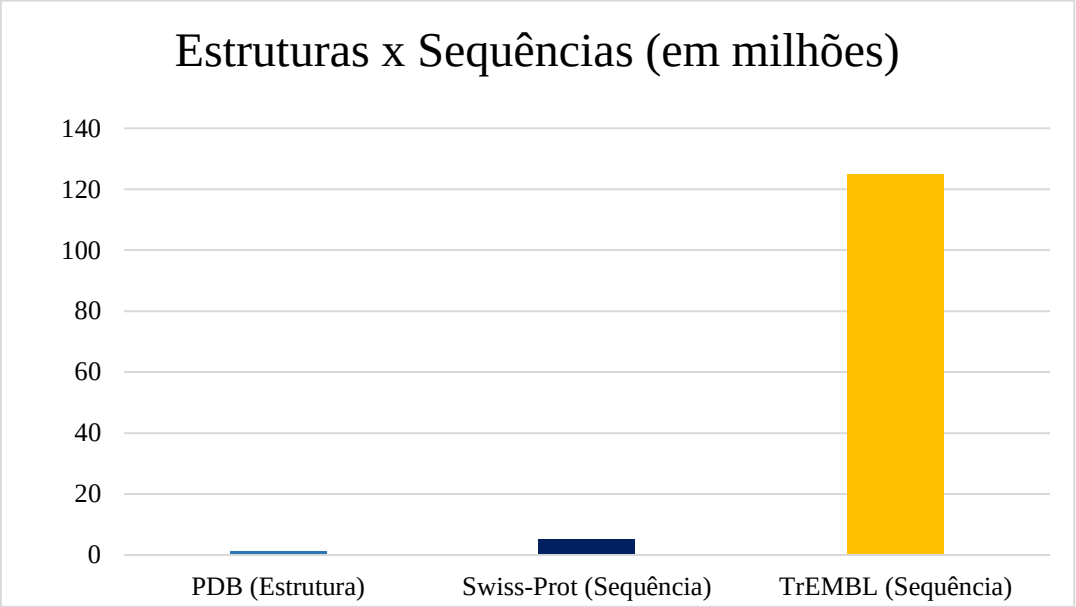

Figura 3. Quantidade de estruturas depositadas no PDB (azul claro) em comparação com o número de sequências manualmente anotadas (Swiss-Prot – Azul escuro) e automaticamente anotadas (TREMBL - Amarelo) até Junho de 2018.

3.2.6. Análises Filogenéticas e Fenéticas

Em biologia evolutiva, os principais métodos utilizados para resolver os problemas de classificação das espécies são a fenética e a filogenética, as quais diferem entre si em conceitos e abordagens (Zander 2013). Na fenética, duas espécies que compartilham mais caracteres similares, são alocadas em posições mais próximas no dendrograma. Este agrupamento é realizado com base em níveis hierárquicos, onde membros mais próximos nos ramos terminais são os que compartilham maior similaridade (Hall 1988; M. Williams and Ebach 2008). Por exemplo, ao analisarmos morfologicamente um crocodilo e um lagarto, observa-se que estes compartilham maior similaridade considerando seus caracteres morfológicos, do que quando se comparam estes táxons com um pássaro. No entanto, sabemos que filogeneticamente isso não ocorre. Assim, no fenograma teríamos um agrupamento entre os dois primeiros, com a ave em um ramo mais externo. Porém, na filogenia “crocodilo e pássaro” estariam mais próximos, uma vez que estes últimos possuem um ancestral comum mais próximo. Contudo, é importante ressaltar que filogenética e

fenética podem concordar entre si, a depender das características comparadas (Ridley 2004; Rahman et al. 2016) (Figura 4).

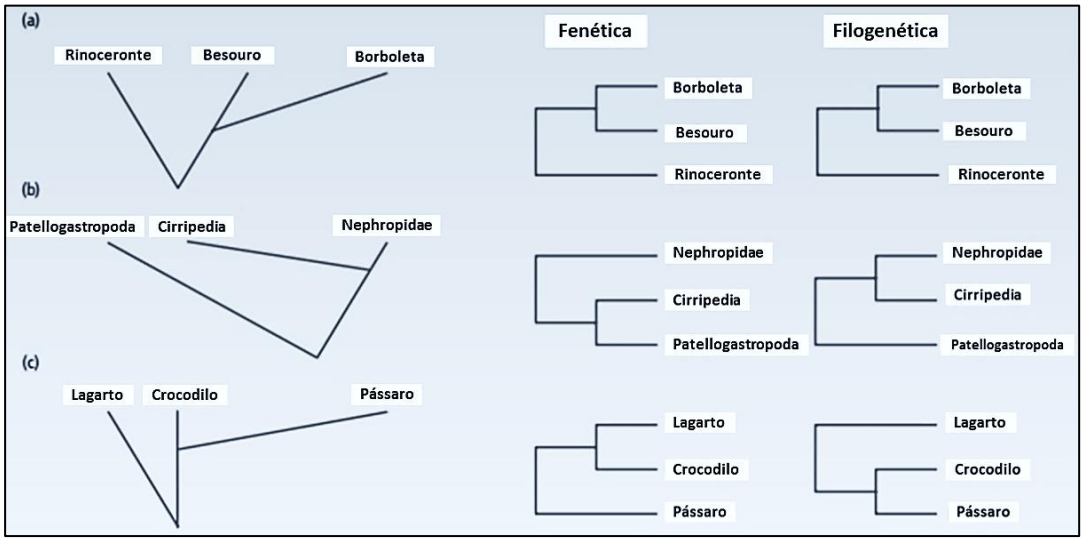

Figura 4. Os princípios de classificação fenética e filogenética podem convergir (a) ou divergir (b e c) entre si (Ridley 2004).

A fenética utiliza algoritmos baseados em matrizes de distância para construir suas representações, onde o *Unweighted Pair Group Method with Aritimetic Mean* (UPGMA) e o *Neighbor Joining* (NJ) recebem maior destaque (Lemey et al. 2009).

O UPGMA é provavelmente o método mais antigo e mais simples usado para reconstruir árvores filogenéticas a partir de dados de distância. O agrupamento é construído utilizando o menor valor fornecido pelas matrizes de distância pareadas. Esta abordagem constrói uma árvore identificando a menor distância na matriz transformando dois taxa em uma única OTU (Unidade Taxonômica Operacional – *Operational Taxonomic Unit*) que será submetida a todos os cálculos subsequentes, calculando uma nova matriz de distância e repetindo essas etapas. Em UPGMA, a distância do cluster recém-formado é a média das distâncias das OTUs que o originaram. O cálculo das médias assume que a taxa evolutiva do nó das duas OTUs agrupadas é idêntica para cada uma das entidades individualmente (Peng 2007).

Por sua vez, o método NJ constrói uma árvore alocando as OTUs vizinhas em único nó interno. O método de clusterização usado por esse algoritmo é bem diferente do UPGMA, porque ele não agrupa as OTUs com as médias mais relacionadas, mas minimiza o tamanho de todos os ramos internos e, portanto, o comprimento da árvore. O algoritmo NJ começa

assumindo uma árvore em formato de estrela que não possui ramificações internas. Na primeira etapa o algoritmo introduz a primeira ramificação interna e calcula o comprimento da árvore resultante. O algoritmo conecta sequencialmente todos os pares OTU possíveis e, finalmente, mantém os pares de OTUs que produzem a árvore mais curta (Saitou and Nei 1987; Lemey et al. 2009).

Por outro lado, a análise filogenética demanda a inclusão de um grupo externo ou grupo irmão, sendo baseada em parâmetros evolutivos, ou seja, leva em consideração a ancestralidade comum das OTUs (Ridley 2004). Em uma árvore filogenética entidades mais relacionadas serão agrupadas mais próximas nos ramos terminais, em contrapartida, à medida que o ancestral comum se torna cada vez mais distante, os agrupamentos se mostrarão cada vez mais separados na classificação (Gregory 2008). Para tal, as matrizes e métodos estatísticos possuem um arcabouço mais complexo do que aqueles utilizados nas análises fenéticas, pois analisam cada carácter das entidades taxonômicas (Peng 2007). Em uma filogenia de proteínas, cada aminoácido é tomado como um carácter a ser analisado e, então, a depender da matriz utilizada, outras variáveis são adicionadas à comparação, tais como: posição, composição química ou mutações. Por sua vez, na análise fenética apenas as diferenças e/ou semelhanças entre as entidades são levadas em consideração (Nei 1996; Lemey et al. 2009).

Entre os métodos aplicados na reconstrução filogenética incluem: máxima parcimônia (*maximum parcimony*), máxima verossimilhança (*maximum likelihood*) e inferência bayesiana (*bayesian inference*)

O método de máxima parcimônia é baseado no principio da navalha de Occam, preconizado pelo filósofo William de Occam (1285-1347), onde sugere que entre duas ou mais hipóteses que possam explicar um mesmo fenômeno, provavelmente a hipóotese mais simples seja a mais adequada (Thorburn 1915; Lemey et al. 2009). Os algoritmos deste tipo funcionam selecionando árvores construídas com o menor número de passos. Para cada *sítio* no alinhamento, todas as árvores possíveis são avaliadas - procedimento que difere daquele adotado em inferências fenéticas. Em comparação com outros métodos filogenéticos, este é menos dependente de dados pré-estabelecidos; no entanto, possui a desvantagem de ser pouco eficiente quando se tem dados muito heterogêneos (Rizzo and Rouchka 2007; Rahman et al. 2016).

A máxima verossimilhança se assemelha ao de máxima parcimônia no que diz respeito à atribuição de um *score* às diferentes topologias a serem comparadas, porém trata-se de um método probabilístico. Originalmente desenvolvido como uma metodologia

estatística para estimar parâmetros desconhecidos em um dado modelo, sendo sua função definida como a probabilidade dos dados em vista dos parâmetros (Yang and Rannala 2012). Para a construção da árvore, calculam-se as probabilidades associadas a diferentes topologias e cada uma delas com as variações nos tamanhos dos ramos, considerando o modelo evolutivo escolhido. , Trata-se do método mais amplamente utilizado na atualidade, devido à melhora do poder computacional (embora ainda oneroso) somado à implementação de programas e modelos estatísticos voltados, exclusivamente, para a análise de sequências. Além do custo computacional, apresenta a desvantagem de não apresentar a correção do modelo estatístico, que pode resultar - a depender dos parâmetros estabelecidos, na obtenção de dados discrepantes (Yang 1996; Lemey et al. 2009; Yang and Rannala 2012).

Por último, a inferência bayesiana - uma abordagem também estatística, que difere da máxima verossimilhança no modo em que tratam os parâmetros (na máxima verossimilhança são constantes fixas e na bayesiana são variáveis aleatórias), tendo como base o modelo criado por Thomas Bayes, o qual foi posteriormente adaptado para aplicação em análises filogenéticas (Yang and Rannala 1997). Antes da análise dos dados, os parâmetros devem ser atribuídos em uma distribuição anterior que é então combinada com os dados, gerando assim a distribuição (ou probabilidade) posterior (Bellhouse 2004; Yang and Rannala 2012). Assim, sua principal desvantagem seria a especificação desses parâmetros, e, apesar de demandar menor custo computacional que a máxima verossimilhança, continua sendo computacionalmente custoso (Holder and Lewis 2003).

Analogamente, outra medida qualitativa aplicada na construção de árvores, utilizada nas outras abordagens é o método de *bootstrapping.* Nesta abordagem, as colunas do alinhamento original são reamostradas aleatoriamente, repetidas vezes. e novas bases de dados (réplicas) são geradas, sendo cada uma delas usada para gerar uma árvore. O algoritmo de construção da árvore é então aplicado a esse novo conjunto de dados, onde todo o procedimento de seleção das colunas e construção da árvore é repetido em uma quantidade de vezes pré-estabelecida (geralmente 1000 vezes). Com isso, tem-se na árvore final a frequência com que um determinado conjunto de dados foi amostrado (Peng 2007).

Em conjunto, todas estas metodologias *in silico* podem ser aplicadas para uma maior compreensão dos diversos aspectos relacionados ao objeto de estudo (a exemplo dos peptídeos antimicrobianos), envolvendo informações a cerca da estrutura primária, diversidade e função, até a sua possível história evolutiva. Além disso, análises com ferramentas computacionais são também essenciais para a manipulação e tratamento de dados, os quais possam, posteriormente, ser validados através de experimentos laboratoriais,

implicando em inferências biotecnológicas. Neste contexto, a escolha do tipo de dado e metodologia de análise dependem da natureza da hipótese evolutiva que se deseja testar.

CAPÍTULO 1

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REVIEW ARTICLE

Snakin: Structure, Roles and Applications of a Plant Antimicrobial Peptide

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Abstract: Snakins are plant antimicrobial peptides (AMPs) of the Snakin/GASA family, formed by three distinct regions: an N-terminal signal peptide; a variable site; and the GASA domain in the C-terminal region composed by twelve conserved cysteine residues that contribute to the biochemical stability of the molecule. These peptides are known to play different roles in response to a variety of biotic (i.e., induced by bacteria, fungi and nematode pathogens) and abiotic (salinity, drought and ROS) stressors, as well as in crosstalk promoted by plant hormones, with emphasis on abscisic and salicylic acid (ABA and SA, respectively). Such properties make snakin/GASA members promising biotechnological sources for potential therapeutic and agricultural applications. However, information regarding their tertiary structure, mode of action and function are not yet completely elucidated. The present review presents aspects of snakin structure, expression, functional studies and perspectives about the potential applications for agricultural and medical purposes.

Keywords: Snakin/GASA domain, cysteine-bridges, stress response, crosstalk.

1. INTRODUCTION

Along evolution plants developed complex defense mechanisms against a broad spectrum of pathogens. However, for many pathogenic microorganisms defense mechanisms are still inefficient, leading to disease development [1]. A common feature in defense strategies of plants and animals is the existence of a wide variety of small antimicrobial peptides (AMPs) that act as effector molecules of non-specific defense [2]. These peptides figure among most effective plant weapons, since they can be both constitutively expressed and stress induced by a wide range of biotic and abiotic stressors [3, 4]. Normally encoded by multigenic families, in which some genes are developmentally regulated, AMPs may act as potent broad-spectrum antibiotics, with potential as novel therapeutic agents.

Usually formed by 20 to 120 amino acid residues, these peptides can be rich in proline, glycine, histidine, arginine, tryptophan or cysteine [5]. Most of the cysteine-rich peptides have a globular structure, which is stabilized by disulfide bridges [6] and represent the major class of antimicrobial proteins found in plants. Among plant cysteine-rich AMPs, snakins are the most recently described peptides, isolated initially from potato and pepper, showing a broad spectrum activity against plant pathogens [7-9]. Studies based on sequence analysis, phenotypic characterization and expression pattern analysis have suggested that these peptides can be involved in diverse processes, both in development and in response to external factors [8, 10].

The present review focuses on the most significant features of this new family of antimicrobial peptides: structure, function, expression and potential applications, exploring its importance based on the current knowledge.

2. SNAKIN/ GASA FAMILY

2.1. Molecular Structure and Characterization

Snakin genes encode small proteins that comprise three distinct domains: (1) a signal peptide with 18-29 residues, (2) a variable region that is highly divergent between family members, both in amino acid composition and sequence length, and (3) the GASA domain, a conserved C-terminal region with approximately 60 amino acids, 12 of them are cysteine residues in conserved positions [10] (Fig. **1**).

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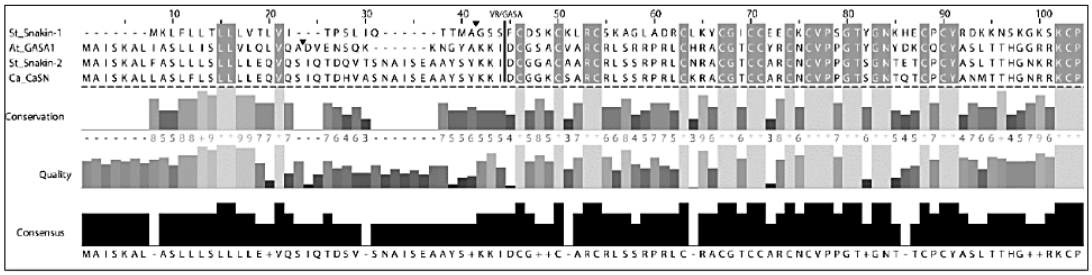

Fig. (1). Multiple sequence alignment of snakin/GASA peptides. The black triangles show de cleavage site for the signal peptide in *Solanum tuberosum* Snakin-1 (St-Snakin-1), *Arabidopsis thaliana* GASA1 (At_GASA1), *Solanum tuberosum* Snakin-2 (St_Snakin-2) and *Capsicum annuum* Snakin (Ca_CaSN), which is in the same position for At_Snakin-1, Ca_Casn and St_Snakin-2. The vertical bar indicates the limit between the variable region (VR) and the start of the GASA domain that exhibits twelve conserved cysteines and other nine conserved residues (dark gray in the alignment). Bars below the alignment indicate the levels of conservation, quality and, at the bottom, the consensus sequence.

Snakin/GASA (Gibberellin acid-stimulated from Arabidopsis) family comprises a group of widely distributed peptides among higher plants (Table **1**) sharing common structural features with many known antimicrobial peptides from eukaryotes, including the N-terminal putative signal sequence and a highly divergent acidic intermediate region. However, the main structural aspect is a cysteine-rich C-terminal signature, which contains 12 cysteine amino acids, in highly conserved positions (XCX3CX3CX8CX3CX2C CX2CXCX11CXCX12CX), forming a globular conformation, through six disulfide bonds (Fig. **1**). This C-Terminal region was described in *Arabidopsis thaliana* by Herzog *et al.* [10] and is called GASA domain. This arrangement is considered important for structure maintenance (specially the 3D conformation) and function of these peptides [11-15].

Studies have revealed that genes from this family may be regulated by plant hormones and participate in hormonal signaling pathways modulating the responses of the plant through their involvement in redox and hormone homeostasis. It is known that the presence of hormones such as Gibberellic Acid (GA) and SA has been associated to an increase in the expression level of snakin/GASA genes of *A. thaliana*, *Fagus sylvatica, Fragaria ananassa, Gerbera hybrida*, *Petunia hybrida*, *Oryza sativa* and *Zea mays* [10].

Genes of this family often encode a basic peptide with a molecular size below 11 kDa. After post-translational processes, the mature peptide basically consists of a structure with approximately 60 amino acid residues, with a molecular weight of approximately 7 KDa, for all snakin isolated so far. The basic isoelectric point (pI < 9.4) was described for Snakin-2 from potato and other GASA homologues, as well as for other antimicrobial peptides [7, 8, 16, 17], revealing that this feature is very conserved among AMPs and their homologues. Their basic character provides an advantage in interacting with negatively charged microbial membrane components [2]. All these features were also observed in a recently isolated snakin from a nematode-resistant pepper cultivar (*Capsicum annuum* cv. Santaka). According to Mao *et al.* [11] the CaSn (*C. annuum* snakin) gene encodes a protein of 104 amino acids residues (11 kDa) and a mature peptide of 66 amino acid residues that corresponds to a molecular mass of 7.03 kDa. Another member of this family was isolated from alfalfa (*Medicago sativa*), sharing many features (as a signal peptide comprising 25 residues) with the peptides of this group, representing a new member of the family [18].

Although there are three different genomic structures for Snakin/GASA members, only two structures were observed in snakins: (1) consisting of two exons and (2) consisting of three exons [11, 19]. Genes with two exons have been commonly described among antimicrobial peptides [6], including Snakin-1 from *Solanum tuberosum*, being considered a component of the constitutive defense against pathogens in both storage and reproductive plant organs [9, 20]. The second structure, also found in the Snakin-2, from potato [7] and in other AMPs [6] was associated with developmental stages and in response to specific pathogens, being considered an important component of both constitutive and induced defense [8, 21, 22]. The number of copies of Snakin-1 and Snakin-2 in the potato genome was estimated by southern blot hybridization, therefore the patterns observed indicated the existence of only one or two copies per genome [7, 9], whereas no CNV (Copy Number Variation) has been reported up to date.

Snakin/GASA proteins are expressed in different plant organs, however little is known about their 3D structure and mode of action [23]. To our knowledge, there is just one theoretical model for Snakin-1, whose putative structure is very similar to two other AMPs: thionins and α-helical hairpins [24, 25], formed by one short helix and two long α-helices. These findings strongly suggest that some classes of cysteine-rich AMPs share a common ancestor [6]. Furthermore, although the cysteine residues may be conserved in sequence and position, the disulfide bonds may be not structurally conserved, showing different patterns among snakin members [15, 26].

2.2. Synthesis, Developmental Process and Relationship to Stress Tolerance

2.2.1. Spatiotemporal Expression

When considering subcellular localization, there is a tendency among different snakin/GASA members to vary,

Table 1. Plant Snakin/GASA peptides previously described.

Sequence name	Species	Family	Reference
GEG	*Gerbera hybrida*	Asteraceae	[52]
GASA1-14	*Arabidopsis thaliana*	Brassicaceae	[10, 11, 12, 36]
GmSN-like	*Glycine max*	Fabaceae	[Oliveira-Lima *et al.*]*
GsGASA	*Glycine soja*		[35]
MsSN1	*Medicago sativa*		[18]
Snakin-like	*Phaseolus vulgaris*		[28]
VuSN-like	*Vigna unguiculata*		[Oliveira-Lima *et al.*]*
FsGASA	*Fagus sylvatica*	Fagaceae	[53]
OsGASR1-2; OsGSR1	*Oryza sativa*	Poaceae	[54, 55]
ZmGSL1-10	*Zea mays*		[31]
FaGAST1	*Fragaria ananassa*	Rosaceae	[30]
CaSN	*Capsicum annuum*	Solanaceae	[8]
Gip1-5	*Petunia hybrida*		[56, 57]
RSI-1; GAST1	*Solanum lycopersicum*		[29, 34]
Snakin1-2	*Solanum tuberosum*		[10, 22]

* Author's data, paper *in prep*.

whereas some representatives (i.e., Snakin-1 from an agroinfiltrated *Nicotiana benthamiana*) had their localization experimentally observed in the plasmatic membrane. Moreover, depending on their structural features (for example, when a signal peptide is present) not all snakin/GASA proteins are secreted to the extracellular matrix possibly due to post-translational modifications. Other interactions may lead their localization as shown by Nahirñak *et al*. [27] reporting that Snakin-1 self-interacts *in vivo*. Another exemplified interaction regarded SN2-like from common bean (*Phaseolus vulgaris*) which associated with a proline-rich protein, resulting in a protein complex named FBCBP (French bean chitin-binding protein) [28].

Currently it is known that the expression of these peptides may vary for each gene and organ or may still be tissue specific. Most information about the function of some Snakin/GASA members was based on phenotypic characterization of mutants and transgenic plants or, still, on expression profiling. Some candidates seem to be involved in processes of arrest or promotion of cell elongation and division in adult plants, root formation (in early stages), flowering time and also fruit ripening [27, 29-33].

Among snakin genes, *Snakin-1* and *2* have different levels of expression along the age of the plant and developmental stage in potato plants. *Snakin-1* had its expression level decreasing along the plant development, being more active in young stages in tissues like carpels, petals, floral buds and tubers [9]. In turn, *Snakin-2* exhibited a pattern varying for both, development and tissues, being found in stems, stamens, flower buds, leaves, shoot apices, carpels, petals and tubers, being also induced by pathogen infection [7].

2.2.2. Hormone Responsiveness

Many Snakin/GASA genes have been reported to have their transcription regulated by hormones [32, 34-36]. The first members of this family showing positive induction by GA and auxin hormones were the tomato *GAST1* (*Gibberellic Acid-Stimulated Transcript* 1) and *RSI-1* genes, respectively [29, 34]. Nevertheless, the positive induction by hormones (especially GA) was not considered a decisive factor for all members. For example, the transcription of potato *Snakin-2* was inhibited by GA, whereas the expression of *Snakin-1* of the same species had no alterations [7, 9]. On the other hand, under abscisic acid (ABA) treatment, the *Snakin-2* was induced, confirming the importance of crosstalk between snakin and this hormone, since ABA is an important wound signaling in Solanaceae species [7].

2.2.3. Redox Homeostasis

All identified members of the Snakin/GASA family presented a signature with twelve cysteine residues in the GASA domain, which is considered to exhibit a significant oxidative power due to their putative catalytic disulfide bonds (redox active cysteines), indicating a participation in redox regulation [15, 37, 38]. Besides, many metabolic pathways are activated by biotic stress which, in turn, trigger crosstalk responses, such as Reactive Oxygen Species (ROS) [10, 14, 39]. It is known that ROS is also involved in wounding where snakin may play a role in the redox regulation [10,

21]. Additionally, Nahirñak *et al.* [28] observed that metabolites that mitigate ROS effects were significantly reduced in *Snakin-1* silenced strains from *S. tuberosum* in comparison to wild type. These lines also exhibited affected cell division, since the silenced lines presented smaller leaf sizes, what might reflect an inhibition of the cell division or growth, suggesting that Snakin-1 might be involved in cell division by modulating some metabolites [27].

2.2.4. Biotic and Abiotic Stress

As previously mentioned, snakins have been associated with a wide range of biotic and abiotic stresses [7, 14, 20, 21, 40, 41]. Interestingly, *Snakin-1* was not induced in potato leaves by either biotic or abiotic stresses, indicating this peptide as a component of the plant constitutive defense [9]. In contrast to *Snakin-1*, *Snakin-2* was positively regulated in potato leaves by wounding and presented a weak response to salinity stress, while drought stress caused no effect [7].

The following section details the existing evidence that *Snakin* genes are induced when plants are challenged by a wide range of microorganisms, whereas their overexpression in several angiosperms resulted in increased resistance against several microbial diseases. These findings, together with their reported *in vitro* antimicrobial activities may indicate a potential role of snakins to act as resistance traits against the main phytopathogens.

3. ROLE IN DEFENSE RESPONSE: *IN VITRO* AND *IN VIVO* BIOLOGICAL ACTIVITIES

Several *in vitro* and *in vivo* antimicrobial activities have been attributed to plant snakins. It has been reported that Snakin-1 (StSN1) and Snakin-2 (StSN2), isolated from *S. tuberosum* cv. Desireé and cv. Caerla, respectively, exhibit *in vitro* antimicrobial activity against important pathogens of potato and other plant species. StSN2 and StSN1 have been effective against the bacteria *Clavivacter michiganensis* subsp. s*epedonicus* and against fungal pathogens such as *Fusarium solani*, *Colletotrichum lagenarium*, *Colletotrichum graminicola*, *Bipolaris maydis* and *Botrytis cinerea,* among others, but not against *Ralstonia solanacearum*, an important potato bacterial pathogen [7, 9]. It was further observed that StSN1 presented an intermediate inhibitory effect on *Listeria monocytogenes*, a foodborne bacteria [42].

Additionally, StSN1 was heterologously expressed in *Escherichia coli,* followed by peptide purification and evaluation of its activity. This recombinant protein showed antibacterial and antifungal activity, inhibiting completely the growth of *Clavibacter miciganensis* subsp. *sepedonicus* and spore germination of *Botrytis cinerea* and *Colletotrichum coccoides* at a concentration of 14 mM [43], also inhibiting the growth of the mycelium of *Gaeumannomyces graminis* var. *tritici* - an important wheat pathogen [44]. A peptide of *Capsicum annuum* (CaSN), with high homology to StSN2 was also produced in *E. coli.* Free-living nematodes, like *Caenorhabditis elegans* were fed with *E. coli* strains expressing CaSN, leading to an inhibition of larval development, reduced growth and mobility, as well as death of most nematodes. In addition, plant-parasitic nematode intoxication was observed in toxicity assays using the supernatant containing the CaSN protein [8].

A possible role *in planta* has been suggested by overexpressing a *snakin* gene resulting in plants with increased resistance against several diseases. Transgenic lines of *S. tuberosum* subsp. *tuberosum* cv. Kennebec that accumulated high levels of SN1 mRNA from a potato wild relative (*Solanum chacoense*) showed heightened resistance against important potato pathogens, such as the fungus *Rhizoctonia solani* and the gram negative bacteria *Erwinia carotovora* [40] and also against *Pectobacterium carotovorum* subsp. *carotovorum* and *Blumeria graminis* [20]. *Snakin-1* also decreased the virulence of the bacteria *Dickeya chrysanthemi* in sensitive tomato mutants [45], while the overexpression of a gene encoding SN2 increased tolerance to *Clavivacter michiganensis* subsp. *michiganensis* [21]. Transgenic wheat expressing StSN1 showed prominent resistance to 'take-all root rot', a wheat root disease caused by *Gaeumannomyces graminis* var. *tritici* [44], indicating a potential *in vivo* antifungal and antibacterial activity.

Plant snakins are induced by a wide range of biotic stresses and by plant hormones involved in stress signaling, also supporting their role in plant defense. In potato *StSN1* gene activation was not observed when young leaves were subjected to biotic and abiotic stimuli [9]. However, a down-regulation of the gene encoding *StSN2* was observed during *Ralstonia solanacearum* and *Erwinia chrysanthemi* infection, whereas an up-regulation was detected after infection with the compatible fungus *Botrytis cinerea* [7]. In *Nicotiana benthamiana* the silencing of *Snakin-2* homologs increased susceptibility to *Clavibacter michiganensis* subsp. *michiganensis* [41], while their over-expression in transgenic tomato plants limited the invasiveness of this tomato pathogenic bacterium [21]. Variation regarding resistance and/or susceptibility to different pathogens indicates possible pathogen-specific associations or still induction mediated by other signaling molecules.

4. SNAKIN BIOTECHNOLOGY: STATE OF ART, APPLICABILITY AND PERSPECTIVES

Due to the identified antimicrobial activities [9] snakins have been considered interesting biotechnological targets. Thus, their biotechnological significance was indeed highlighted after the recognition of their involvement in the regulation of developmental processes in plants and also by the possibility to obtain considerable amounts of this peptide by expression in *E. coli* [10, 46]. Although their limited exploration in relation to other AMPs, the potential of snakins has been analyzed mainly by methods of transgeny and cisgeny (artificial gene transfer between organisms that could otherwise be conventionally bred).

In this regard, Almasia *et al.* [40] generated transgenic potato plants (*S. tuberosum* subsp. *tuberosum* cv. Kennebec) transformed via *Agrobacterium tumefaciens* with a construct encoding the gene SN1 of *S. chacoense* under the control of the CaMV 35S promoter. The resulting transgenic lines were evaluated under inoculation with *Erwinia carotovora* and *Rhizoctonia solani,* both relevant bacterial and fungal potato pathogens, respectively, whereas the transformed lines showed high rates of survival when compared to wild-type controls, indicating that StSN1 is involved in the resistance process against these pathogens.

In turn, Balaji & Smart [43] obtained genetically engineered tomato (*Solanum lycopersicum*) plants overexpressing *StSN2* or *ELP* (*Extensin like protein*) genes of the own species. The generated OGMs were evaluated under infection with the bacteria *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causal agent of bacterial wilt and canker, one of the most important tomato diseases throughout the world. In the transformed plants, the bacterial population was significantly reduced (100–10,000 fold) when compared to the wild type, indicating that StSN2 and ELP products limit the action of this pathogen, suggesting a potential *in vivo* antibacterial activity.

Additionally, Mohan *et al.* [22] obtained transgenic lines of potato (cv. Iwa) overexpressing the gene *Gibberellin Stimulated-Like 2* (*GSL2*; also known as *Snakin-2*) from the same species. Three trials performed independently showed that the modified strains exhibited a significant induction of *GSL2* transcriptional activity resulting in increased resistance to *Pectobacterium atrosepticum* (previously known as *Erwinia carotovora* subsp. *atroseptica*) the causal agent of potato blackleg, strengthening the role of this AMP in the defense against pathogens in potato.

Evaluations addressed to other environmental factors have shown that snakins may have a wide range of functions, highlighting their biotechnological potential. For example, Almasia *et al.* [47] characterized the promoter region (*PStSN1*) of the potato gene *StSN1*. Using bioinformatic approaches the authors identified 55 possible target regulation motifs associated to tissue specificity, abiotic stresses (high and low temperatures), defense and response to hormones, among others. To confirm the indicated functional dynamism the authors evaluated transgenic lines of *A. thaliana* expressing a reporter gene under control of the identified promoter (PStSN1:GUS). Contrary to expectations, there was no correlation between bioinformatic simulations (which indicated their potential functions) and the activation by the expected stressors, including phytohormones, light, dark, UV radiation or infection by *Pseudomonas syringae*. However, plants transformed with the identified promoter presented induction of responses associated to high or low temperatures, and mechanical damage (wounding), providing interesting targets for development of plants tolerant to extreme temperatures, insectivory [48], attack by pathogenic fungi [49] and viruses [50].

In addition to the antimicrobial role in biotic and abiotic stresses, recent data on snakins have increased the range of their potential biotechnological applications. Zare-Zardini *et al.* [51] observed that a protein named Snakin-Z presented, in addition to the already known antioxidant role, inhibitory effects over acetylcholinesterase (AChE) and butyrylcolinesterase (BChE) enzymes. This was considered a very important finding, since there are evidences that the inhibition of these enzymes may be applicable for the treatment of Alzheimer's disease, indicating that snakins may be a potential target for pharmacological trials in animal models to confirm these properties by *in vivo* essays [51].

The range of functions regarding snakins may still be underestimated, since several proteins bearing the Snakin/GASA domain play important roles in response to various types of stress. Due to the observed properties, snakins may have broad applications in different sectors: from agriculture, by obtaining tolerant/resistant transgenic plants to a range of stressful conditions; pharmaceutical industry, since their antifungal and antibacterial effects can be exploited for the development of antibiotics or antimicrobial substances, as well as a possible role in medicine, in the field of neurological diseases, due to its proposed ability to inhibit target enzymes involved in Alzheimer's disease [51]. Therefore, additional efforts are necessary to identify additional representatives of these interesting AMPs and their possible biological roles, especially considering their functional plurality.

CONCLUDING REMARKS

Among the diverse families of plant antimicrobial peptides, snakins deserve a special attention, considering that, at one side, they share many features with other AMPs and, by the other side, they possess peculiar features, like the number of cysteines (twelve) in the C-terminal region, which is the most important GASA domain signature, an important player of redox homeostasis.

Snakins seem to represent different roles in diverse plant developmental stages and tissues, from young to differentiated tissues, some induced by stress, exhibiting activity against a variety of bacteria, fungi and also nematodes. It has been also associated to hormones specially the GA, auxin and SA, being also constitutively expressed with participation in key events like cell division. Based on all these evidences, the broad potential of snakins is still to be explored, expanding the knowledge of their diversity, evolution, mode of action and its correlation with the structure, which constitute its main knowledge gap.

LIST OF ABBREVIATIONS

ABA	=	Abscisic acid
AMP	=	Antimicrobial peptide
CaMV	=	Cauliflower mosaic virus
CaSN	=	*Capsicum annuum* snakin
ELP	=	Extensin-like protein
FaGASA	=	Fragaria ananassa GASA
FBCBP	=	French bean chitin-binding protein
FsGASA	=	Fagus sylvatica GASA
GA	=	Gibberellic acid
GASA	=	Gibberellin acid-stimulated Arabidopsis
GAST	=	Gibberellic acid-stimulated transcript
GEG	=	Gerbera homolog of *GAST1* gene
Gip	=	GA-induced proteins
GmSN	=	*Glycine max* snakin
GsGASA	=	*Glycine soja* GASA
GSL	=	Gibberellin stimulated-like
MsSN	=	Medicago sativa snakin

OsGASR = *Oryza sativa* GA-stimulated transcript-related gene

ROS = Reactive oxygen species

RSI-1 = Root system inducible-1

SA = Salicilic acid

StSN = Solanum tuberosum snakin

VuSN = Vigna unguiculata snakin

ZmGSL = *Zea mays* gibberellin-stimulated like

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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CAPÍTULO 2

Artigo a ser submetido à revista: ***Peptides***
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Structural and functional analysis of Snakin/GASA members in soybean (*Glycine max*) and description of a new member (*Gm*SN2)

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Abstract

Several members of Snakin/GASA family have been described in plant species, all of them with a C-terminal conserved motif of 12 cysteines and six disulfide bonds. They are reported to be expressed in different tissues, developmental stages and under given environmental conditions - sometimes constitutively or induced by pathogens. This work focuses on the characterization of Snakin/GASA (SNK) representatives in soybean based on transcriptomic data, available at GENOSOJA (Brazilian Soybean Genome Consortium). Thirty-three non-redundant snakin sequences were identified in the soybean transcriptome of which 20 exhibited the complete GASA-domain. They share many characteristics with other (SNK) members, like isoelectric point ranging from 5.53 to 9.32, low molecular weight (6.88-18.04 kDa) and putative antimicrobial activity showed by *in silico* approaches. All sequences were addressed to the extracellular environment. A new snakin member (GmSN2) is here described for the first time, including gene isolation, genome sequencing, expression validation by RT-qPCR and analysis of the peptide structure and stability. This new SNK (GmSN2) presented an α-hairpin structure that is common in other AMPs, and it is also stabilized by disulfide bonds (six) with the pattern: Cys1-7, Cys2-5, Cys3-4, Cys6-12, Cys8-11, and Cys9-10. The validation by RT-qPCR showed that GmSN2 is constitutively expressed in leaves, maintaining the same expression level also after inoculation with the rust fungus *Phakopsora pachyrhizi*. The genome-wide analyses revealed that GmSN2 has 40 homologs distributed along 16 of 20 soybean chromosomes and also in six chromosomes of *P. vulgaris* and *M. truncatula*. This study provides an important source of information to map potential defense genes of biotechnological importance.

Keywords: antimicrobial peptides, molecular modeling, molecular dynamics, annotation.

Running title: Snakin/GASA in soybean

1. Introduction

Cysteine-rich small peptides (CRP) are effective components of eukaryotic genomes. In plants, they are constitutively expressed or induced under biotic and abiotic stresses and have emerged as an efficient alternative to mitigate the effects of stress factors [1,2]. These peptides can be classified into different families, such as thionins, defensins, cyclotides and nakins (SNKs) (Benko-Iseppon et al. 2010; Nawrot et al., 2014).

SNK proteins are members of the Snakin/GASA protein family [5] which exhibit three distinct domains: (i) a signal peptide with 18-29 residues, (ii) a variable region that is highly divergent between family members, both in amino acid composition and sequence length, and (iii) a conserved C-terminal region with approximately 60 residues, of which 12 are cysteine residues in conserved positions connected by six disulfide bonds which stabilize the mature peptide - the GASA domain [6–8]. Studies based on sequence analysis, phenotypic characterization and expression pattern have suggested that these peptides may be involved in plant growth and development, in response to abiotic or biotic stresses [9–12]. Concerning biotic stresses, a recent study (Oliveira-Lima et al. 2017) showed that Snakin-2 could cause membrane pores in fungi and bacteria, also participating in crosstalk promoted by phytohormone signaling.

SNK proteins have been identified in several plant species, including tomato (*Solanum lycopersicum*) [13], petunia (*Petunia hybrida*) [14], *Arabidopsis thaliana* [15], potato (*Solanum tuberosum*) [6,7], gerbera (*Gerbera hybrida*) [16], strawberry (*Fragaria ananassa*) [17], common bean (*Phaseolus vulgaris*) [18], rice (*Oryza sativa*) [19], beechnut (*Fagus sylvatica*) [20], maize (*Zea mays*) [21], pepper (*Capsicum annum*) [10], alfalfa (*Medicago sativa* L.) [22], rape (*Brassica napus*) [23], in wild soja bean (*Glycine soja;* Li et al. 2011) and, most recently, in soybean (*Glycine max*; He et al. 2017). The listed works report the expression of SNK coding genes under abiotic or biotic stresses.

In view of the importance of soybean and the limiting factors to the increase in its production, such as long periods of drought and susceptibility to pathogens, the GENOSOJA consortium was created aiming at the search for transcripts involved in biotic and abiotic stresses useful to increase soybean productivity (Benko-Iseppon et al. 2012). The GENOSOJA database was constructed with millions of soybean transcripts identified via RNAseq, DeepSuperSAGE, miRNA, and ESTs, under different biotic and abiotic stress

situations. Detailed information about available libraries, accessions, and experimental conditions are described in Benko-Iseppon et al. (2012) and do Nascimento et al. (2012).

The present work intends to answer the following questions: (1) How many SNKs exist in the soybean genome, what is their structure and how many are expressed? (2) Expression of these SNKs is tissue or specific stage or both, depending on the SNK analyzed? (3) Is the distribution of SNKs in the genome of soybeans and other legumes clustered or dispersed by several chromosomes? SNK expression is preferably constitutive or induced after stress? Are SNKs also induced under abiotic stress? The genomic and transcriptomic data available for soybean (with emphasis on those generated by the GENOSOJA consortium, which includes 29 libraries of different tissues and situations) allow us to shed light on these issues, helping to understand the function and range of action of SNKs.

2. Material and Methods

2.1. Screening for snakin seed sequences and soybean homologs

Complete sequences of experimentally validated SNKs (StSN1 and StSN2), previously identified in potato (*Solanum tuberosum* cv. Desireé; Segura et al. 1999; Berrocal-lobo et al. 2002) were used to retrieve homologs by BLASTp search [28] on PhytAMP (http://phytamp.pfba-lab-tun.org/about.php; Hammami et al. 2009) database. UniProt identifiers (IDs) of the retrieved homologs and both potato SNKs were used as seed sequences to retrieve orthologs with the complete GASA domain using SeedServer (http://biodados.icb.ufmg.br/seedserver/; Guedes et al., unpublished). The Batch CD-Search [30] was used in order to identify the conserved domain. Next, a tBLASTn (cut-off e-value $\leq e^{-04}$) was performed on the GENOSOJA database (http://lge.ibi.unicamp.br/soybean; do Nascimento et al. 2012) to identify soybean homologs. Redundant matches were eliminated, and the non-redundant were annotated to their putative homology performing a BLASTx against the NCBI nr database. The sequences identified were translated with ORF-Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the conserved domains were annotated via Batch CD-Search. The predicted subcellular localization, the putative antimicrobial activity, the signal peptide, the molecular weight (MW) and the isoelectric

point (pI) (for the mature peptide) were analyzed using Protcomp V.9 [31], $CAMP_{R3}$ algorithms [32] SignalP [33] and JVirgel [34], respectively.

2.2. In silicoprofiling of soybean snakins

The expression profile of putative SNKs was traced using transcripts downloaded from GENOSOJA databank (http://lge.ibi.unicamp.br/soybean; Benko-Iseppon et al. 2012; do Nascimento et al. 2012). After counting on different EST libraries, the evaluation of reads frequencies that compose each contig was submitted to the Hierarchical Clustering Method [35], which creates a matrix that compares the set of genes pair to pair to build a Neighbor-Joining tree. The dendrograms were visualized using Java TreeView [36]. Additionally, these contigs were used for anchoring the three comparisons (case x control) of the six DeepSuperSAGE libraries.

*2.3. Gm*SN2 *isolation and Neighbor-Joining analysis*

Soybean genomic DNA was extracted using CTAB method (Weising et al. 1995). The genomic sequence of *Gm*SN2 was obtained by PCR, using specific primers (F) AGCTTGTCTTTGGCACCCTA and (R) ACATTTGCACTCAGCACAGC, designed based on the genomic homology of the transcript Contig14826 soybean genomic data obtained from Phytozome v.9 [37] to amplify the complete GASA domain.

PCR amplification was performed with 10 ng DNA, 10X PCR buffer, 1,25 µL of 25 mM $MgCl_2$, 1.5 µL of 10 mM dNTPs, 1.0 µL of each primer (10 mM), 1.0 U of Taq DNA Polymerase, in a total reaction volume of 25 µL. PCR reactions were performed using the following cycling conditions: 95°C for 10 min, 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, followed by an extension step at 72°C for 10 min. The reactions were performed on a Techne TC-412 Thermocycler (Barloworld Scientific). The PCR products were separated in 1.5% (w/v) agarose gel, quantified by fluorometry (Qubit-Invitrogen) and its identity was confirmed by sequencing (from both directions: forward and reverse, in triplicate) using the BigDye Terminator v3.1 Cycle Sequence Kit (ThermoFisher) in a Genetic Analyzer 3500 (Applied Biosystems) at the Sequencing Platform LABBE (Universidade Federal de Pernambuco, Recife, Brazil).

The *Gm*SN2 sequences (in triplicate) were assembled using BioEdit, v.7 (Hall, 1999) and the contig was annotated using BLASTx against the NCBI nr database and translated

using ORF-Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The conserved domain, signal peptide, pI, and MW were predicted using CD-Search [30], SignalP v. 4.1 [33] and JVirgel [34], respectively. SNK protein sequences described in previous works, the trancripts retrieved from GENOSOJA databank and the *Gm*SN2 sequence was aligned using ClustalW [38] of the MEGA6 package [39], followed by a Neighbor-Joining tree construction with bootstrap (1,000 replicates) and Dayhoff model [40].

2.4. Genomic distribution and gene structure of snakin homologs

The peptide sequence from *Gm*SN2 was aligned against soybean, *Medicago truncatula* and *Phaseolus vulgaris* genomes at Phytozome v.10.3 [37] and NCBI using tBLASTn. Alignment matches that shared the GASA domain were evaluated to identify their relative position in the respective virtual chromosomes, the gene structure, as well as their abundance. The graphic representation of the Snakin candidates in the virtual chromosomes was generated with Circos software package [41].

2.5. Molecular modeling

In order to select the best template to perform the comparative modeling for GmSN2 and the soybean transcripts, a BLASTp was performed against the Protein Data Bank [42] using the respective sequences as seeds. Using MODELLER 9.15 [43] one hundred of molecular models were constructed with snakin-1 structure as template (PDB ID: 5E5Q), whose final model was selected according to the discrete optimized protein energy (DOPE) score (a measure which evaluates the energy of the model and indicates the best one). Additionally, the final models were evaluated through ProSA II [44] which accesses to the folding quality of the protein and evaluated by ProFunc [45] to check the stereochemical quality of the model, using Ramachandran plot. Reliable models are expected to have more than 90% of the amino acid residues in the most favored and allowed regions. Structure visualization was carried out using PyMol v.1.6 (The PyMol Molecular Graphics System, Schrödinger, LLC).

2.6. Validation of GmSN2 gene expression by RT-qPCR

Soybean plants inoculated with the fungus *Phakopsora pachyrhizi* were used for expression analysis of *Gm*SN2 gene. Details on the soybean accession studied as well as growing conditions are available in Kulcheski et al. 2010. Three biological replicates were evaluated for controls and treatments. Leaf tissues from each plant were collected at 1, 12, 24, 48, 96 and 192 hai (hours after inoculation), frozen in liquid nitrogen and used for RNA extraction. Samples from 48 hai (hours after inoculation), including mock treatments, were chosen for expression analysis of *Gm*SN2 gene. Total RNA of leaf tissue was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The first-strand cDNA synthesis reaction was done with 1 µg of total RNA (DNA-free) using the ImPro-II Reverse Transcription System (Promega) and stored at -20°C until expression analysis by RT-qPCR.

The expression profile of *GmSN2* was analyzed by RT-qPCR using soybean cDNA samples (48 dai, including inoculated and mock treatments, with biological triplicates). A set of seven genes (previously identified as potential reference genes) were included in this approach (Supplementary Table S1). Firstly, the amplification efficiency (E) of each primer was calculated according to the equation: $E\ (\%) = (10^{-1/slope} - 1) \times 100$ (Rasmussen, 2001), using five serially diluted cDNA (concentrated, 1:10, 1:100, 1:1000 and 1:10,000).

All RT-qPCR amplifications were performed on the LineGene 9660 model (Bioer), using SYBR Green detection chemistry (USB). Each reaction mixture comprised 1 µL of template cDNA, 5 µL of HotStart-IT SYBR Green qPCR Master Mix 2x (USB), 0.05 µL of ROX, 1.95 µL of water, and 1 µL primer (500 nM each) to a final volume of 10 µL. The reactions were denatured at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 15 s in 96-well reaction plates, with the detection of the fluorescence signal at the end of each extension step. After amplification, dissociation curves were produced (60°C to 95°C at a heating rate of 0.1°C/sec and acquiring fluorescence data every 0.3°C) to discriminate the main reaction products from other nonspecific ones or primer-dimers. Each PCR always included an NTC (no template control) and reverse transcription negative controls.

The relative expression analysis was performed using the REST 2009 software package [47]. The expression stability of the reference genes was evaluated using the geNorm v. 3.5 software [48] which is based on the assumption that expression of two ideal reference genes will always have the same ratio among samples regardless of the experimental conditions before the qPCR.

3. Results

3.1. Snakin seed-sequences and homologs in soybean

Using the potato SN1 (ID: Q948Z4.1) and SN2 (ID:Q93X17.1) as seeds sequences on PhytAMP database, the BLASTp recovered sequences from four plant families as best matches: Orchidaceae (*Gymnadenia conopsea*, ID: A3F8U7), Brassicaceae (*Arabidopsis thaliana*, ID: Q1G2Y4), Rosaceae (*Fragaria ananassa*, ID: O49134) and Asteraceae (*Gerbera hybrida*, ID: Q9XGJ3). Afterward, these UniProt IDs were used as input in the SeedServer. This analysis retrieved 52 SNK candidate sequences, 47 of which showed complete GASA domain, distributed on 10 plant families: Brassicaceae (11 genes); Fabaceae (7 genes); Solanaceae (6 genes); Selaginellaceae (5 genes), Pinaceae and Poaceae (both 4 genes); Vitaceae, Salicaceae and Euphorbiaceae (both 3 genes); Orchidaceae (1 gene) (Supplementary Fig.1)**.**

The tBLASTn performed on GENOSOJA database (using the 47 SNK candidates as queries) recovered 33 non-redundant sequences within the established parameters (cut-off e^{-4}) (data not shown), of which, 20 sequences exhibited the complete GASA-domain. A subsequent BLASTx search on nr NCBI revealed that 14 sequences described as "unknown" were all from *G. max*. Sequence lengths ranged from 481 to 965 bp and from 65 to 138 amino acid residues (**Table 1**).

The virtual 2D-electrophoresis evaluation revealed signal peptides with isoelectric point (pI) varying between 4.30 (Contig15060) and 9.58 (Contig7785), while for the mature peptides, pI values ranged from 5.53 (Contig14720) to 9.32 (Contig9321) (**Table 1**). Regarding the molecular weight (MW), there was a discrete variation between the values in the signal peptide (from 2.37 to 2.99 kDa in the contigs Contig22342 and SJ01-E1-C06-026-A02-UC.F, respectively). For the mature peptides, this variation was between 6.88 (Contig15060) and 18.04 (Contig9293) kDa. For contig14826, only the MW value for the mature peptide is presented, due to the absence of the signal peptide (**Table 1**).

The subcellular localization analysis showed that all sequences possessed the signal peptide addressing to the extracellular environment, a result supported by optimal scores (between 8.9 and 9.6; data not shown), regarding the antimicrobial activity prediction all candidates were classified as AMPs.

Table 1 - Soybean clusters identified through tBLASTn tool in the GENOSOJA database. A search conducted with seed sequences (*e-value* ≤ e^{-4}) reported by the SeedServer (only those with complete GASA-domain) and annotated via BLASTx tool (GenBank).

Reference ID	Cluster	nr NCBI BLASTx best matches		Size		MW	pI
		Accession	Description	nt	aa		
GmSN-like1	**Contig1367**	ACU15001.1	Unknown	749	99	8.07	8.53
GmSN-like2	**Contig1373**	XP_003549639.1	Snakin-2-like	965	115	10.36	7.93
GmSN-like3	**Contig14720**	XP_003528161.1	Unc Prot	804	119	10.77	5.53
GmSN-like4	**Contig14826**	XP_003528161.1	Unc Prot	802	65	6.92	8.39
GmSN-like5	**Contig15060**	XP_003525918.1	Snakin-1	696	88	6.88	8.10
GmSN-like6	**Contig16419**	ACU14488.1	Unknown	608	106	9.00	8.50
GmSN-like7	**Contig18282**	ACU14567.1	Unknown	792	107	9.33	8.75
GmSN-like8	**Contig2059**	ACU14224.1	Unknown	481	106	9.11	7.72
GmSN-like9	**Contig20912**	ACU15584.1	Unknown	809	138	12.33	8.78
GmSN-like10	**Contig22342**	ACU14458.1	Unknown	637	115	10.31	7.93
GmSN-like11	**Contig23713**	ACU16624.1	Unknown	619	90	7.18	8.37
GmSN-like12	**Contig26289**	XP_003536595.1	Snakin-1-like	554	90	7.24	8.50
GmSN-like13	**Contig3375**	ACU13692.1	Unknown	735	99	8.10	8.80
GmSN-like14	**Contig3731**	ACU14995.1	Unknown	791	88	6.92	9.28
GmSN-like15	**Contig627**	ACU14995.1	Unknown	783	88	6.87	9.09
GmSN-like16	**Contig7139**	XP_003523021.1	GRP	798	106	9.01	8.90
GmSN-like17	**Contig7785**	ACU13226.1	Unknown	847	117	10.59	6.89
GmSN-like18	**Contig9293**	ACU16056.1	Unknown	935	191	18.04	9.29
GmSN-like19	**Contig9321**	ACU13162.1	Unknown	701	110	9.38	9.32
GmSN-like20	**SJ01-E1-C06-026-A02-UC.F**	ACU15001.1	Unknown	542	94	7.46	8.66

UncProt: Uncharacterized protein; MW: molecular weight; pI: isoelectric point (pI).

3.2. Analyses of snakin based on ESTs and DeepSuperSAGE data

Hierarchical clustering of the EST libraries was carried out, including 29 soybean libraries from different tissues and developmental stages. Among the organs and tissues evaluated, the most representative number of libraries regarded seed tissues (six libraries, with 1,988 reads, representing 82% of total 2,415). The remaining reads were present in tissues as leaves (129 reads), hypocotyls (61 reads), flowers (50 reads), roots (31 reads) and epicotyls (8 reads). Moreover, read frequency obtained from normalized data revealed the prevalence of transcripts in the early stages of development (**Figure 1**).

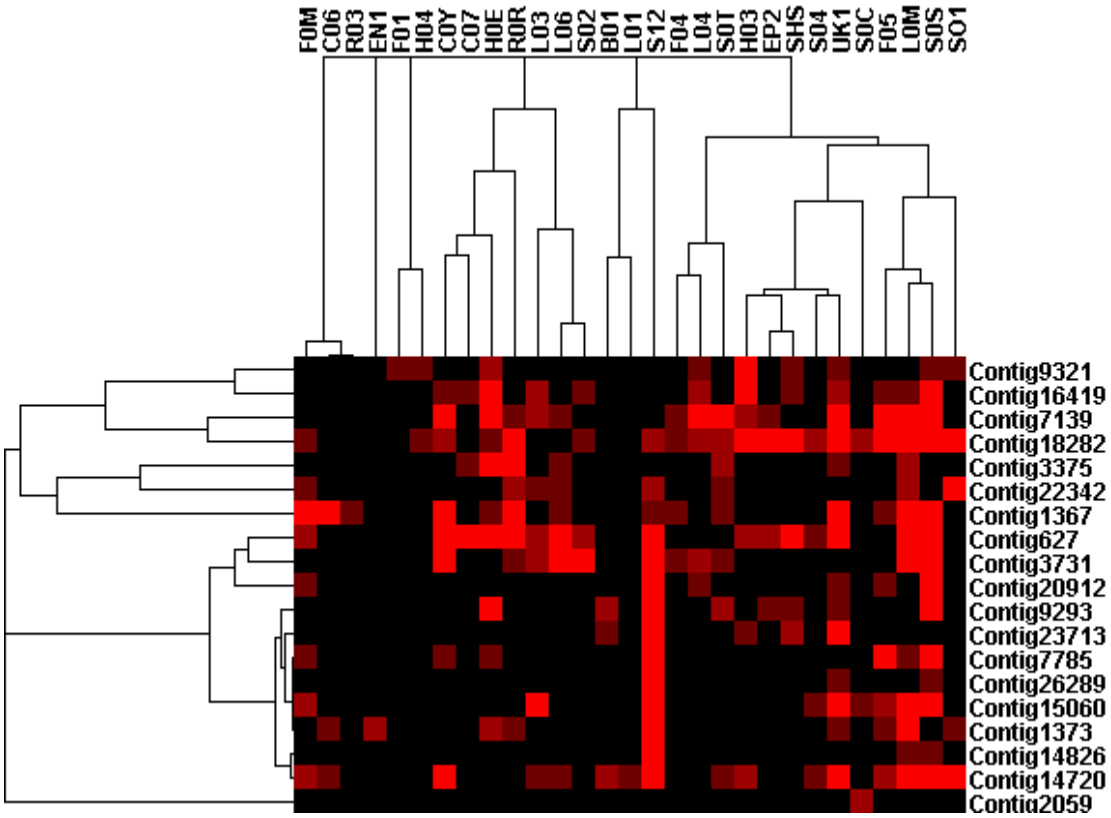

Figure 1 - Hierarchical clustering of transcriptional profile in soybean performed using the CLUSTER software. The red color indicates higher prevalence; the dark red indicates intermediate prevalence and black means absence of SNKs in the respective treatment. Libraries: B01 (vegetable buds; field-plants); C0Y (young cotyledons); C06 (wounded cotyledons); C07 (degenerating cotyledons; 10 days old, etiolated seedlings); EN1 (endosperm - developing seeds); EP2 (seedling epicotyl); F01 (floral meristem); F0M (mature flowers); F04 (floral meristem mRNA); F05 (immature flowers; field-plants); H03 (hypocotyl/plumule; germinating seeds); H04 (*P. sojae;* infected hypocotyl); H0E (etiolated hypocotyls); L01 (senescing leaf; greenhouse-plants); L03 (fully expanded leaves; greenhouse-plants); L04 (immature leaves; greenhouse-plants); L0M (mature leaves); L06 (leave; drought stressed); R0R (roots); R03 (seedling roots); S02 (mature seed pods; greenhouse-plants); S04 (young seeds); S0C (seed coats); S0S (seedlings); S12 (seeds; globular stage embryos); SHS (shoots); SO1 (somatic embryos; cultured on MSD 20); S0T (stem) and UK1 (unknown tissue).

The analysis of the DeepSuperSAGE data allowed the identification of 81 tags (out of 4,000,000 in the database), of which, 20 regarded the root dehydration comparison in the drought-tolerant contrast (DT *vs.* TC; **Figure 2A**), 16 were associated with the same

treatment in the drought-sensitive contrast (DS *vs*. SC; **Figure 2B**), whereas 45 tags occurred in the leave tissue contrast (RR *vs*. RC; i.e., rust-resistant *vs*. non-inoculated control; **Figure 2C**). Regarding exclusive tags in each stress condition, 22 tags were exclusive in the comparisons regarding root tissues (comparisons 12 TD *vs*. TC; 9 SD *vs*. SC; and one tag in both), while 34 tags were exclusive to the leave tissue contrasts (RR *vs*. RC). Among 81 tags obtained, 31 (38 %) showed up/down-regulation (UR/DR, respectively): 10 tags in the contrast TD *vs*. TC (1 UR and 9 DR); 1 DR tag in the contrast SD *vs*. SC and 20 tags (6 UR and 14 DR) in the contrast RR *vs*. RC.

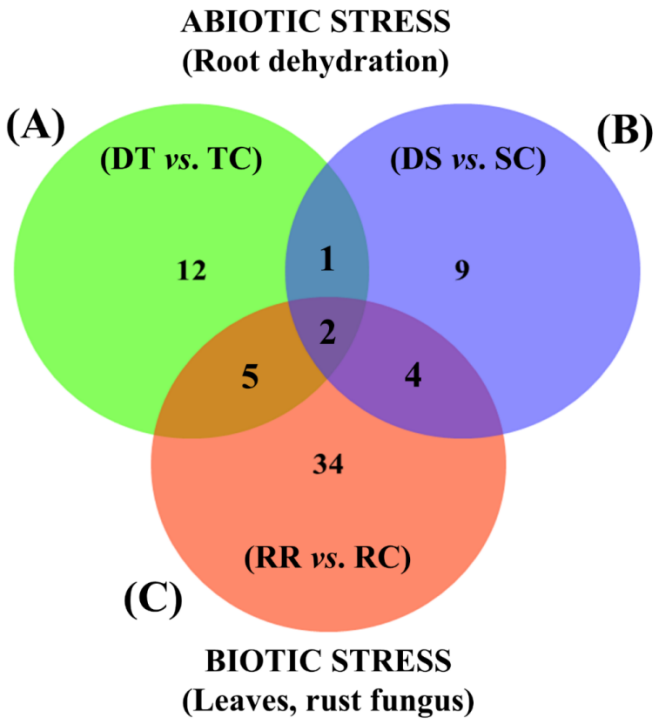

Figure 2 - Venn diagram showing the number of DeepSuperSAGE UR tags matching soybean SNK exclusive in each contrast. (**A**) DT *vs*. TC, roots of drought-tolerant accession (Embrapa 48); stressed *vs*. non-stressed control; (**B**) DS *vs*. SC, roots of drought-susceptible accession (BR16); stressed *vs*. non-stressed control and (**C**) RR *vs*. RC, leaves of rust-resistant accession (PI561356); inoculated *vs*. non-inoculated - Mock.

*3.3. Analysis of the snakin Gm*SN2

The *Gm*SN2 genomic sequence contains an ORF (215 bp), which encodes a peptide with a variable region (1-11 residues), followed by a 60-residue sequence corresponding to

the conserved GASA domain (**Figure 3**). This mature peptide is basic (pI = 8.52) with a predicted molecular mass of 6.32 kDa.

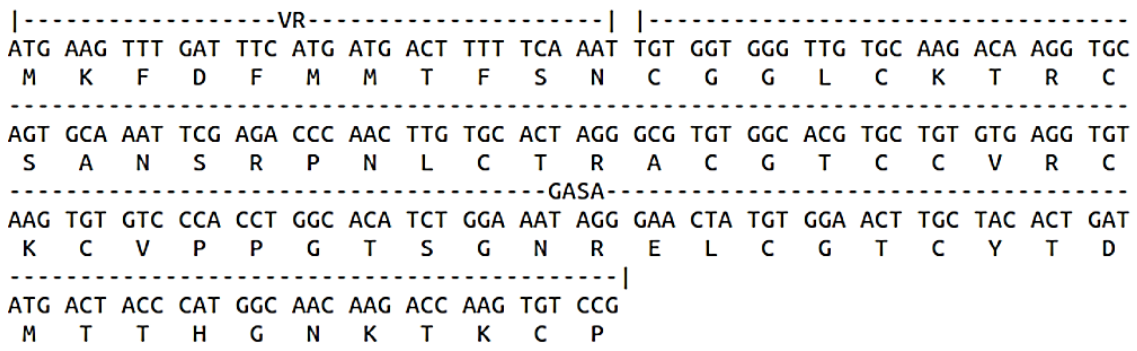

Figure 3. *Gm*SN2 gene sequence and the putative amino acid sequence. VR: Variable region; GASA: GASA domain.

3.4. Neighbor-Joining analysis

The alignment showed that GmSN2 shares a similarity of 75% in the amino acid sequence with StSN2 (snakin-2 of *S. tuberosum*). Additionally, 21 high conserved residues were observed, containing 12 cysteine motifs and other eight residues (R, V, P, G, G, Y, K, P), performing the general motif: CX3CX2RCX8CX3CX2CCX2CXCVPXGX2GX4CXCYX10/13KCP. This pattern was observed in all sequences (**Figure 4**) and regards the typical conserved structure of the Snakin/GASA family.

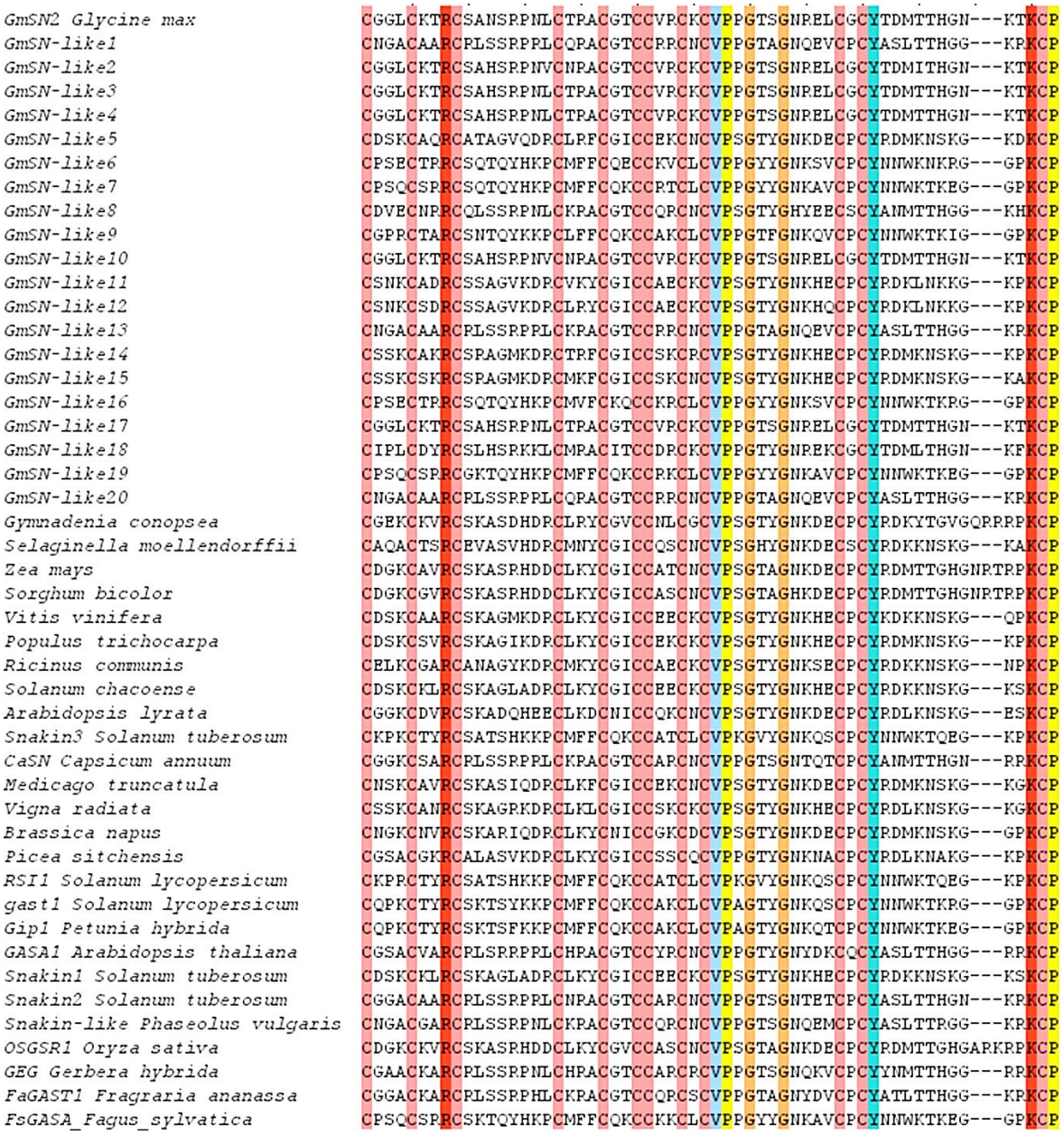

Figure 4 - Multiple sequence alignment performed with peptide sequences of Snakin/GASA family members, the *Gm*SN2 and the other soybean peptides (GmSN-like) identified showing the main conserved residues represented by the 12 cysteine motif (blue) and other eight residues, Arginine (R) – yellow; Lysine (K): pink; Valine (V): light green; Proline (P): red; Glycine (G): green and Tyrosine (Y): grey, that compose the general motif "CX3CX2RCX8CX3CX2CCX2CXCVPXGX2GX4CXCYX10/13KCP".

The Neighbor-Joining tree showed that Snakin/GASA family can be divided into three subfamilies (I-III). The subfamily I comprises the GmSN-like5, 11, 12, 14 and 15 with 15 other homologues, the general pattern of the group is: CX2A/KCX2RCX2AX3D/EXCX3CG/NI/VCCX2CXCVPP/SGH/TA/YGH/NKX2CP/SCYR/KDX2N/TX2GX/5KCP, where X is any of the 20 proteinogenic amino acids, the subfamily II comprises the GmSN2 and 10 other soybean transcripts (GmSN-like1-4, 6, 16-20) and the general pattern of the subfamily is: CX3CX2RCX8CX3CX2CCX2CXCVPP/SGT/YXGX4CXCYX4T/NX5KCP, finally the subfamily III which is the smallest group with 10 sequences with the transcripts GmSN-like6, 7, 9, 16 and 19 with the general pattern: CX3CT/SXRCSXTS/QXK/HKPCM/LF/VFCQ/KXCCX2CLCVPXGXF/YGNKX2CPCYNNWKN/TK/QXGK/GPKCP (**Figure 5).**

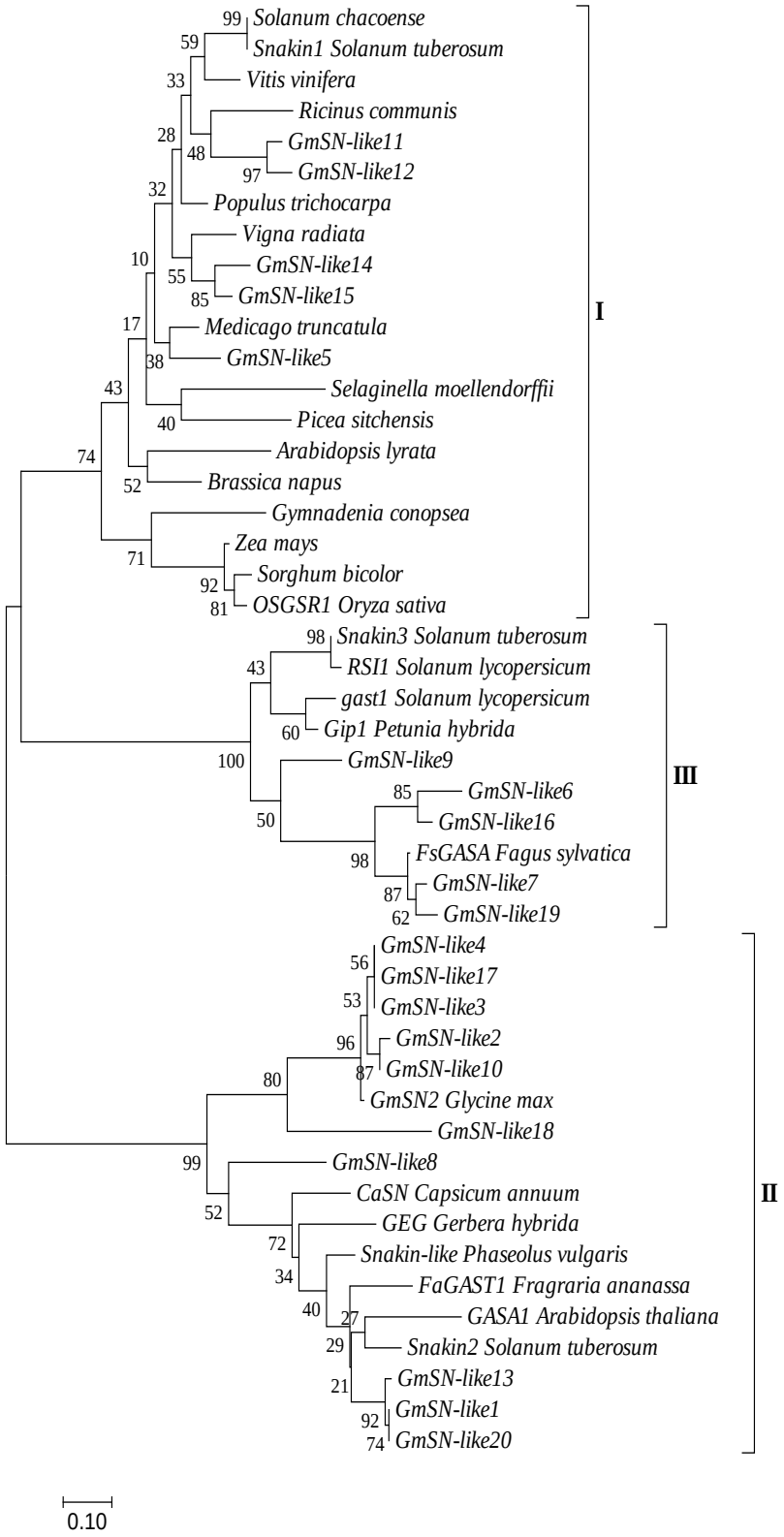

Figure 5 - Phenetic analysis of Snakin/GASA proteins including *Gm*SN2 and the GmSN-like peptides. The branches are supported by the bootstrap values showed in percent (%). The three subfamilies are evidenced by the bracket, at the right side. The scale length (0.1) indicates distance.

3.5. Genomic distribution and gene structure of snakin candidates

A tBLASTn search was carried out to investigate the distribution and abundance of *Gm*SN2 homologous in soybean virtual chromosomes and their homologs in other legumes. The chromosomal localization was plotted on a circular genome map with their predict gene structure which it was shown that possesses two, three or four exons (**Figure 6**).

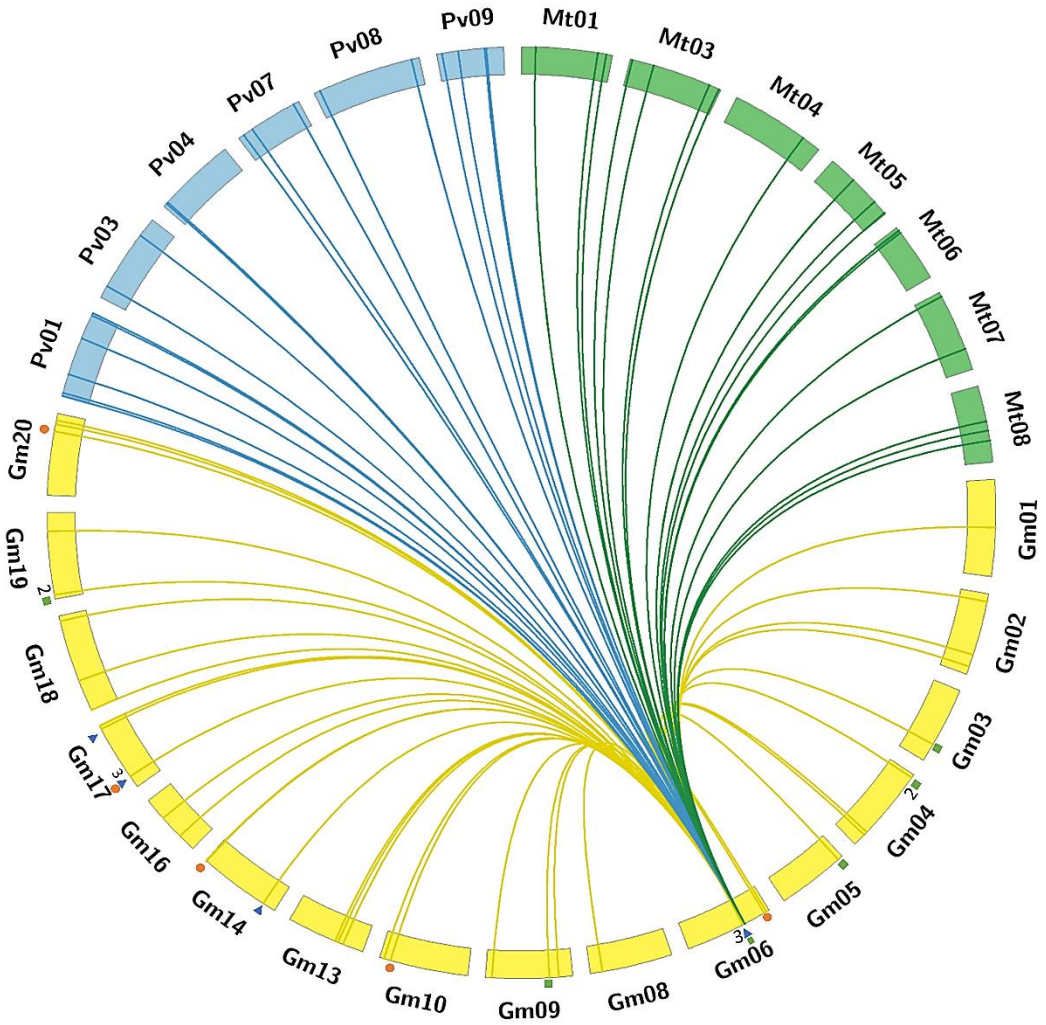

Figure 6 - Genomic distribution of *Gm*SN2 homologs in soybean (GM), *M. truncatula*, (Mt) and *P. vulgaris* (Pv). The orange circles, blue triangles and green squares indicate the soybean transcripts mapped and their gene structures with two, three and four exons respectively.

The anchoring based on identity and e-value showed that *GmSN2* has three exons and it is in the chromosome 6 with three links to mapped transcripts. Some transcripts may have the same position in the chromosomes due to alternative splicing variants. The

chromosomes Gm06 and Gm17 were the most representatives for the mapped transcripts with five links each, in the Gm06 all the gene structures were found, in total were mapped five genes with two exons and eight genes with three and four exons each (Supplementary Table S2). Counting all the links 41 homologues sequences had a heterogeneous distribution among 16 of the 20 chromosomes of soybean haploid set, 28 homologs in six of the 11 chromosomes in *P. vulgaris* and 26 distributed in seven of eight of the *M. truncatula* haploid chromosome set. In general, the distribution was diversified to all compared organisms with few clusters in soybean (e.g., Gm13 and Gm20), *P. vulgaris* (Pv01) and *M. truncatula* (Mt06).

3.6. Molecular Modelling

For molecular modeling, the snakin-1 (PDB ID: 5E5Q) was chosen as template. It shares 47% of identity with GmSN2 and from 50-74 % with the soybean transcripts (Supplementary Table S3). The three-dimensional modeling of GmSN2 showed an overall structure composed by two α-helix (residues ^{2}Gly to Ser^{10} and from ^{15}Pro to Cys^{29}), like an α-hairpin, a 3_{10} helix ^{40}REL^{42} and another α-helix in the C-terminal region composed by residues ^{43}GTCYTD^{48}. The disulfide bridges (**Figure 7A**, in yellow) showed a general pattern as follows: Cys1-7, Cys2-5, Cys3-4, Cys6-12, Cys8-11, and Cys9-10. In the Ramachandran plot for GmSN2, 91.5 % of the residues occurred in favored regions, 6.4 % in the additional allowed regions and 2.1 % in the generously allowed regions for GmSN2 (**Figure 7B**), the Z-score on ProSA (-3.79) indicated a valid model (**Figure 7C**). The variation of the Ramachandran plot to the different regions was from 91.3-96 % to the favored regions, 4-8.7 % to additional allowed regions and 0-2.1 % to generously allowed regions. Only the structure Contig3731 (GmSN2) had one amino acid in a disallowed region and the Z-Score varied from -5.64 to -3.72 (Supplementary Table S2).

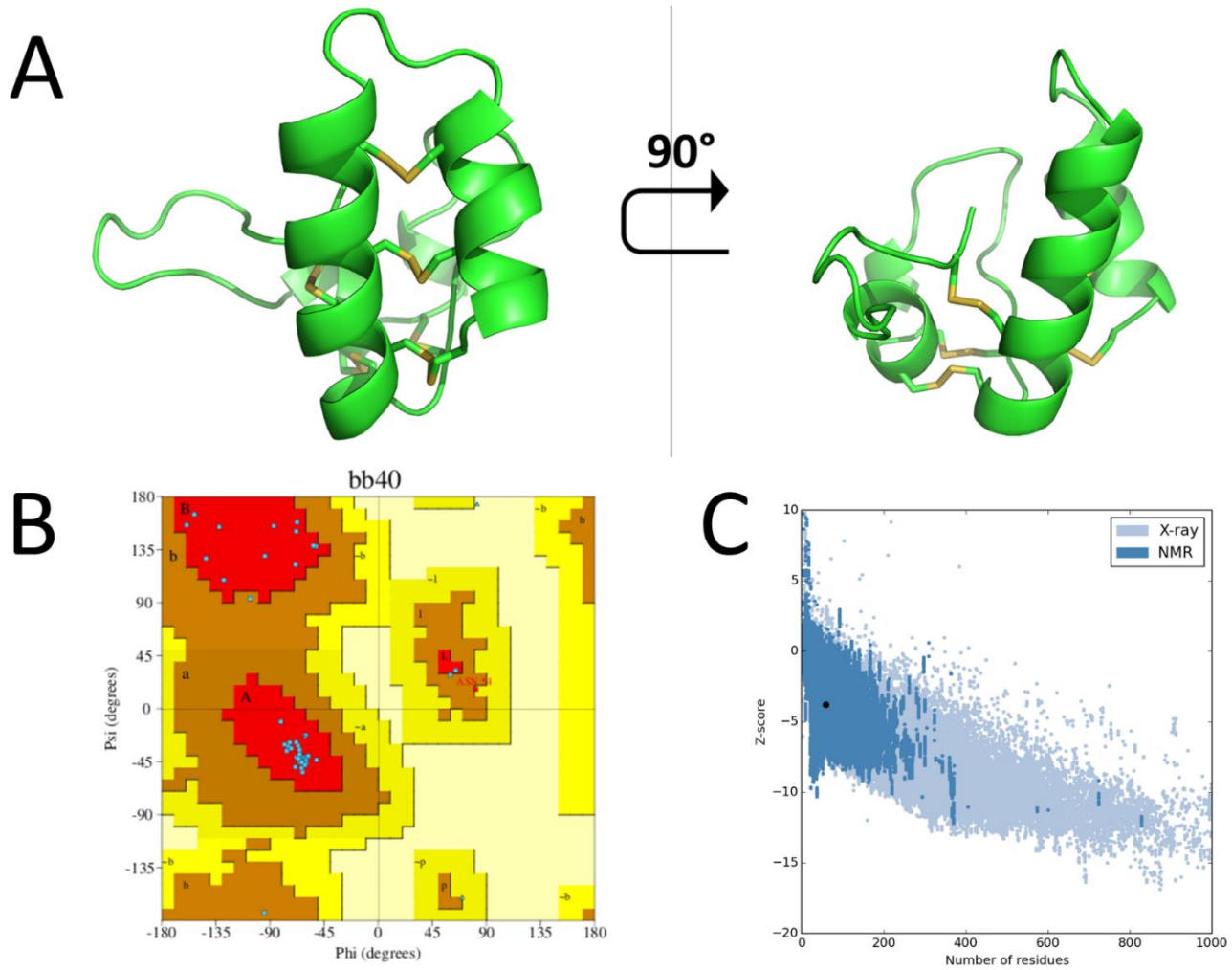

Figure 7 – GmSN2 structure showing the disulfide bonds (in yellow) (A). (B) Ramachandran plot where the blue dots are the aminoacids, the red regions are the most favored regions, in brown the additional allowed regions and in yellow the generously allowed regions and (C) Quality of folding showed by the Z-Score where the black spot is the GmSN2 model.

3.7. Expression analysis of GmSN2 in response to P. pachyrhizi

The RT-qPCR method was employed to analyze the expression profile changes of *Gm*SN2 in response to biotic stress (soybean, 48 h after inoculation with *P. pachyrhizi*). The primer pairs of the target and reference genes tested by melting curve and agarose gel electrophoresis revealed product specificity (data not shown). All primers (target and reference genes) showed amplification efficiency (E) higher than 90%, ranging from 90.25 to 96.06% for CYP2, CHLP, CWC15 and *Gm*SN2; and from 100.08 to 107.23% for ACT, TUA, ZMET, and 26P. The detection limit of the analyzed primer set (represented by the *y-intercept value*) ranged from 33.17 (CWC15) to 38.01 (CHLP) for the reference genes and 36.09 for *Gm*SN2 gene. The linear regression (R^2) analysis, in turn, revealed value ≥ 0.990, for all genes (see Supplementary Table S1).

In order to choose the best reference genes, geNorm software [48] was used. The most stable genes were CYP2 and 26P. The M value obtained for these two genes was 1.307 and 1.431, respectively (Supplementary Fig. 2A). Similarly, the pairwise variation ($V_{2/3}$) was 0.103 (Supplementary Fig. 2B)**,** that is, below the cut-off (0.15), suggesting the combination of CYP2 and 26P was adequate to normalize gene expression. However, in accordance with Vandesompele et al. (2002), we consider three reference genes as a minimal number for expression analysis. So, a third gene (ZMT) was used in order to normalize the relative expression of *Gm*SN2 **(**Supplementary Fig. 2).

After confirmation of the genomic localization and molecular characterization, the *Gm*SN2 gene was analyzed by RT-qPCR to explore the expression pattern of this antimicrobial peptide in soybean leaves (PI561356 accession) 48 hai (hours after inoculation) with *P. pachyrhizi*, in comparison with those in the mock-inoculated plants (control). The real-time PCR analysis revealed no change in the expression of *Gm*SN2 expression in the soybean leaves in response to biotic stimulus caused by the *P. pachyrhizi* (Supplementary Fig. 3)**.**

4. Discussion

The use of six Uniprot SNK homologs (from *S. tuberosum*, *A. thaliana*, *Ge. hybrida*, *Gy. conopsea* and *F. ananassa*) as seeds in the SeedServer allowed the identification of SNK candidates in different taxa, most of which (90%) with the expected structural features (Snakin/GASA domain). The search for homologs in the SeedServer

also permitted inferences about the possible origin of the SNK family in the Lycopodiophyta clade (Selaginellaceae), where the most basal SNK sequence was found (Supplementary Fig.1). It is likely that this family of antimicrobial peptides underwent events of duplication during evolution, being subsequently settled in upper clades, as it has been observed for other peptides [49,50], although no functional annotation to this gene was available in the searched databases.

The seeds allowed the identification of 20 putative SNKs in the soybean transcriptome, considering the adopted cut-off e^{-4}, plus the GmSN2 isolated. All sequences presented the complete GASA domain, shared by all SNK members, characterized by a cysteine-rich signature (C-terminal, 12 cysteine residues) (Figure 4), in highly conserved positions, which is essential for their biochemical activity, being also responsible for their protein structure [15,51–53], in addition 41 putative snakins were mapped in the genome, while *M. truncatula* and *P. vulgaris* showed 26 and 28 genes mapped respectively, experimental data of Nahirñaki et al 2016 showed 16 snakin members in potato, this data confirms that soybean and the other legumes are important sources to study this gene, being soybean, so far, the organism that has more predict snakins mapped in the genome than any other.

…

Regarding the subcellular localization, our data showed that all putative soybean SNKs were addressed to the extracellular environment, a role similar to *GASA5*, that acts as a regulator of flowering time and stem growth [54,55]. Moreover, it is known that SNK genes are also involved in other signaling pathways, i.e., hormonal [9] and stress response, as already reported for other AMPs [56]. Considering this evidence, a putative divergence of roles may be recognized in these peptides, depending on plant physiological state and/or stress condition imposed. However, all the peptides retrieved reported putative antimicrobial activity as it was already reported for other SNKs. Thus, the soybean putative SNK member emerged as an important candidate to study the antimicrobial activity of these peptides like He et al. 2017 showed to the GmSN1.

Although a high similarity among these peptides with “unknown” and “uncharacterized” sequences from soybean has been recognized, they exhibited conservation in structure, size, molecular weight, and pI very close to those found in other members of the family [10,57]. Besides the most conserved region described for these peptides, the C-terminal domain (GASA) [58] demonstrates strict conservation, which

can be a strong evidence of functional specialization, also highlighting their importance to the maintenance of the plant homeostasis.

Soybean SNK candidates were found in all the reads libraries, prevailing in tissues at early developmental stages (leaves, roots, seeds, and cotyledons) counting 29 different situations in total, this is the most complete *in silico* profile for this gene so far, where the cellular activity and the action of effectors that regulate the cell division (as gibberellin) are stronger. This pattern was also observed for other SNK members [59]. This fact may indicate that soybean SNK in some cases presented here follows the same model, as it was observed for homologs involved in shoot elongation [13], corolla and carpel [16], as well as in seed and root development [21,60]. Besides, it should be highlighted that these tissues are also prime targets of many pathogens and nematodes [7,10]. Therefore, the expression of antimicrobial peptides, like SNKs, have been associated as part of the permanent and inducible defense against microbial assault [61–63], in addition we also found that they may be involved in responses related to wounded tissues, from early from to late stages.

The analysis of DeepSuperSAGE libraries revealed a significant number of tags (81) with homology to SNKs. This may indicate the critical role of SNK in response to both biotic and abiotic stresses in soybean. As expected, most DeepSuperSAGE unitags (45 unitags) were identified in the biotic stress condition (after rust fungus inoculation). It is known that rust fungus often infects leaves, a tissue which has a ubiquitous expression of SNKs, both constitutive and induced (Balaji and Smart 2012; Nahirñak et al. 2012). However, to our knowledge, there are no reports of SNK transcripts associated with this pathogen. In concordance with the constitutive expression observed of *St*SN1 [6,64] in potato, as well as, *Ms*SN1 in alfalfa (Garcia et al, 2014), the expression pattern of *Gm*SN2, obtained by RT-qPCR, shows to be a component of the constitutive defense barrier like Snakin-1 from *S. tuberosum* [6].

Regarding libraries submitted to abiotic stress (root dehydration), the considerable number of tags may indicate constitutive expression and/or crosstalk responses, since water deficit causes osmotic and oxidative disturbs, triggering responses via reactive oxygen species (ROS), reflecting in the induced expression of SNKs, as reported for *A. thaliana*, where the GASA14 modulates the accumulation of ROS. It was also known that members of this family, such as StSN2, GIP4, and GIP5, respond to effectors responsive to this stress as the hormone ABA [9]. Nevertheless, most of these transcripts are downregulated, indicating that the factors involved in growth tend to be

suppressed in this situation [65]. In soybean the snakin play roles in many different situations, the reads counting data showed a diverse pattern of expression for stages of development, treatments and tissues, however the DeepSuperSAGE evidenced that they are related more to biotic stresses by fungus even, they are related as well to abiotic stresses.

The isolated *Gm*SN2 exhibits a structure like other typical SNK members: the cysteine motifs, molecular weight, isoelectric point, subcellular localization and the structure of the peptide. Thus, it is plausible to affirm that it is a new cysteine-rich antimicrobial peptide of *G. max*. Based on the amino acid conservation, the phenetic tree confirmed the division of SNK family into three subfamilies, as proposed by Berrocal-lobo et al. (2002). According to this classification, *Gm*SN2 belongs to subfamily II, which includes GEG (*G. hybrida*), FaGAST1 (*F. ananassa*), GASA1 (*A. thaliana*) and Snakin-2 (*S. tuberosum*). This C-terminal conservation (especially for the cysteine residues) might be required not only for the maintenance of the 3D structure, as it was shown in our data, but also to the interaction with other proteins [66]

In comparison to the genome-wide analysis performed in potato (where 16 SNK family members were identified in nine of the 12 chromosomes by Nahirñak et al. (2016) or rice (which has nine homologs distributed over six of the 12 chromosomes according to Zimmermann et al., 2010), our study identified 40 SNK homologs to *Gm*SN2 in the genome using *in silico* approaches, 26 in *M. truncatula* and 28 in *P. vulgaris* the pattern of distribution is very distributed and there is no clear relation between the distribution of the subfamilies or the gene structure once one chromosome like Gm06 has all the structures found for this gene (two, three or four exons) and has snakin genes form different subfamiles. The higher amount of genes can be justified by the paleotetraploid condition of the soybean genome (2n = 40 chromosomes), also identified previously for other gene families [68,69].

Nevertheless, the genomic distribution of the SNK members follows a similar pattern among different organisms, being present in an isolated form or with few neighboring SNK members, presenting, therefore, a distinct distribution as compared to other AMPs that are usually present in gene clusters [70]. However, the positions in the chromosomes may suggest that they are conserved among syntenic blocks in the compared genomes, and since the positions of the links are similar among them it is likely to conjecture that duplication and genomic rearrangement events lead the distribution of this family, similar events are known in the R genes [69,71,72].

The identified soybean candidates exhibited three gene structures related to the SNK family: two, three and four exons [67], regarding probably neofunctionalized candidates (paralogs). This structural variation reflects their functional scope, and this is shown by the broad spectrum of activities like development, biotic and abiotic stress [62,73]. Thus, soybean arises as a promising organism to study these genes, since its genome comprises significant structural and functional variations.

The overall structure of *Gm*SN2 and the other putative soybean peptides show similarities with plant thionins and α-helical hairpin [74,75], peptides that share a hairpin stabilized by disulfide bridges. This reinforces the Silverstein's hypothesis which proposes that some cysteine-rich AMPs have a common ancestor, based on structural features, especially the cysteine pattern and connectivity [2].

5. Conclusions

The results from this investigation were able to indicate 20 transcripts retrieved from Genosoja and also 40 links to snakin in the soybean genome, this amount indicates the highest number of putative genes from this family tagged in a single organism, in addition we demonstrated that their distribution is heterogeneous not following the pattern for defense genes, gene structure that has two, three or four exons or subfamily divisions that are divided by different sequence motifs. The snakin/GASA peptides in *G. max* are related to biotic and abiotic stresses, being induced or constitutively expressed, moreover they are also related to the basal metabolism in both early and late stages of the plant

The isolated GmSN2 is a new member of this subfamily in soybean, this gene is constitutively expressed against the phytopathogen *P. pachyrhizi*. The gene was mapped in the chromosome 6 and has three exons in its genic structure, in addition we mapped potential paralogues in *M. truncatula* and *P. vulgaris.* The peptide structure is composed by an alpha-hairpin and two small helixes all stabilized by six disulfide bridges, this may corroborate the hypothesis of common ancestrality among the cysteine rich AMPs.

The SNK candidates retrieved from GENOSOJA database are related to biotic and abiotic stresses, but also to the developmental stages, furthermore, the comparative distribution of the homologs in soybean, *M. truncatula,* and *P. vulgaris* evidenced that even for a defense related gene they are not distributed like them. Regarding the new

GmSN2, our study provided significant elucidations from the structural point of view to the functional, since it possesses structural and potential functional aspects that mayshares structural and potential functional aspects with other AMPs. This study is an important source to map potential defense genes in soybean with biotechnological importance.

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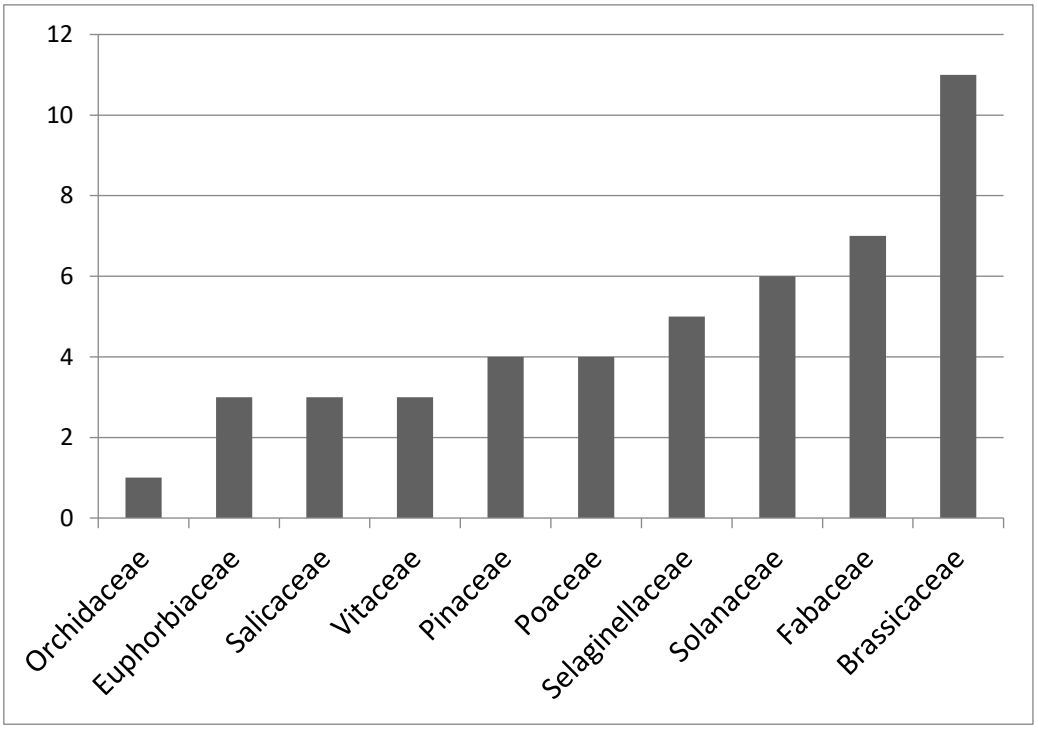
SUPPLEMENTARY MATERIAL

Table S1. Primer sequences of the seven candidate reference genes and of the gene of interest (*Gm*SN2) used in the RT- qPCR analysis

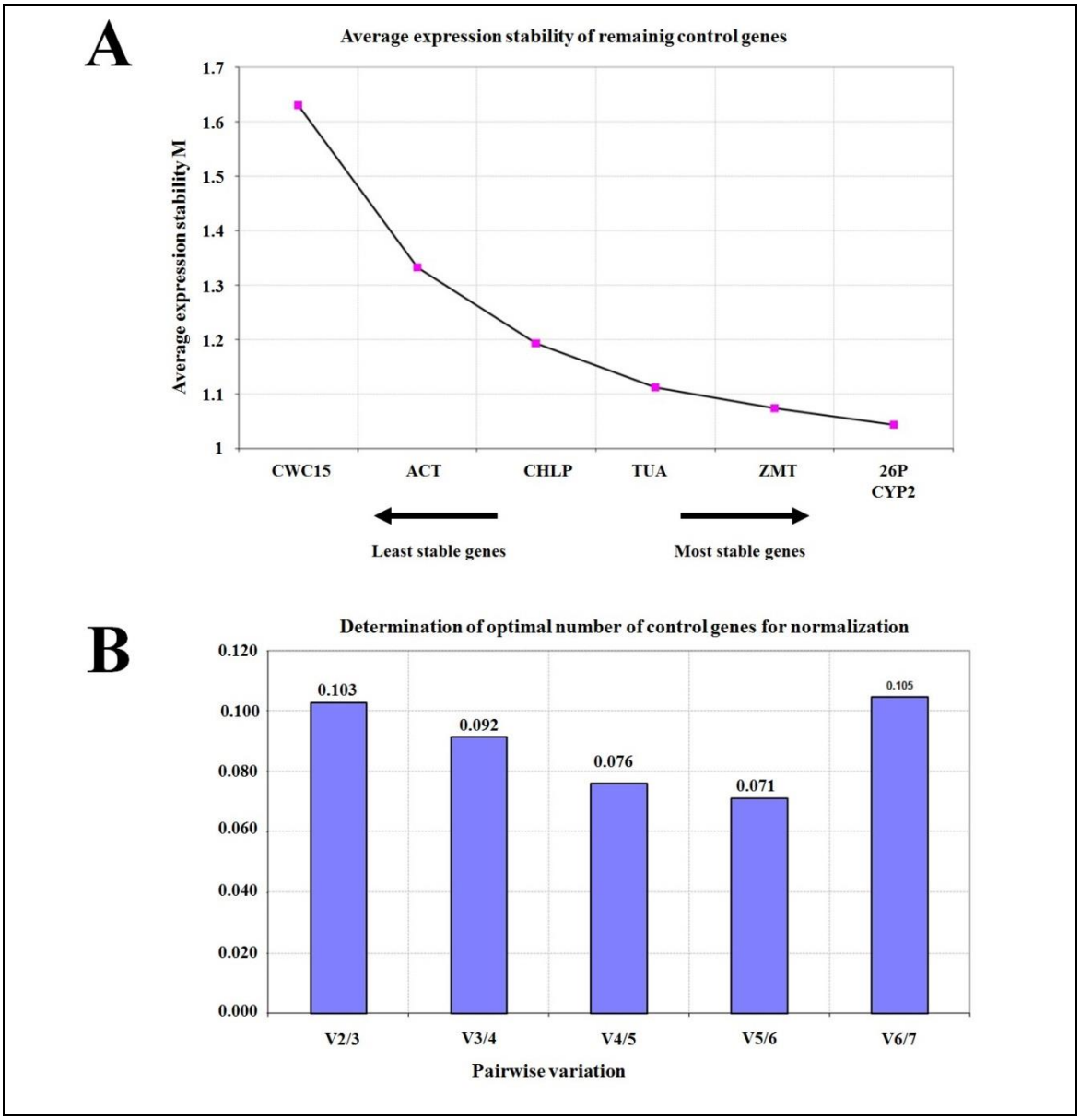
Gene Name (Acronym)	Accession Number	Unigene ID	Gene (Function)	Primers Sequence (5'-3')	Amplicon (bp)	Efficiency (%)	R^2
Actin (*ACT 11)*	BW652479	Gma.32186	Cytoskeletal structural protein	(F) CGGTGGTTCTATCTTGGCATC (R) GTCTTTCGCTTCAATAACCCTA	144	100.08	0.989
Alpha Tubulin *(TUA5)*	CA801144.1	Gma.13580	Structural constituent of cytoskeleton	(F) AGGTCGGAAACTCCTGCTGG (R) AAGGTGTTGAAGGCGTCGTG	159	107.23	0.999
Zinc metallopeptidase (*ZMET)*	XM_003521127.3	Gma.7635	Unknown	(F) GCAACCAACCTTTCATCAGC (R) GCCTCGACCCTTTGCTCAAT	150	102.65	0.998
26S proteasome *(26P)*	XM_003530886.3	Gma.59029	Protein degradation	(F) ATGGCTGTGGATGAGGAACC (R) TCAAGTGGGCAACAGAGCAG	169	107.23	0.999
Geranylgeranyl diphosphate reductase *(CHLP)*	XM_003524728.3	Gma.1279	Reduction of geranylgeranyl diphosphate to phytyl diphosphate	(F) GAGTCGCCAAGTCTATTGAT (R) CCGTAGAAATCTGGAGAAAC	145	96.06	0.998
Spliceosome-associated protein *(CWC15)*	XM_003549867.3 XP_003549915.1	Glyma.17G141500	Spliceosome-associated protein CWC15	(F) GCTCAAAGTAAAGGAGGCAGAGC (R) CCACGGGCTTGGTTCTTAAAC	120	96.06	0.999
cytochromes P4502 gene family *(CYP2)*	CF806591	Gma.31599	Biosynthetic and detox pathways	(F) CGGGACCAGTGTGCTTCTTCA (R) CCCCTCCACTACAAAGGCTCG	154	90.25	0.999
GmSN2			*G. max* Snakin-2	(F) AGACGCGAACAGAAGGCTAA (R) TGCACCTTGTCTTGCACAAT	215	96.06	0.999

R^2, Regression coefficient

Supplementary Fig.1 Taxonomic distribution of representative gene-candidates of Snakin/GASA family identified using SeedServer. The y-axis represents the number of genes, and the x-axis represents the plant family.



Supplementary Fig. 2 - geNorm analysis, indicating the average expression stability (M value) (A) and pairwise variation (B), calculated by pair-wise variation analysis between normalization factors (NF_n and NF_{n+1}).



Supplementary Fig. 3 - Expression profile of GmSN2 gene from soybean plants (PI561356 accession) in response to *Phakopsora pachyrhizi*, 48 hai (hours after inoculation) in comparison with those in the mock-inoculated plants (control). Soybean genes encoding CYP2 (cytochromes P4502 gene family, 26P (26S proteasome) and ZMT (Zinc metallopeptidase) were used as reference genes. The error bars represent the set of three independent biological replicates and two technical repeats.

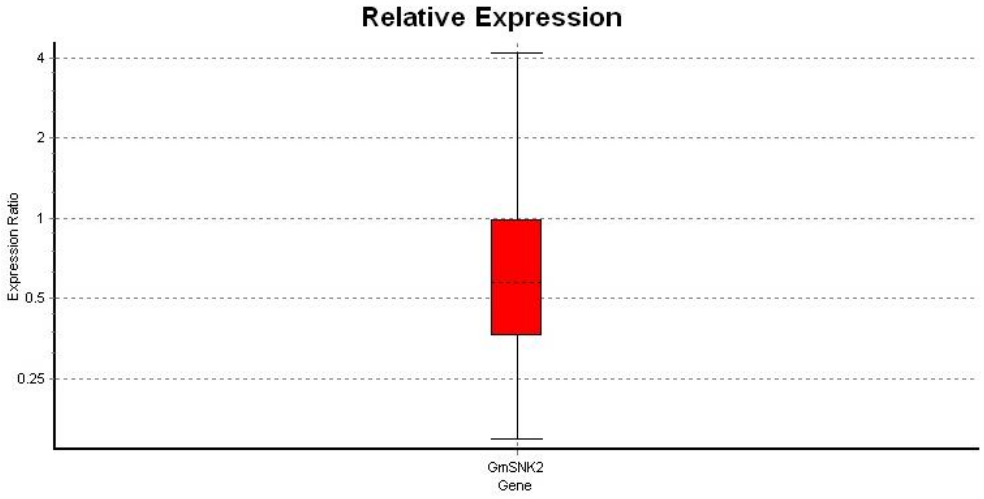


Table S2. Soybean transcripts anchored in the virtual chromosomes

Transcript	Chromosome	Identity (%)	Start	End	Evalue	Exons
GmSN2	6	95.361	16099119	16098926	1.08E-82	3
GmSN-like1	17	100.000	41223285	41222787	0.00E+00	3
GmSN-like2	17	100.000	7253935	7253516	0.00E+00	3
GmSN-like3	6	99.789	16099107	16098634	0.00E+00	3
GmSN-like4	6	99.859	16099369	16098660	0.00E+00	3
GmSN-like5	6	98.622	3382377	3381874	0.00E+00	2
GmSN-like6	6	99.699	17279227	17279558	5.77E-168	4
GmSN-like7	9	99.078	46048084	46047651	0.00E+00	4
GmSN-like8	17	100.000	41213214	41213527	6.25E-160	3
GmSN-like9	3	99.002	34663864	34664364	0.00E+00	4
GmSN-like10	5	100.000	3022907	3023335	0.00E+00	4
GmSN-like11	20	99.502	37179214	37178813	0.00E+00	2
GmSN-like12	10	93.484	48562512	48562864	6.80E-154	2
GmSN-like13	14	98.936	48414359	48413797	0.00E+00	3
GmSN-like14	14	99.824	7800129	7799562	0.00E+00	2
GmSN-like15	17	99.445	39246408	39246948	0.00E+00	2
GmSN-like16	4	99.838	42390534	42389919	0.00E+00	4
GmSN-like17	4	100.000	44455449	44455978	0.00E+00	4
GmSN-like18	19	100.000	1247143	1247613	0.00E+00	4
GmSN-like19	19	100.000	2576710	2577165	0.00E+00	4
GmSN-like20	17	100.000	41223285	41222960	2.17E-166	3

Table S3. Qualitative aspects of the soybean homolog structures, the Z-Score and the Ramachandran plot in % comparing with snakin-1 model (PDB ID 5e5q)

Reference ID	Z-Score	Favored regions (%)	Additional allowed regions (%)	Generously allowed regions (%)	Disallowed regions (%)
GmSN-like1	-4.88	96	4	0	0
GmSN-like2	-4.4	95.7	4.3	0	0
GmSN-like3	-3.73	91.5	8.5	0	0
GmSN-like4	-4.36	96	4	0	0
GmSN-like5	-4.28	91.5	8.5	0	0
GmSN-like6	-5.14	91.8	6.1	0	2
GmSN-like7	-3.95	91.5	8.5	0	0
GmSN-like8	-4.62	95.9	4.1	0	0
GmSN-like9	-3.77	91.5	6.4	2.1	0
GmSN-like10	-4.59	92	8	0	0
GmSN-like11	-4.12	91.3	8.7	0	0
GmSN-like12	-3.8	93.6	6.4	0	0
GmSN-like13	-3.8	94	6	0	0
GmSN-like14	-5.64	91.5	8.5	0	0
GmSN-like15	-3.96	91.5	8.5	0	0
GmSN-like16	-3.85	91.5	8.5	0	0
GmSN-like17	-4.04	91.3	8.7	0	0
GmSN-like18	-3.72	91.5	6.4	2.1	0
GmSN-like19	-4.92	93.9	6.1	0	0
GmSN-like20	-4.4	95.7	4.3	0	0

6. CONCLUSÕES GERAIS

- ✓ Nesta análise foi possível identificar 20 esnaquinas-candidatas no transcriptoma da soja e 40 homólogos no genoma distribuídos em 16 dos 20 cromossomos. Todas apresentaram 12 cisteínas, conservadas em posição, independente de sua subfamília (I, II ou III);

- ✓ As esnaquinas identificadas em soja estão relacionadas ao metabolismo de desenvolvimento, bem como a estresses bióticos e abióticos;

- ✓ Sua distribuição genômica é heterogênea, não dependendo da estrutura do gene que pode possuir dois, três ou quatro éxons ou subfamília.

- ✓ O isolado GmSN2 é um novo membro que é expresso constitutivamente contra o fungo *P. pachyrhizi*, este gene possui três éxons, está localizado no cromossomo 6 da soja e possui homólogos em P. vulgaris e M. truncatula;

- ✓ A estrutura do peptídeo isolado bem como dos transcritos identificados está relacionada a outros AMPs previamente descritos, como alfa-hairpins e tioninas

- ✓ Este estudo provê uma importante fonte para o estudo da função e mapeamento destes genes em leguminosas

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ANEXOS

Anexo I. Instruções para autores – Revista: *Peptides*

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All journal information and instructions compiled in one document (PDF) in just one mouse-click Author information pack

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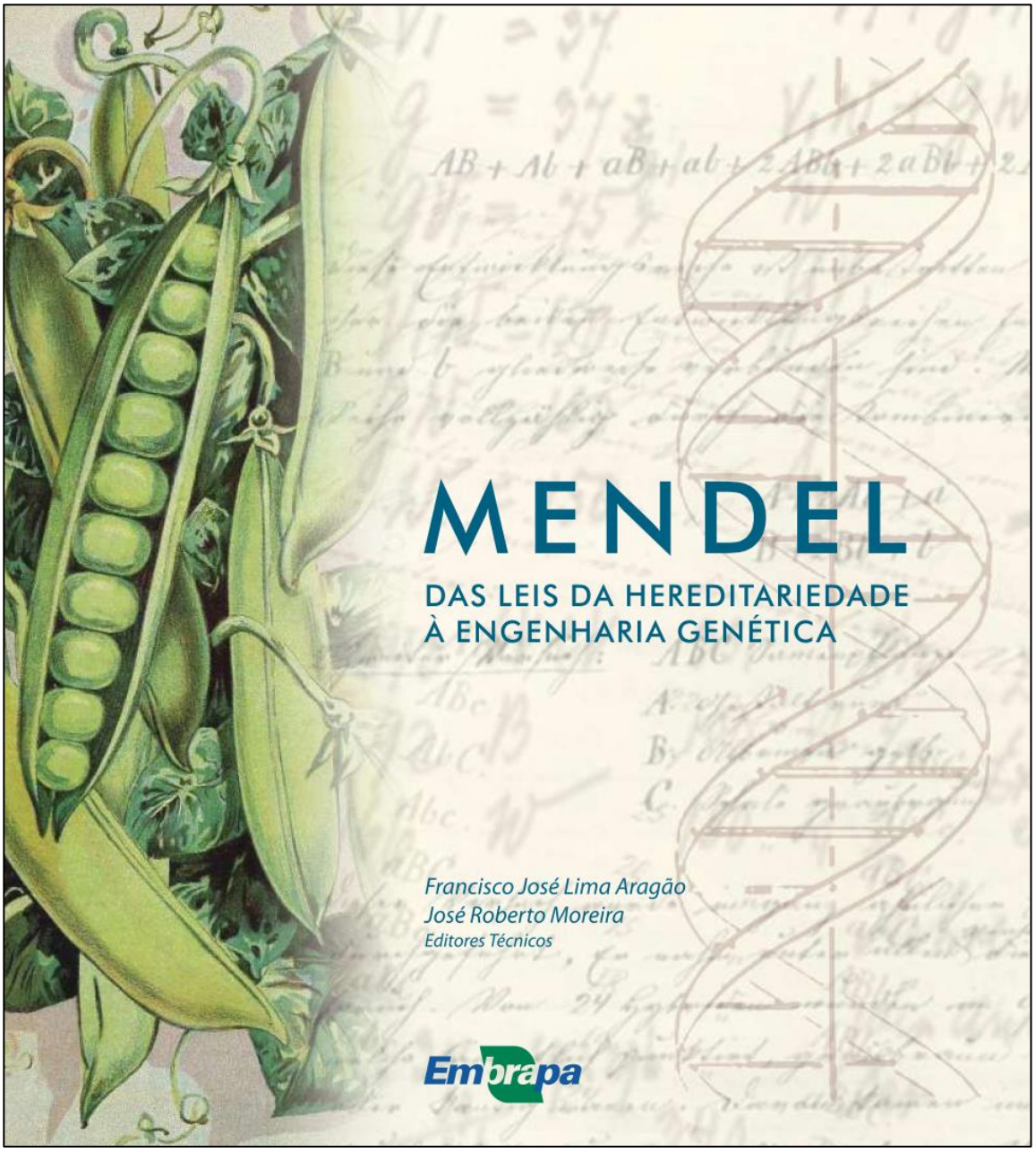

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DAS LEIS DA HEREDITARIEDADE À ENGENHARIA GENÉTICA

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Capítulo 12

Mendel e suas exceções à luz das ômicas e da biologia de sistemas

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Introdução

Em vida, Gregor Mendel nunca recebeu reconhecimento por seus estudos, nem mesmo prêmios ou homenagens. Apesar disso, hoje é praticamente impossível estudar até mesmo os aspectos mais básicos da biologia sem encontrar seus – agora famosos e polêmicos – experimentos com plantas de ervilha (espécie botânica *Pisum sativum* L. da família Fabaceae).

Um dos aspectos que distinguiram Mendel de outros naturalistas de sua época foi o seu excelente conhecimento de matemática. Embora a matemática envolvida na genética mendeliana básica não seja profunda ou mesmo remotamente difícil, merece destaque o fato de que Mendel guardava meticulosas anotações sobre suas experiências [estima-se que tenha cultivado mais de 33.500 plantas de ervilhas no período de estudos mais intensos, entre 1856 e 1863 (HARTL; JONES, 1998)]. Ele documentava experimentos que outros possivelmente considerariam rotineiros, mas que lhe permitiram reconhecer padrões que o levaram à sua descoberta. Assim, pode-se dizer que os experimentos de Mendel estão entre os mais bem desenhados, acuradamente executados e elegantemente interpretados da história da ciência experimental e que sua redescoberta lançou as bases para uma moderna ciência: a genética.

Entre suas principais conclusões, Mendel mostrou que, em cruzamento de linhagens puras de ervilhas (uma com característica dominante e outra, recessiva), o híbrido era idêntico ao parental dominante (não havia mistura das características). Da mesma forma, quando cruzados os híbridos, a descendência era constituída tanto por indivíduos dominantes quanto por recessivos.

Mendel não encontrou evidência de que os fatores (genes) hereditários fossem modificados por sua associação nos híbridos parentais. Cada caráter seria condicionado por um par de fatores (genes), que segregavam e recombinavam-se

Anexo III - Aceito Para Publicação

Antimicrobial peptides as plant defense weapons: characterization and functional genomics

Chapter 7 of the book entitled:
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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 rich in cysteines, capable of preventing the establishment of invading pathogens. Some of these peptides are shared with other eukaryotes such as defensins and cyclotides, or, still, restricted to the plant kingdom (as snakins). In turn, other AMP classes are specific to some plant taxonomic groups (such as heveins). Even when shared with other groups of organisms, a given AMP class presents a much higher number of isoforms in plants, due to gene duplications or genomic redundancy, an occurrence possibly also associated to the sessile habit of plants, which prevents them from evading biotic or environmental stresses. Therefore, plants are the resource of new AMP molecules. This chapter compiles information on their structural features at genomic, transcriptomic and protein level. Since AMPs are often difficult to recognize, based on simple BLAST alignments, a description of database resources and bioinformatics tools available for their identification is provided. Finally, we highlight the still almost unexplored biotechnological potential of AMPs in the generation of both transgenic plants resistant to pathogens, and new drugs or bioactive compounds for 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 aa. Peptides are conventionally understood as having less than 50 aa (Marmiroli and Maestri 2014). Proteins, on the other hand, would be any molecule presenting higher amino acid content, being widely studied in plants. A data-mining at the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed) was carried out in April/2018 using the keywords "Plants AND Proteomics", returned about 5900 articles. However, when using the keywords "Plants AND Peptidomics" only 30 publications returned. This difference evidences the discrepancy between systematic research on plant proteins and peptides, despite the relevant roles of both categories.

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. In animals, peptides are prevalent in intercellular communication, performing as hormones, growth factors and neuropeptides (Germain, Chevalier, and Matton 2006).

In several pathogenic microorganisms, peptides can serve as classical virulence factors which disrupt the epithelial barrier, damage cells and activate or modulate host immune responses. Example of this performance is verified with Candidalysin (Moyes et al. 2016), a fungal cytolytic peptide toxin found in the pathogenic fungus *Candida albicans*. This secreted toxin directly damages epithelial membranes, triggers a danger response signaling pathway and activates epithelial immunity. There are also reports of fungal peptides helping in the organism defense. For example, the Copsin, a peptide-based fungal antibiotic recently identified in the fungus *Coprinopsis cinerea* (Essig et al. 2014) acts killing bacteria by inhibiting their cell wall synthesis. Regarding some bacterial peptides, certain species from the gastrointestinal microbial community can release low-molecular-weight peptides, which trigger immune responses (Singh et al. 2009). Another example includes probiotic bacteria-derived proteases that can degrade cow milk casein and thereby generate peptides with suppressive effects on the lymphocyte proliferation in healthy individuals (Singh et al. 2009). There are additionally peptides that function as bacterial "hormones" that allow bacterial communities to organize multicellular behavior such as biofilm formation (Flaherty, Freed, and Lee 2014).

Plant peptides, in turn, can also be multifunctional, and according to Farrokhi, Whitelegge, and Brusslan (2008) they could be classified into two main categories (**Figure 7.1**):

- 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 (Higgins and Payne 1982). 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), based on the type of precursor, divided BPs into three groups (**Figure 7.1**):

- 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’);

In turn Farrokhi, Whitelegge and Brusslan (2008) present a more intuitive classification of BPs, according to their intracellular role (**Figure 7.1**):

- Phytohormone peptides: the characteristic feature of these peptides is the regulation of fundamental 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 (**Figure 7.1**), 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 (Takahashi et al. 2018). 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 (J. Liu et al. 2013). Regarding the second group, it includes peptides with signaling roles in plant defense, comprising at least four subgroups, including SYST (systemin) (**Figure 1-1**). SYST peptides were identified in Solanaceae members, as tomato and potato (Pearce, Bhattacharya, and Chen 2008) acting on the signaling response to herbivory. SYST leads to the production of a plant protease inhibitor that suppresses insect´s proteases (Ryan 1990). Stratmann (2003) 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 (García-Olmedo et al. 1998). 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 (Benko-Iseppon et al. 2010).

Plant AMPs are the central focus of this chapter, 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.

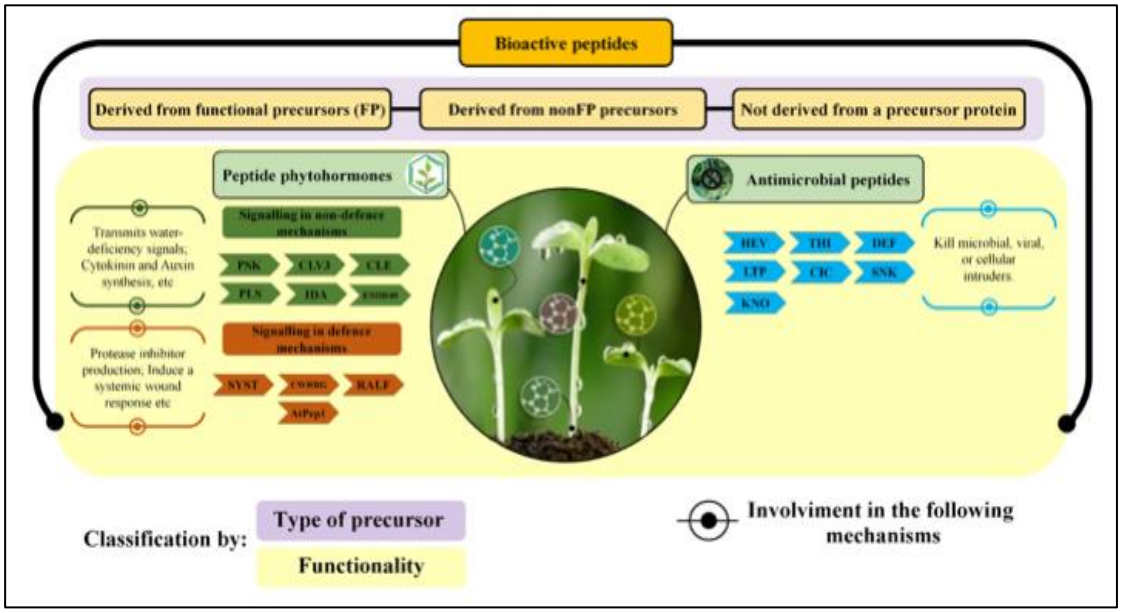

Figure 1-1. Plant bioactive peptides (BP): classification system and some biological processes involved. Tavormina et al. (2015), considering the BP precursors, classified them into: (1) derived from functional precursors or (2) nonfunctional precursors, and (3) not derived from a precursor protein. Farrokhi, Whitelegge, and Brusslan (2008), based on functional groups, classified them into peptide phytohormones (with signaling in defense or non-defense mechanisms) and antimicrobial peptides.

Overall Features of Plant AMPs

AMPs are ubiquitous host defense weapons against microbial pathogens. The overall plant AMP characterization regards the following variables (**Figure 1-2**): electrical charge, hydrophilicity, secondary and three-dimensional structures, and the abundance or spatial pattern of cysteine residues (Benko-Iseppon et al. 2010). These features are primarily related to their defensive role(s) as membrane-active antifungal, antibacterial or antiviral peptide(s).

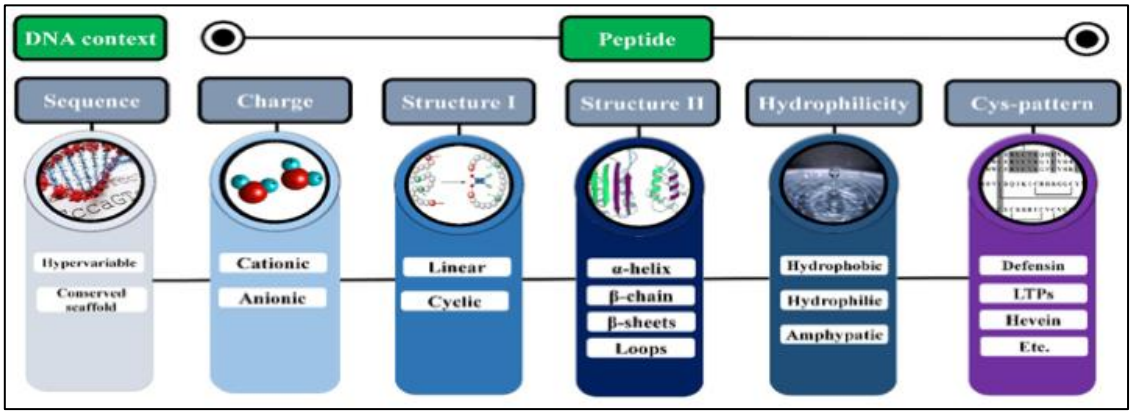

Figure 1-2. Plant antimicrobial peptides features considering DNA sequence level, protein structure and physicochemical properties.

Regarding the nucleotide sequence, plant AMPs are hypervariable (**Figure 1-2**). This genetic variability provides diversity and the ability to recognize different targets. Regarding their charges, AMPs can be classified as cationic or anionic (**Figure 1-2**), but most of the plant AMPs have positive charges, which is a fundamental feature for the interaction with membrane lipid head groups of pathogens (Kaas, Westermann and Craik 2010). Concerning hydrophilicity, AMPs are generally amphipathic, i.e., they exhibit molecular conformation with both hydrophilic and hydrophobic domains (Yu et al. 2013). In regard to their tridimensional structure, AMPs can be either linear or cyclic (**Figure 1-2**). Some linear AMPs adopt an amphipathic α-helical conformation whereas non-α-helical linear peptides generally show one or two predominant amino acids (Zanetti 2004). 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 (Zanetti 2004).

Considering the secondary structures, AMPs may exhibit α-helices, β-chains, β-pleated sheets and loops (**Figure 1-2**). Wang (2010) classified plant AMPs into four families (α, β, αβ, and non-αβ), based on the protein classification of Murzin et al. (1995), with some modifications. AMPs of the 'α' family present α-helical structures (Wang, Wacklin and Craik 2012), whereas AMPs from the 'β' family contains β-sheet structures usually stabilized by disulfide bonds (Willem F. Broekaert et al. 1997; Wang, Wacklin and Craik 2012). Some plant AMPs showing a α-hairpinin motif formed by antiparallel α-helices are stabilized by two disulfide bridges (Terras et al. 1992). Such AMPs present a higher resistance to enzymatic, chemical or thermal degradation (Vriens, Cammue and

Thevissen 2014). 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 (Bruix et al. 1995). Finally, AMPs that do not belong to the ‘αβ’ group exhibit no clearly defined ‘α’ or ‘β’ structures (Wang, Wacklin and Craik 2012).

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 (Tam et al. 2015). Therefore, plant AMPs are commonly grouped as thionins, defensins, heveins, knottins (linear and cyclic), lipid transport proteins, snakins and cyclotides (Broekaert et al. 1997; Tam et al. 2015). Some of these AMP groups will be detailed in the next sections, with comments on their functions, tissue-specificity, and scientific data availability.

Defensin

Both plants and animals produce defensins in response to microbial challenges (Wong, Xia and Ng 2007), being present also in microorganisms. Such peptides comprise a well-known group of plant AMPs showing membranolytic function (Tam et al. 2015). They are abundant and known for their antimicrobial activities even at low concentrations against bacteria (gram-positive and negative), fungi, viruses and parasitic protozoa (Brogden 2005). Usually, defensins are located in plant parts often exposed to pathogens (Holly, Diaz and Smith 2017), such as leaves, roots, barks, pods, tubers, fruits and floral tissues (Lay 2003).

A data mining performed in the PubMed database, with the keywords "plant AND defensins" presented 790 scientific publications covering diverse approaches, from genetic diversity, gene identification/functional analysis, and protein isolation/purification methods. The current knowledge of plant defensins reflects the importance of this AMP family and it’s potential for biotechnological manipulation considering new therapeutic properties or the host fighting against microbial pathogens.

Lipid Transfer Protein (LTP)

A data-mining performed in the PubMed database using the keywords "Plant AND Lipid Transfer Proteins" revealed more than 750 scientific manuscripts.

LTPs are encoded by all land plants, being expressed in the majority of plant tissues (Salminen, Blomqvist and Edqvist 2016). LTPs are considered important proteins also for the plant establishment, colonization and survival on the soil. Generally, LTPs are present in extracellular spaces; however, many LTPs are also found in the cell wall (Tsuboi et al. 1992; Carvalho et al. 2004). Additionally, they can be localized in cellular organelles, such as the glyoxysomes (Tsuboi et al. 1992) and vacuoles (Carvalho et al. 2004). The role of LTPs is not entirely understood. However, published data suggest their importance in the assembly of physical barriers against water by depositing monomers that form waxes (such as cutin and suberin) in plant cell walls (Domínguez, Heredia-Guerrero and Heredia 2015). Scientific data also suggest the association of LTPs with signaling against infection by fungi, bacteria, and viruses (Tsuboi et al. 1992; Maldonado et al. 2002; Carvalho and Gomes 2007). Recently, the LTP participation in tolerance mechanisms to abiotic stresses was also reported (Edqvist et al. 2018).

Hevein

As suggested by the nomenclature, heveins were initially isolated from the rubber tree *Hevea brasiliensis* (Archer 1960). To date, they have been identified in more than 20 different plant species (Slavokhotova et al. 2017). Heveins are commonly expressed in plant tissues such as leaves (Games et al. 2016), latex (Archer 1960), and seeds (Gijzen et al. 2001), among others. There are no reports of their identification in other organisms, except plants.

The hevein action is mainly reported against plant pathogens that are rich in chitin, such as fungi. These microorganisms may bind to the structural components of the chitin-binding site present on heveins (Slavokhotova et al. 2017). It is worth noting that heveins may additionally affect microorganisms devoid of these polysaccharides. Hevein action against oomycetes and bacteria has been reported (Cândido et al. 2014). Besides, heveins have attracted the interest of scientists, with a growing number of researchers in recent years; their mechanism of action, however, remains unsolved. A PubMed data mining performed with the terms "Plant AND Heveins" revealed 215 scientific manuscripts, covering several aspects of heveins.

Cyclotide

Cyclotides are peptides found in all kingdoms (Park et al. 2017). A fundamental characteristic regards their cyclic structure, stabilized by three disulfide bridges (Craik et al. 1999). These features make them functional peptides that tolerate high temperatures, and also support some chemical degradation (Colgrave and Craik 2004). Their involvement in defense functions is also well known, since cyclotides exhibit diverse biological activities, including antifungal, insecticidal (Jennings et al. 2001), antibacterial (Pränting et al. 2010), anthelmintic (Colgrave et al. 2008), molluscicidal (Plan et al. 2008), and anti-HIV (Gustafson et al. 1994).

The cyclotide "[T20K] Kalata B1", isolated from *Oldenlandia affinis* (Rubiaceae) leaves showed to be active during *in vivo* assays against multiple sclerosis, preventing the disease progression without adverse effects (Henriques et al. 2015). These results indicate plant cyclotides as potential candidates for drug development (Thell et al. 2016). Additionally, they are relatively small, what facilitates their production by chemical synthesis and heterologous expression in bacterial, yeast or animal cells. All these features turn them into ideal candidates for new candidates and research tools based on peptides (Camarero 2017). Besides their importance, the mechanisms of action of cyclotides remain not yet fully elucidated (Park et al. 2017). However, more than 400 cyclotides have been already described from several plant species, which are available in the CyBase (http://cybase.org.au/), a database dedicated to cyclic peptides. A data-mining in the PubMed database applying the keywords “cyclotides AND plants” revealed 286 scientific articles, covering different subjects, from their chemical characterization (Hashempour et al. 2013), involvement in the dynamics of cell membranes (Wang 2012), and other issues.

Snakin

Snakins are also plant-exclusive AMPs. They were first described in potato (*Solanum tuberosum*), showing 12 conserved cysteine residues, forming six disulfide bridges (Segura et al. 1999; Nahirnak et al. 2012). These AMPs were also described in several crops such as rice (Furukawa, Sakaguchi and Shimada 2006), tobacco (Kovalskaya, Zhao and Hammond 2011), wheat (Rong et al. 2013), tomato (Herbel, Schäfer and Wink 2015), and in the medicinal plant *Peltophorum dubium* (Fabaceae) (Rodríguez-Decuadro et al. 2018).

They were observed expressing in diverse plant tissues as inflorescences (Boonpa et al. 2018), tubers (Segura et al. 1999), leaves, stems and roots (García et al. 2014) and seedlings (Rodríguez-Decuadro et al. 2018). Regarding their subcellular location, the presence of snakins in potato cell walls (Nahirnak et al. 2012) suggested a role in the plant cell defense as a physical obstacle, preventing the invasion of some pathogens (Segura et al. 1999). According to Nahirnak et al. (2012), the silencing of potato snakin gene (StSN1) affected the plant metabolism and the cell wall composition. This result together with those from the scientific literature suggests that snakins may play other functions in plant growth and development, and also in hormonal crosstalk (Nahirnak et al. 2012), besides response to biotic stress (García et al. 2014). They can act against some fungal and bacterial pathogens at micromolar concentrations (EC 50 <10 μM), as observed by snakin SN1 performance in potato plants (Segura et al. 1999). Only a few studies covering the snakin mechanism of action against plant pathogens were published so far, regarding only 36 articles after a search at the PubMed database using the terms "plant AND snakin".

Structural features of AMPs at gene and protein levels

Defensin

The first plant defensins were isolated from wheat (Colilla, Rocher and Mendez 1990) and barley grains (Mendez et al. 1990), initially called γ-hordothionins. Due to some similarities as 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 (Broekaert et al. 1995).

Plant defensins belong to a diverse protein superfamily called cis-defensin (Parisi et al. 2018) and exhibit cationic charge, consisting of 45 to 54 aa with two to four disulfide bonds (W. F. Broekaert et al. 1995; Janssen et al. 2003; Pelegrini and Franco 2005). The 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 (Shafee et al. 2017) (**Figure 1-3**).

Defensins are known for their antimicrobial activity at low micromolar concentrations against Gram-positive and -negative bacteria (Kraszewska et al. 2016),

fungi (Oddepally and Guruprasad 2015), parasitic viruses and protozoa (Brogden 2005). Additionally, they present inhibitory, insecticidal, and antiproliferative activity, acting as an ion-channel blocker, being also associated with protein synthesis (Carvalho and Gomes 2009).

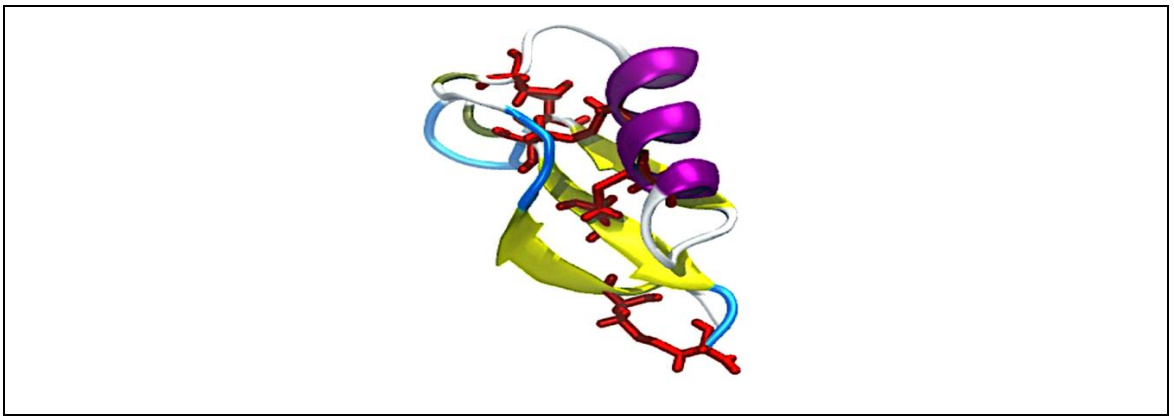

Figure 1-3. Three-dimensional structure NaD1 defensin from *Nicotiana alata* (Solanaceae) (1mr4.pdb available in https://www.rcsb.org/structure/1mr4). In yellow the three β-sheets; in purple the α-helix and in red the three disulfide bonds.

Lipid Transfer Protein (LTP)

Non-specific Lipid Transfer Proteins (nsLTPs) were first isolated from potato tubers (Mazliak et al. 1975) and are actually identified in diverse terrestrial plant species. They concern a large gene family, are abundantly expressed in most tissues, and are absent in most basal plant groups as chlorophyte and charophyte green algae (Edstam et al. 2011). They generally include an N-terminal signal peptide that directs the protein to the apoplastic space (Salminen, Blomqvist and Edqvist 2016). 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 (unspecifically, the list includes phospholipids, fatty acids, their acylCoAs or sterols). They have approximately 100 aa and are relatively larger in size than other AMPs, as defensins.

Depending on their molecular mass LTPs may be classified into two subfamilies: LTP1 and LTP2 with relative molecular weight of 9 kDa and 7 kDa, respectively (Kader 1996; Castro et al. 2003). 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) (Edstam et al. 2011). 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 (**Figure 1-4)**. Although this latter classification system is the most recent, the conventional classification of types LTP1 and LTP2 has been maintained by most working groups.

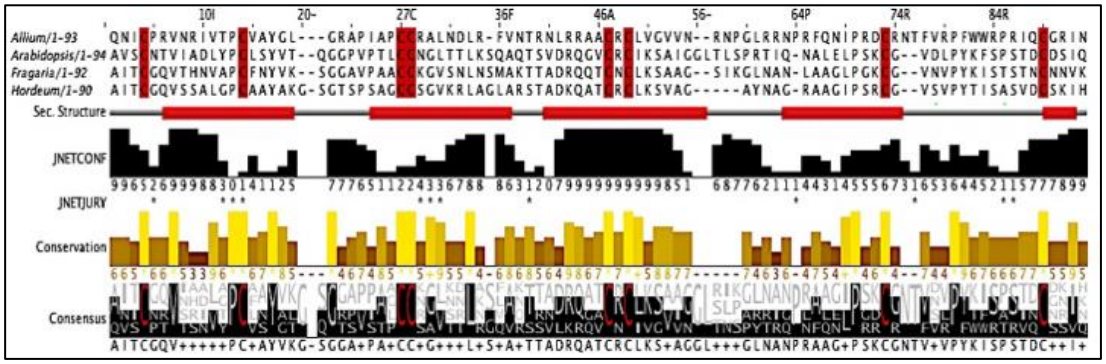

Figure 1-4. Alignment of plant LTP1 sequences from onion, arabidopsis, strawberry and barley. Cysteine residues are highlighted in red in the alignment. Below the alignment, the secondary structure prediction generated by JalView. Bars in yellow/brown show sequence conservation. Amino acid consensus sequence is shown at the bottom of the figure.

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 naming system sometimes made it quite difficult, extremely time-consuming and sometimes frustrating to compare LTPs with different roles (Salminen, Blomqvist and Edqvist 2016).

An additional nomenclature was also proposed by Salminen et al. (2016), that named the LTPs as follows: AtLTP1.3, OsLTP2.4, HvLTPc6, PpLTPd5 and TaLTPg7, with the first two letters indicating the species of plants (At = *Arabidopsis thaliana*, Pp = *Physcomitrella patens*, etc.), 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 1-5**), 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 (Lindorff-Larsen and Winther 2001; Berecz et al. 2010; Edstam and Edqvist 2014). These foldings provide a different specificity of lipid binding at the LTP binding site, where the LTP2 structure is relatively more flexible and present a lower lipid specificity when compared to LTP1 (Goyal and Mattoo 2016).

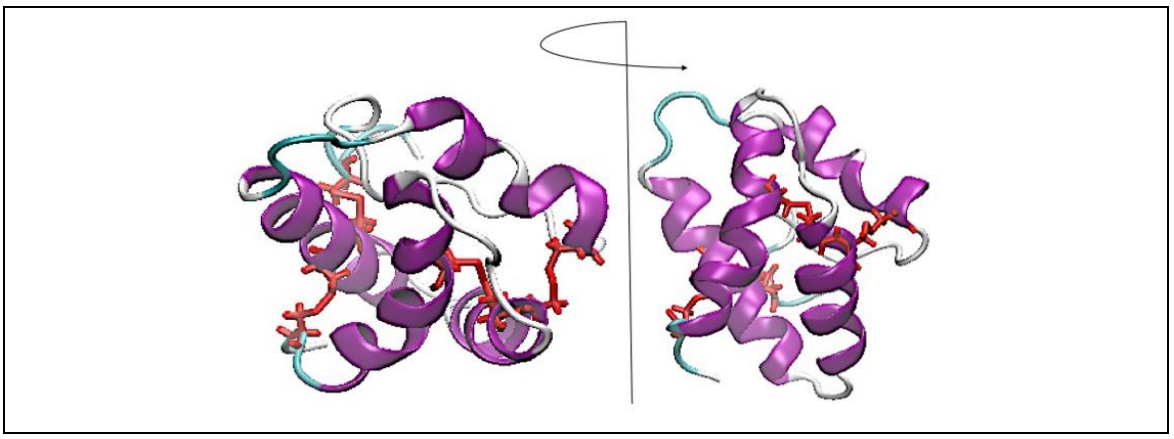

Figure 1-5. Three-dimensional structure of TaLTP1.1 purified from wheat seeds. Model available at: Protein Data Bank (identifier PDB ID: 1GH1). Four helices of TaLTP1.1 are colored pink, while the four conserved disulfide bridges are colored red.

The first three-dimensional structure of an LTP was established for TaLTP1.1 (**Figure 1-5**) based on 2D and 3D data of 1H-NMR, purified from wheat (*Triticum aestivum*) seeds in aqueous solution (Simorre et al. 1991; Gincel et al. 1994). Currently, several three-dimensional structures of LTPs have been determined, either by RMN 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. (Peumans, De Ley and Broekaert 1984) with eight cysteine residues forming a typical Cys motif (Slavokhotova et al. 2017).

The structure of the hevein consists of 29 to 45 aa, positively charged, with abundant glycine and cysteine residues ranging from 6, 8 to 10 C (Slavokhotova et al. 2017) and aromatic residues (Asensio et al. 2000; Tam et al. 2015). 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 (Wong et al. 2016; Slavokhotova et al. 2017). 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 (Tam et al. 2015). Antiparallel β chains form the central β sheet of the hevein motif with two long coils stabilized by disulfide bonds (**Figure 1-6**).

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 (Raikhel, Lee and Broekaert 1993). 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 (Asensio et al. 2000; Slavokhotova et al. 2017). 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 (Beintema 1994; Rogozhin et al. 2015). It may also occur in other proteins that bind to polysaccharide chitin (Beintema 1994), 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 (Broekaert et al. 1992). Among the several classes of proteins mentioned, the proteins with a high degree of similarity to hevein are classes I and IV of chitinases (Slavokhotova et al. 2017).

Chitinases are known to play an essential role in plant defense against pathogens (Iseli, Boller and Neuhaus 1993), also inhibiting fungal growth *in vitro* (Broekaert et al. 1989), especially when combined with β-1,3-glucanases (Leah et al. 1991) and interferes

with the growth of hyphae, resulting in abnormal ramification, delay and swelling in their stretching (Rogozhin et al. 2015). 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 (Van Parijs et al. 1991). 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 (Koo et al. 1998), evidencing the statement raised by Van Parijs et al. (1991). 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 (Koo et al. 1998; Kaas et al. 2010). Therefore, heveins have good antifungal activity, and only a few are active against bacteria, most of them with low activity (Lipkin et al. 2005; Rogozhin et al. 2015).

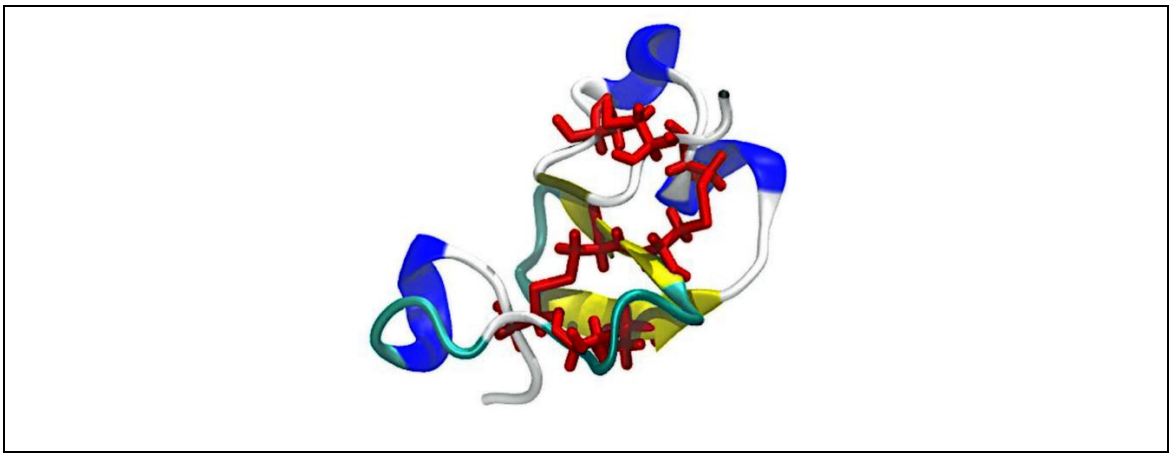

Figure 1-6. Three-dimensional structure of hevein peptide from *Hevea brasiliensis* latex (1hev.pdb available in http://www.rcsb.org/structure/1HEV). In yellow the three β-sheets; in blue three α-helix and in red the three disulfide bonds.

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 (Molesini et al. 2017). Their structure was initially identified by crystallography of carboxypeptides isolated from potato, showing the cystine-knot motif, 39 aa and six cysteine residues (Rees and Lipscomb 1982). 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 (Molesini et al. 2017). 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 2 disulfide bonds (Chiche et al. 2004). 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 (Molesini et al. 2017) (**Figure 1-7**).

In addition, the knottins have different functions, such as signaling molecules (Murphy, Smith and De Smet 2012), response against biotic and abiotic stresses (Li and Asiegbu 2004), root growth (Iyer and Acharya 2011), symbiotic interactions as well as antimicrobial activity against bacteria (Aboye et al. 2015), fungi (Göransson et al. 2012), virus (Gustafson et al. 1994), and insecticidal activity (Jennings et al. 2005), among others. The 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 (Göransson et al. 2012). The accumulation of peptides on the surface of the membrane results in the weakening of the pathogen membrane (Burman et al. 2011) resulting in transient and toroidal perforations (Göransson et al. 2012).

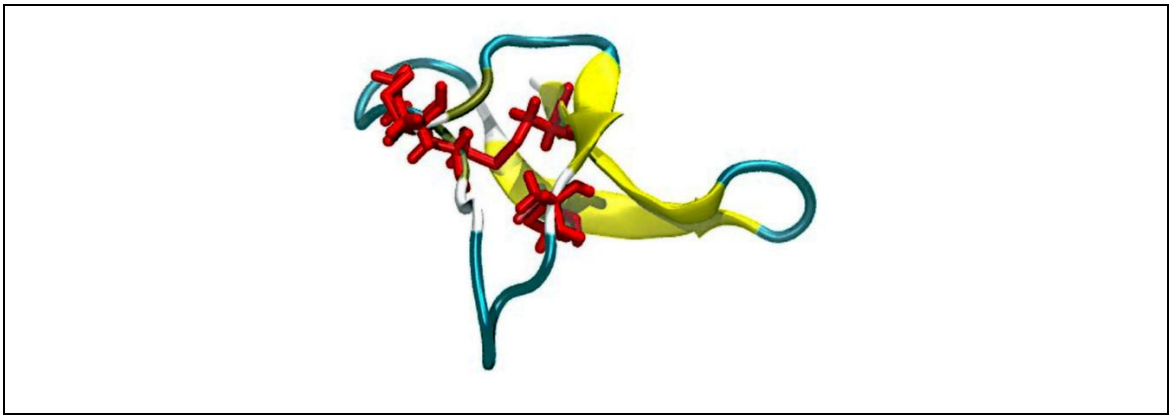

Figure 1-7. Three-dimensional structure of cysteine-knot (or knottin) peptide (Ep-AMP1) from *Echinopsis pachanoi* (Cactaceae; source: 2mfs.pdb available in https://www.rcsb.org/structure/2mfs). In yellow the three β-sheets and in red the three disulfide bonds.

Macadamia (β-barrelins)

In the course of a large-scale survey to identify novel antimicrobial peptides from Australian plants (Marcus et al. 1997; McManus et al. 1999), 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 6 cysteine residues. Its three-dimensional structure was determined using NMR spectroscopy (McManus et al. 1999) 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 1-8**). 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 (McManus et al. 1999). This structural fold resembles a superfamily of proteins called *γ-crystallin-like* characterized by the precursors *βγ- crystallin* (Ohno et al. 1998). This family includes AMPs from other organisms, for example, WmKT a toxin produced by the wild yeast species *Williopsis mraki* (Antuch, Güntert, and Wüthrich 1996).

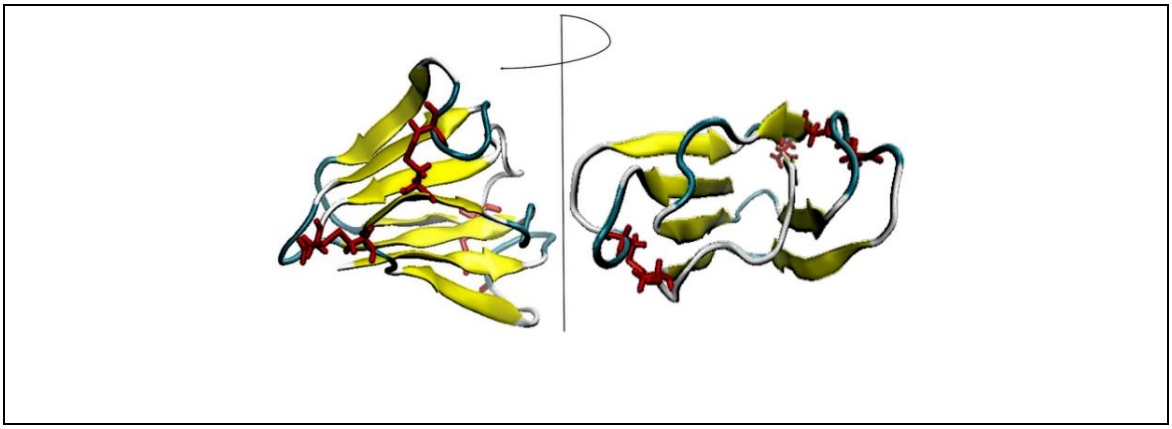

Figure 1-8. Three-dimensional structure of MiAMP1 purified from *Macadamia integrifolia* (source *Protein Data Bank*, PDB ID: 1C01). In yellow the eight beta leaves and in red the three disulfides conserved bridges.

MiAMP1 exhibited *in vitro* antimicrobial activity against various phytopathogenic fungi, oomycetes and gram-positive bacteria (Marcus et al. 1997) 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* (Kazan et al. 2002), 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 (Manners 2009) 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 present 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. The predicted Ib-AMP precursor protein consists of a pre-peptide followed by six mature peptide domains, each flanked by propeptide domains ranging from 16 to 35 aa in length (**Figure 1-9**). This primary structure with repeated domains of alternating basic peptides and acid propeptide domains was reported before in plants (Tailor et al. 1997).

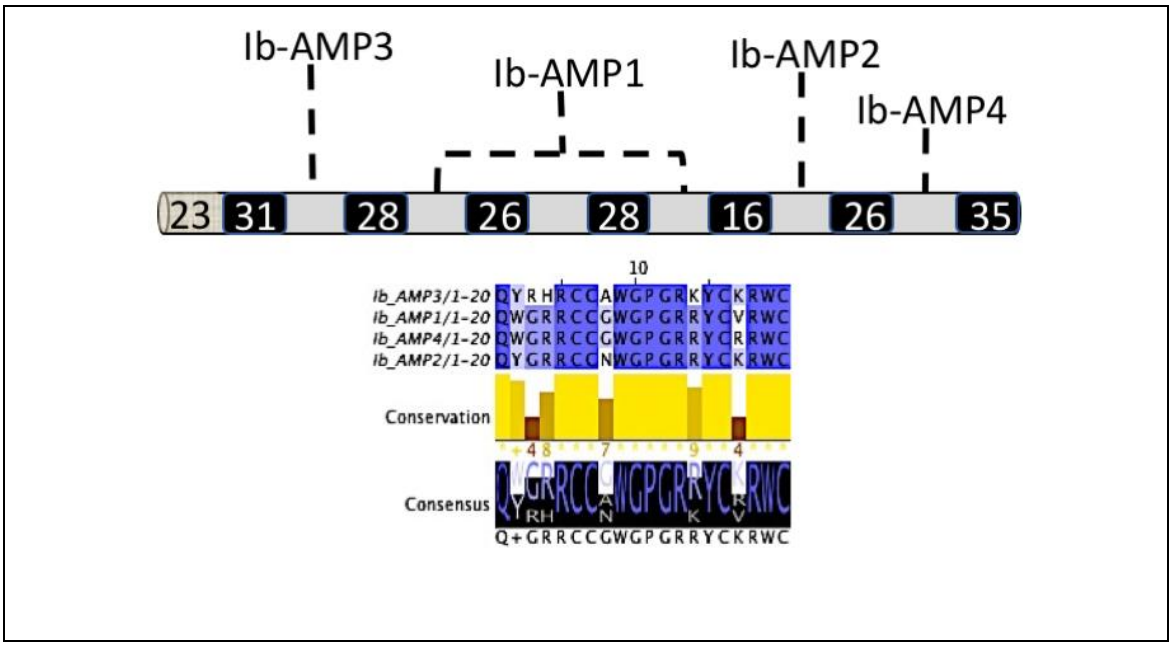

Figure 1-9. General gene structure and alignment of peptide sequences. Figure based on Tailor et al. 1997. (A) Structure of the predicted translation product of 333 amino acids with the individual 20 amino acid domains representing the mature Ib-AMP peptides (in gray), with the Ib-AMPs indicated above 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.

Circular dichroism (CD) and two-dimensional proton nuclear magnetic resonance (1H NMR) were performed in IbAMP1 by Patel et al. (1998) indicating that the peptide presents a turn but shows no evidence of helical or sheet structure over the analyzed temperature and pH range (**Figure 1-10**). 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 (Patel et al. 1998) .

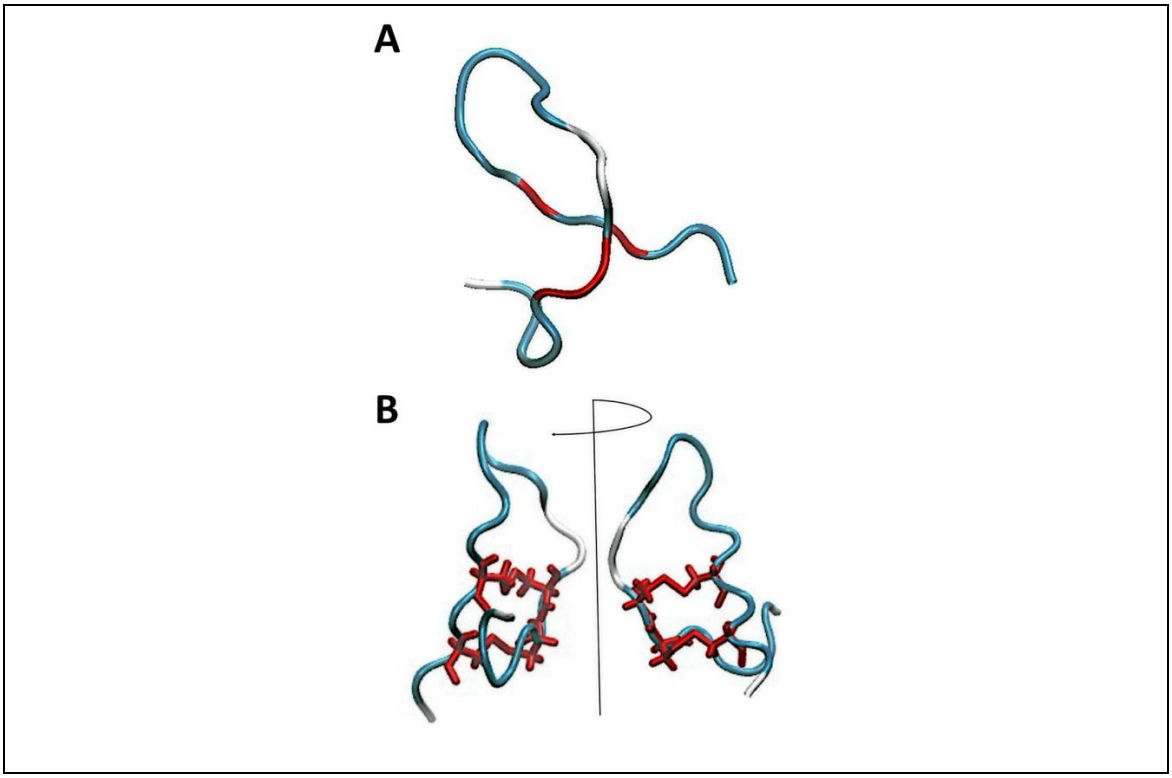

Figura 1-10. (**A**) Tridimensional structure of Ib-AMP1. Alternative model generated by *ab initio* modelling with Rosetta algorithm, evidencing the four conserved cysteines (in red). (**B**) An alternative ab initio model generated with the aid of the Rosetta algorithm, presenting two disulfide bridges.

Little is known about the mode of action of Impatiens-like AMPs. Lee et al. (1999) 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 analyzes 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 substitution of amino acid residues by arginine or tryptophan may improve by more than

twice the antifungal activity (Thevissen et al. 2005). 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 (P. Wang et al. 2009).

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 (Gautier et al. 1994).

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 (Gautier et al. 1994) (**Figure 1-11**).

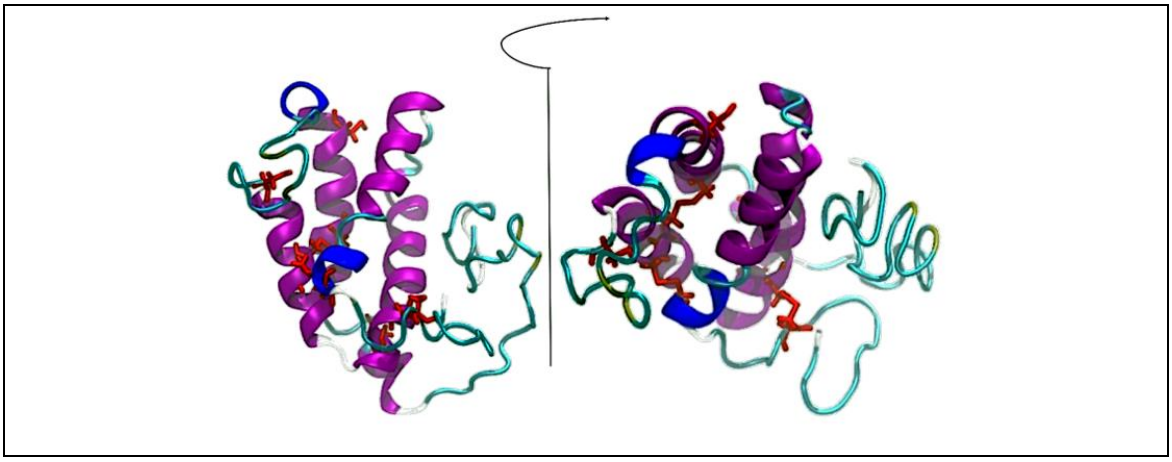

Figure 1-11. Tridimensional structure of PIN-a from *Aegilops kotschyi* (Poaceae). Alternative model generated by *ab initio* modeling using the Rosetta algorithm, showing in pink the four β-sheets and in red conserved cysteine bridges.

The conformation of the two PIN isoforms was studied by infrared and Raman spectroscopy. The results showed that 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 (Le Bihan et al. 1996). There is no high-resolution structure for any of the PIN isoforms, bringing challenges to understanding the function of their hydrophobic regions, with some evidence coming only from homolog peptides (Gautier et al. 1994).

PINs are proposed to be functional components of wheat grain hardness loci, control core texture, besides antifungal activity (Giroux et al. 2003; Bhave and Morris 2008; Dhatwalia et al. 2009; J. Zhang et al. 2011). 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) (Douliez et al. 2000; Morris 2002; Marion, Bakan, and Elmorjani 2007).

Puroindolines form protein aggregates in the presence of membrane lipids, and the organization of these aggregates is controlled by the 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) 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*) (Segura et al. 1999; Berrocal-Lobo et al. 2002). Due to their sequence similarity to GASA (Gibberellic Acid Stimulated in Arabidopsis) proteins, the snakins were classified as members of the snakin/GASA family (Oliveira-Lima et al. 2017). 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 (Segura, et al. 1999; Berrocal-Lobo et al. 2002; Mao et al. 2011; Yeung et al. 2016) (Figure 1-12).

Snakins may be expressed in different parts of the plant, like stem, leaves, flowers, seeds and roots (S. Zhang et al. 2009; Almasia et al. 2010; R. Zimmermann, Sakai, and Hochholdinger 2010; Guzmán-Rodríguez et al. 2013), both constitutive or induced by biotic or abiotic stresses. *In vitro* activity was observed against a variety of fungi, bacteria and nematodes, acting as destabilizer of the plasma membrane (Segura et al. 1999; Faccio et al. 2011; Herbel, Schäfer, and Wink 2015). They were reported as important agents in biological processes such as cell division, elongation, cell growth, flowering, embryogenesis, and signaling pathways (Kotilainen et al. 1999; Furukawa, Sakaguchi, and Shimada 2006; Roxrud et al. 2007; Lucau-Danila et al. 2010).

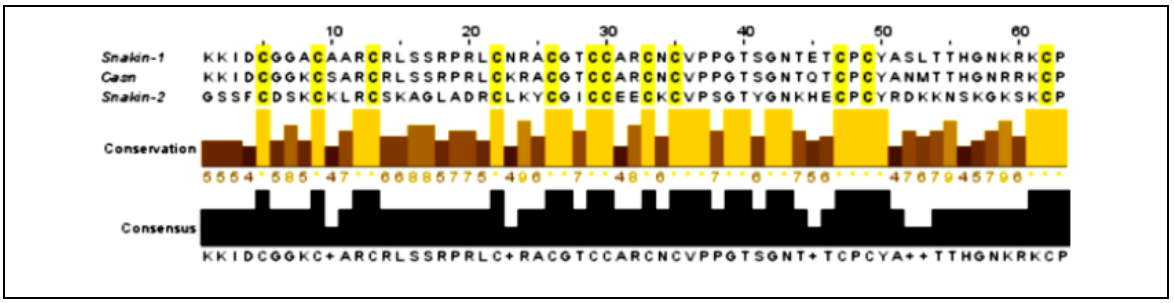

Figure 1-12. Comparative alignment of three Snakins from *Solanum tuberosum* (Snakin-1 and Snakin-2) and *Capsicum annuum* (Casn), evidencing the GASA domain with the conserved motif of twelve cysteines (yellow). The yellow bars show conservation between the sequences and below the consensus sequence is presented.

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) (Craik et al. 1999). 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 (Lima et al. 2016). 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 (Weidmann and Craik 2016; Park et al. 2017) (**Figure 1-13**).

To date, several cyclotides were identified in eudicot families such as, Rubiaceae (Gran 1973), Violaceae (Claeson et al. 1998), Fabaceae (Poth et al. 2011) and Solanaceae (Poth et al. 2012), in addition to some monocots of Poaceae family (Nguyen et al. 2011). 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 (Gran 1973), antibacterial (Pränting et al. 2010), anti-HIV (Gustafson et al. 1994) and anticancer (Lindholm et al. 2002). All these characteristics added to the stability conferred by the CCK motif turn these peptides into excellent candidates for drug development (Craik, Clark, and Daly 2007; Northfield et al. 2014).

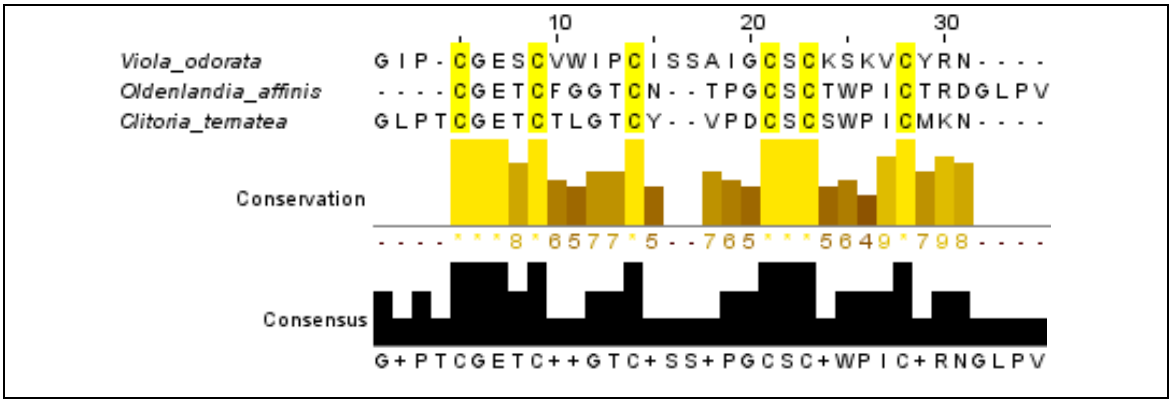

Figure 1-13. Alignment of cyclotides from three plant species: *Viola odorata* (Violaceae), *Oldenlandia affinis* (Rubiaceae) and *Clitoria ternatea* (Fabaceae) evidencing the conserved motif of eight cysteines (yellow). The yellow bars show conservation between the sequences. Consensus sequence is presented at the bottom of the figure.

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 (Liu, Sturrock and

Ekramoddoullah 2010). TLPs are abundant in the plant kingdom, being found in angiosperms, gymnosperms and bryophytes (Liu, Sturrock, and Ekramoddoullah 2010), being also identified in other organisms, including fungi (Grenier, Potvin, and Asselin 2000; Sakamoto et al. 2006), insects (Brandazza et al. 2004), and nematodes (Kitajima and Sato 1999).

TLPs are known for their antifungal activity, either by permeating fungal membranes (Batalia et al. 1996) or by binding and hydrolyzing β-1,3-glucans (Trudel et al. 1998; Grenier et al. 1999). In addition, they may act to inhibit fungal enzymes, such as xylanases (Fierens et al. 2007), α-amylases or trypsin (Schimoler-O'Rourke, Richardson and Selitrennikoff 2001). Besides, the expression of TLPs is regulated in response to some stress factors, such as drought (Jung et al. 2005), injuries (Ruperti et al. 2002), freezing (Hon et al. 1995), and infection by fungi (Kumar and Kirti 2011; Rudd et al. 2015), viruses and bacteria (Breiteneder and Radauer 2004).

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 (Jami, Anuradha, and Guruprasad 2007; Tachi et al. 2009). Most of the TLPs have molecular mass ranging from 21 to 26 kDa (J.-J. Liu, Sturrock, and Ekramoddoullah 2010), possessing 16 conserved cysteine residues (**Figure 1-14**) involved in the formation of eight disulfide bonds (Breiteneder 2004), which help in the stability of the molecule, allowing a correct folding even under extreme conditions of temperature and pH (Fierens et al. 2009). TLPs also contain a signal peptide at the N-terminal which is responsible for targeting the mature protein to a particular secretory pathway (J.-J. Liu, Sturrock, and Ekramoddoullah 2010).

The tertiary structure, in turn, 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 (Leone et al. 2006). 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; **Figure 1-14**), being very relevant for specific receptor binding of antifungal activity (Batalia et al. 1996; Koiwa et al. 1999; Min et al. 2004).

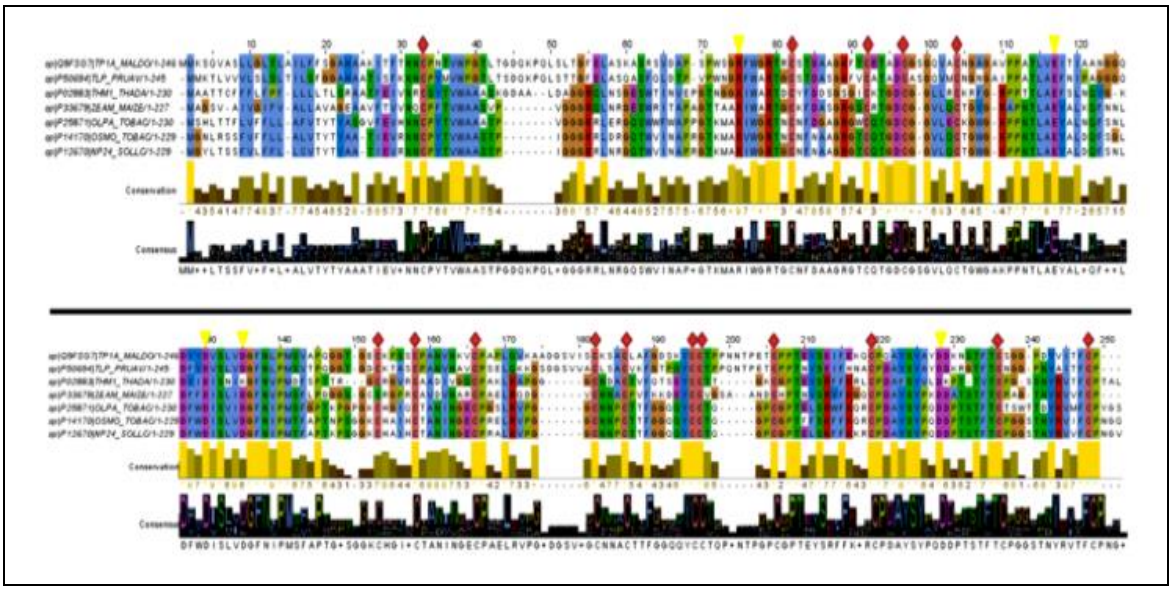

Figure 1-14. Alignment of thaumatin-like amino-acid sequences of six different species (*Malus domestica*, *Prunus avium*, *Thaumatococcus daniellii*, *Zea mays*, *Nicotiana tabacum*, and *Solanum lycopersicum*). Sequence comparison was carried out with Clustal Omega software with standard parameters. Colored bars show conserved residues and motifs. Pink diamonds indicate the cysteine residues that form the 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.

Crystallized structures were determined for some plant TLPs, such as thaumatin (Ogata, Gordon, and Vos 1992; **Figure 1-15A)**, zeamatin (Batalia et al. 1996; **Figure 1-15B)**, tobacco PR-5d (Koiwa et al. 1999) and osmotin (Min et al. 2004; **Figure 1-15C)**, the cherry allergen PruAv2 (Dall'Antonia et al. 2005), banana allergen Ba-TLP (Leone et al. 2006; **Figure 1-15D)** and tomato NP24-I (Ghosh and Chakrabarti 2008; **Figure 1-15E)**, among other TLPs.

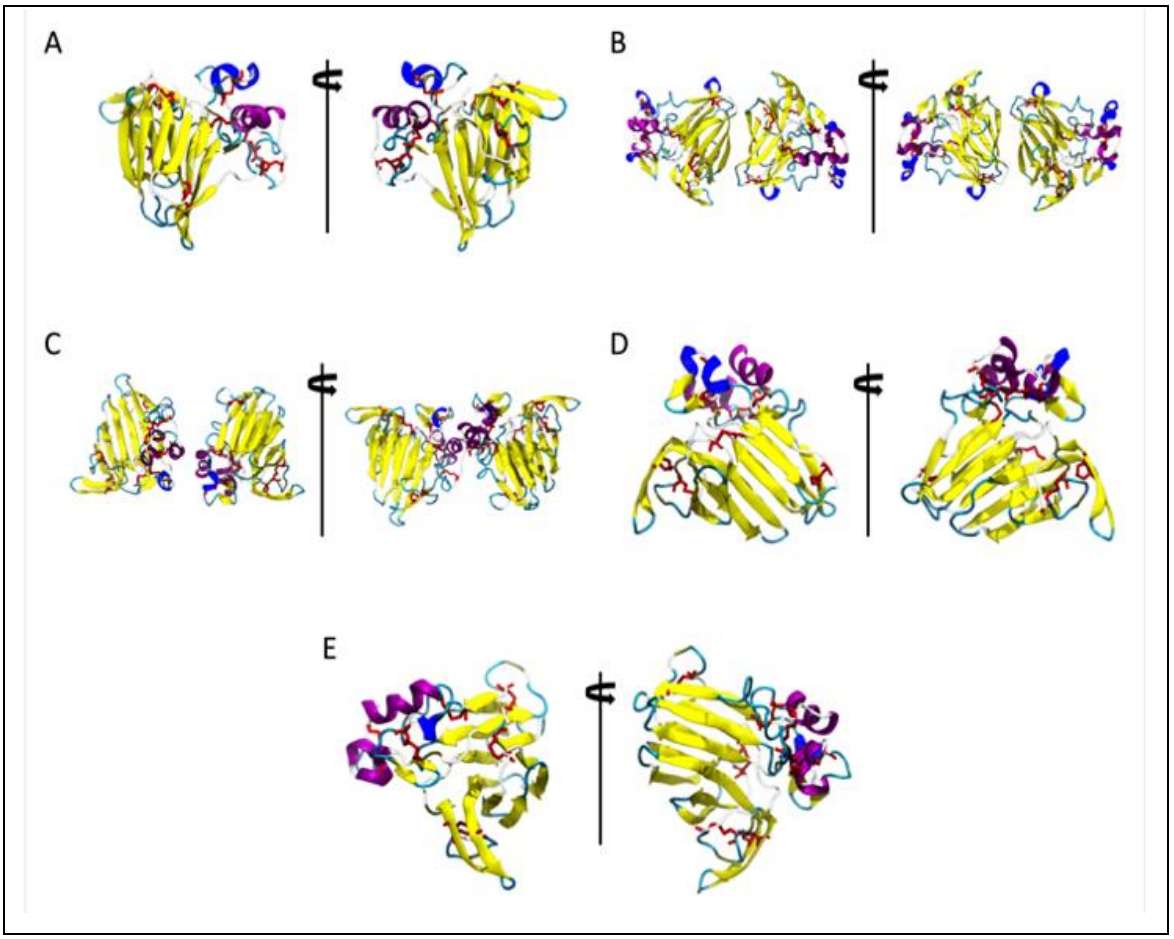

Figure 1-15. Crystal structure of representative plant TLPs available at PDB and visualized and viewed at two angles through the software VMD. **(a)** Thaumatin (1RQW), *Thaumatococcus daniellii*; **(b)** Zeamatin (1DU5), *Zea mays*; **(c)** Osmotin (1PCV), *Nicotiana tabacum*; **(d)** Allergen with antifungal properties (1Z3Q), *Musa acuminata*; **(e)** NP24-I (2I0W), *Solanum lycopersicum*.

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 (Osherov et al. 2002; Greenstein et al. 2006; Liu, Sturrock, and Ekramoddoullah 2010). Other TLPs exhibit an extracellular TLP domain and an intracellular kinase domain, being known as PR5K (PR5-like receptor kinases) (X. Wang et al. 1996) and are present in both monocots and dicots. For example, *Arabidopsis* contains three PR5K genes while rice has only one (Liu, Sturrock, and Ekramoddoullah 2010).

Bioinformatics tools and databases including 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 (Apweiler et al. 2004; Torrent et al. 2012). In this sense, the construction of databases that deal directly with AMPs was an important step to organize the data.

During the last 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, according to Porto, Pires, and Franco (2017). 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) (**Table 1-1**). 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 (Thomas et al. 2010; Waghu et al. 2014, 2016). The current version includes structures and signatures specific to families of prokaryotic and eukaryotic AMPs (Waghu et al. 2016). The platform also includes some tools for AMP prediction.

The antimicrobial peptide database (APD) (Z. Wang 2004; G. Wang, Li, and Wang 2016) 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 (Zhao et al. 2013).

DBAASP (Gogoladze et al. 2014) 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 (Gogoladze et al. 2014).

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, pattern of covalent bonds, 3D structure and molecular targets (Jorge, Lourenço and Pereira 2012; H.-T. Lee et al. 2015).

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 (Seebah et al. 2007; S. Liu et al. 2017).

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 (Mulvenna , Wang and Craik 2006).

Finally, the PhytAMP is a database designed solely dedicated to plant AMPs based on information collected from the UniProt database and from the scientific literature through PubMed (Hammami et al. 2009).

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/

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 (Eltabakh, Ouzzani, and Aref 2006). Considering biological data, information can be classified into: (i) primary (sequences), (ii) secondary (structure, expression, metabolic pathways, types of drugs, etc.) and (iii) specialized, e.g. containing information on a species or class of protein (Mehboob-ur-Rahman, Mahmood-ur-Rahman and Zafar 2016). Within this third group some references to AMPs can be mentioned, such as $CAMP_{R3}$ (Waghu et al. 2014) and APD (G. Wang, Li, and Wang 2016) that compile sequence data and structure retrieved from diverse sources, and also the Defensin knowledgebase (Seebah et al. 2007) and the Cybase (Mulvenna , Wang and Craik 2006) which are dedicated to specific classes of peptides (defensins and cyclotides respectively), in addition to PhytAMP (R. Hammami et al. 2009), 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 (Altschul et al. 1990) and FASTA (Pearson 1990), but also by searching for specific patterns with Regular Expression (REGEX) or Hidden Markov Model (HMM) (Porto, Pires, and Franco 2017). 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 (Polyanovsky, Roytberg, and Tumanyan 2011). This tool splits the sequence into small pieces (words), comparing it with the database. However, this approach has an important 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 (J. Tam et al. 2015; Porto, Pires, and Franco 2017).

In order to reduce the effects of local alignment limitations, other strategies based on the search for specific patterns were introduced, such as REGEX (Thompson 1968) and HMM (Eddy 1998). 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 (Yoon 2009; Porto, Pires, and Franco 2017).

Predicting antimicrobial activity

The design of new AMPs led to the development of methods for the discovery new peptides, thus allowing new experiments to be done by the 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 (Zhou et al. 2013; Zhou and Huang 2015; Guangshun Wang, Li, and Wang 2016).

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 (Niarchou et al. 2013; Waghu et al. 2016). 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 (Lin and Jeon 2006; Karasuyama and Takeuchi 2010; Ding et al. 2013).

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 (Dobslaff et al. 2012). The identification of novel AMPs in databases is primarily dependent on knowing about specific AMPs together with a sufficient sequence similarity (Fedders and Leippe 2008). However, orthologs may be divergent in terms of sequence, mainly because they are under strong positive selection for variation in many taxa (Tennessen 2008), leading to extremely 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 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 (Wilkins et al. 1996). Protein extraction, purification, and identification methods have significantly advanced our capacity to elucidate many biological questions using proteomic approaches (Wasinger and Corthals 2002; Sheoran et al. 2009). 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 (J. D. Thompson, Schaeffer-Reiss and Ueffing 2008).

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 own limitations (Cho 2007; Baracat-Pereira et al. 2012). Obtaining native proteins is a challenge in proteomics or peptidomics, 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 (Kolodziejek and van der Hoorn 2010).

Based in 2D gel, Al Akeel et al. (2017) 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 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 (Baracat-Pereira et al. 2012).

Additionally, peptides are most often cleaved from larger precursors by various releasing or processing enzymes (Menschaert et al. 2010).

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 (Nägele et al. 2004). 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 (Mitulovic and Mechtler 2006).

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 proteolytic agent, generating a set of unique peptides whose masses are determined by mass spectrometry (Blueggel, Chamrad, and Meyer 2004; McHugh and Arthur 2008). These methodologies are widely adopted for large-scale identification of peptide from MS/MS spectra (Hughes, Ma and Lajoie 2010). 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 (Eng et al. 2008) and MASCOT [Matrix Science, Boston, MA (www.-matrixscience.com)] (Perkins et al. 1999). They rank peptide matches based on a cross-correlation to match the hypothetical spectra to the experimental one.

MASCOT is widely used for peptidomics and proteomics analysis, including AMP identification in many organisms or to evaluate the antibacterial efficacy of novel antimicrobial peptides. Evaluating new AMP against multidrug-resistant (MDR) *Salmonella enterica*, Tsai et al. (2016) 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.

In a similar way, Umadevi et al. (2018) 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 (G. Wang, Li, and Wang 2016) 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 (G. Wang, Li, and Wang 2016), together with PhytAMP (R. Hammami et al. 2009) and $CAMP_{R3}$ (Waghu et al. 2016). To enrich the characterization parameters, isoelectric point, aliphatic index and grand average of hydropathy were also used (Gasteiger et al. 2005) (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 peptidomics associated with the innate immunity against *Phytophthora*, evidencing de usability of proteomics/peptidomics data for AMP characterization in any taxa, including plant AMPs, aiming the exploitation of these peptides as next-generation molecules against pathogens (Umadevi et al. 2018).

In the literature, other search tools are described using database searching algorithms, such as X!TANDEM (Craig and Beavis 2004), OMSSA (Geer et al. 2004), ProbID (N. Zhang, Aebersold and Schwikowski 2002), RADARS (Field, Fenyö and Beavis 2002), 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, Vitek and Aebersold (2007). 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 (Gazzana and Borlak 2007; Pestana-Calsa, Ribeiro and Calsa Jr. 2010). 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 (Hughes, Ma and Lajoie 2010; Menschaert et al. 2010).

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 (Weiner et al. 2017). 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 (G. Wang 2010).

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 (Jothi 2012). Experimental techniques for determining structures, such as X-ray crystallography, NMR, electron paramagnetic resonance and electron microscopy, require significant effort and investments (Kaufmann et al. 2010).

All methods mentioned have their own 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 (Jothi 2012). 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 (Moult et al. 2018).

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 (Källberg et al. 2012). 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 (Martí-Renom et al. 2000).

There are several servers and computer models that automate the comparative modeling process, with SWISS-MODEL and MODELLER figuring as the most used (Biasini et al. 2014; Webb and Sali 2016). 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 (Webb and Sali 2016). 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, and that similarity elements in evolutionarily distant or unrelated proteins are often found. The most used servers based on this approach are MUSTER (Wu and Zhang 2008), SPARKS-X (Yang et al. 2011), RaptorX (Källberg et al. 2012), ProSa-II (Wiederstein and Sippl 2007) and most notably the I-TASSER (Yang Zhang 2008). 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 (Yang Zhang, Kolinski and Skolnick 2003; Wu, Skolnick and Zhang 2007). 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 (Jothi 2012).

Among the most used servers and programs for *ab initio* modeling, we highlight the ROSETTA (Kaufmann et al. 2010), and TOUCHSTONE II (Yang Zhang, Kolinski and Skolnick 2003). The accuracy of the models calculated by many of these methods is evaluated by CAMEO (Continuous Automated Model Evaluation) (Haas et al. 2018) and

by CASP (Critical Assessment of protein Structure Prediction) experiment (Moult et al. 2018). 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 (Moult et al. 2016).

Molecular Dynamics Simulation

Molecular dynamics (MD) is a computational simulation technique that predicts the changes of 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" (Robustelli, Piana and Shaw 2018). 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 (Freddolino et al. 2010). 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 (Karplus and McCammon 2002). 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 are conducted for a variety of purposes, such as determining the folding of a structure to a native structure and analyzing the dynamic stability of this structure (Mitsutake and Takano 2018).

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 (Freddolino et al. 2010). The considerable application potential led to the implementation of MD simulation in many software packages, including GROMACS (Hess et al. 2008; Pronk et al. 2013; Abraham

et al. 2015), AMBER (Case et al. 2005), NAMD (Phillips et al. 2005), CHARMM (Brooks et al. 1983), LAMMPS (Plimpton 1995) and Desmond (Bowers et al. 2006). In addition to those mentioned, there are other types of simulation available, such as the Monte Carlo Method, Stochastic Dynamics and Brownian Dynamics (Abraham et al. 2015).

In the last decades, molecular dynamics simulation has become a standard tool in theoretical studies of large biomolecular systems, such as proteins or DNA, 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 large as possible (Hess et al. 2008).

Pathogen interaction studies and molecular docking

Currently, the control of bacterial pathogens is a great challenge, due to 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 (Neu and Gootz 1996; Hong, Zeng, and Xie 2014; Cheng et al. 2016). 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 (Nikaido 1994; Sun, Deng, and Yan 2014; Munita and Arias 2016). The acquisition of these mechanisms has led to a worsening of clinical conditions, and new alternative methods have emerged to improve antibiotics efficacy (Tam et al. 2015). In this context, plant-derived AMPs have great potential for combating pathogens (Chandra et al. 2017), especially because plants have unique AMP classes, not present in other organisms (Benko-Iseppon et al., 2010). The differences of repertoire and composition in an amino acid of plant AMPs reveal structural and functional alterations of their protein families (Bolouri Moghaddam, Vilcinskas, and Rahnamaeian 2016) 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 (Nawrot et al. 2014).

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 (Botero et al. 2018). 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* (Schneider and Collmer 2010; Peyraud et al. 2016). Studies of protein-protein interactions to understand the regulatory process are essential (Ramakrishnan, Chandra, and Srinivasan 2014) 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 (R. Hammami and Fliss 2010).

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 (Sousa, Fernandes and Ramos 2006). 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 (London, Raveh and Schueler-Furman 2013; Pagadala, Syed and Tuszynski 2017). 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 (Pierce et al. 2014).

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 (Petrey and Honig 2014; van Zundert et al. 2016).

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 (Pierce et al. 2014), the rDOCK (Ruiz-Carmona et al. 2014) and the HEX (Ghoorah et al. 2013) 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 (Pagadala, Syed and Tuszynski 2017). 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 (Andrusier et al. 2008).

The best example is the HADDOCK software (van Zundert et al. 2016), 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 (Schneider et al. 2010).

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 (Corbeil, Williams and Labute 2012), GOLD (Jones et al. 1997), Glide (Friesner et al. 2006), FlexX (Sousa, Fernandes and Ramos 2006) and Surflex (Spitzer and Jain 2012), 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 (Rentzsch and Renard 2015; Pagadala, Syed and Tuszynski 2017).

Despite the existing 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 (Audie and Swanson 2013). While both ‘small molecule docking methods’ and ‘custom protocols’ can be used, short peptides are difficult targets because of their high torsional flexibility (Rentzsch and Renard 2015). 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 (Audie and Swanson 2012; London, Raveh and Schueler-Furman 2013; Sousa et al. 2013). 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 (Wilson 2009; Unal, Gursoy and Erman 2010).

Despite the number of existing docking programs, the algorithms are not accurate and 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 (Lensink, Velankar and Wodak 2017). 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 (Lensink and Wodak 2013).

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 (Lensink, Velankar and Wodak 2017). 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.

5. 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 1-2**), but also regarding other plant AMPs (**Table 1-3**). The first transgenic plant expressing a defensin was generated by Gao et al. (2000) 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) 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.

In another experiment, Chen, Liu, and Zou (2006) 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) 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) 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), 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* (Pérez-Bernal et al. 2017). 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 (Portieles et al. 2010).

Thao et al. (2017) 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 promoter p35S 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 (H.-H. Lee et al. 2018).

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 1-2**).

In regard to snakins, Almasia et al. (2008) 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) 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) 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.

With respect to alpha-thionines, the first record of a transformed plant was carried out by Carmona et al. (1993) 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 (Iwai et al. 2002).

The alpha-thionin *Thi2.1* was used for transformation of tomato and resistance evaluation of symptoms after inoculation with *F. oxysporum* and *R. solanacearum* (Chan et al. 2005). 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) 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) 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* (R. Shukurov et al. 2012). 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) 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, (Chen et al. 1999) 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) 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) 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) 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 analyzes also confirmed disease resistance, demonstrating inhibition and malformation in the development of hyphae in leaf tissues of transgenic plants.

Aghazadeh et al. (2017) 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 (Ojola et al. 2018).

Gaspar et al. (2014) 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, Kalinina and Schmid (2013) 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 (Lacerda et al. 2014).

Table 1-1. Plant defensins used for transformation of various plant species, with their donator species, transformed plants and *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*)	Choi et al. 2009
alfAFP (MsDef1)	*Medicago sativa*	Potato and tomato	Antifungal action against *Verticillium dahliae* (potato*), Fusarium oxysporum* (tomate) and antibacterial against *Ralstonia solanacearum* (tomato)	Gao et al. 2000; Chen et al. 2006; Abdallah et al. 2010
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*)	Kanzaki et al. 2002; Khan et al. 2006; Sjahril et al. 2006; Ntui et al. 2010; Khan et al. 2011; Darwishet et al. 2014
BjD	*Brassica japonica*	Tabacco and peanut	Antifungal action against *Phytophthora parasitica* and *Fusarium moniliforme* (tabacco), *Phaeosariopsis personata* and *Cercospora arachidicola* (peanut)	Anuradha et al. 2008
NaD1	*Nicotiana alata*	Cotton	Antifungal action against *Fusarium oxysporum* and *Verticillium dahliae*	Gaspar et al. 2014
Sm-AMP-D1	*Stellaria media*	Banana	Antifungal action against *Fusarium oxysporum* f. sp. *cubense* (Foc)	Ghag et al. 2014
Rs-AFP2	*Raphanus sativus*	Tabacco, wheat and peanut	Antifungal action against *Alternaria longipes* (tabacco), *F. graminearum* and *Rhizoctonia cerealis* (wheat), *Phaeoisariopsis personata* (peanut)	Terras et al. 1995; Li et al. 2011; Vasavirama and Kirti 2012
BSD1	*Brassica campestris*	Tabacco	Antifungal action against *Phytophthora parasitica*	Park et al. 2002

Table 1-2 continued...

Name	Origin	Transformed plants	*in vivo* activity	Reference
PhDef1	*Petunia hybrida*	Banana	Antifungal action against *Fusarium oxysporum* f. sp. *cubense* (Foc)	Ghag et al. 2012
PhDef2	*Petunia hybrida*	Banana	Antifungal action against *Fusarium oxysporum* f. sp. *cubense* (Foc)	Ghag et al. 2012
DDR30	*Pisum sativum*	Canola	Antifungal action against *Leptosphaeria maculans*	Wang et al. 1999
DmAMP1	*Dahlia merckii*	Papaya and rice	Antifungal action against *Phytophthora palmivora* (papaya), *Magnaporthe oryzae* and *Rhizoctonia solani* (rice)	Zhu et al. 2007; Jha et al. 2009
J1-1	*Capsicum annuum*	Pepper and tobacco	Antifungal action against *Colletotrichum gloeosporioides* (Antracnose) in pepper and *Phytophthora parasítica* in tabacco	Seo et al. 2014; Lee et al. 2018
MtDef4.2	*Medicago truncatula*	*Arabidopsis thaliana*	Antifungal action against *Hyaloperonospora arabidopsidis* Noco2 and *Fusarium graminearum*	Kaur et al. 2012
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)	Portieles et al. 2010; Pérez-Bernal et al. 2017
VrDEF1	*Vigna radiata*	Tabacco	Inhibitor insecticide of caruncho α-amylase	Thao et al. 2017

Table 1-2. Plant Snakins, LTPs, α-Thionins, Heveins and Thaumatins used for transformation of various plant species, with their donator species, transformed plants and *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)	Almasia et al. 2008; Faccio et al. 2011
Snakin-2 (SN-2)	*Solanum lycopersicum*	Tomato	Antibacterial against *Clavibacter michiganensis*	Balaji and Smart 2012
Ace-AMP1 (ns-LTP)	*Allium cepa*	Rice	Antifungal action against *Magnaporthe grisea*, *Rhizoctonia solani* and antibacterial against *Xanthomonas oryzae*	Patkar and Chatoo 2006
α-Thionin	Cevada	Tabacco	Antibacterial against *Pseudomonas syringae*	Carmona et al. 1993
α-Thionin	Aveia	Rice	Antibacterial against *Burkholderia plantarii* and *B. glumae* (rice)	Iwai et al. 2002
α-Thionin (Thi2.1)	Arabidopsis	Arabidopsis and tomato	Antifungal action against *Ralstonia solanacearum* (tomato) and Antifungal action against *Fusarium oxysporum* (arabidopsis and tomato)	Epple et al. 1997; Chan et al. 2005
α-HordoThionin (α-HT)	Cevada	Sweet potato	Antifungal action against *Ceratocystis fimbriata*	Muramoto et al. 2012
Pn-AMP-h2 (Hevein)	*Pharbitis nil*	Tabacco	Antifungal action against *Phytophthora parasitica*	Koo et al. 2002
SmAMP1 (Hevein)	*Stellaria media*	Tabacco and Arabidopsis	Antifungal action against *Bipolaris sorokiniana* (arabidopsis) and *Thielaviopsis basicola* (tabacco)	Shukurov et al. 2012

Table 1-3, 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)**	**Datta et al. 1999; Chen et al. 1999; Velazhahan and Muthukrishnan 2003; Mahdavi et al. 2012; Aghazadeh et al. 2017; Ojala et al. 2018**
Thaumatin	*Thaumatococcus daniellii*	Tabacco	Antifungal action against *Pythium aphanidermatum* and *Rhizoctonia solani* and increased tolerance to water and saline stress	Rajam et al. 2007
VaTLP (Thaumatin)	*Vitis amurensis*	Grape	Antifungal action against *Plasmopara viticola*	He et al. 2017
ObTLP1 (Thaumatin)	*Ocimum basilicum*	Arabidopsis	Antifungal action against *Scleretonia sclerotiorum* and *Botrytis cinerea* and increased tolerance to water and saline stress	Misra et al. 2016
CsTLP (Thaumatin)	*Camellia sinensis*	Potato	Antifungal action against *Macrophomina phaseolina* and *Phytophthora infestans*	Acharya et al. 2013

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 (Benko-Iseppon and Crovella 2010; Benko-Iseppon et al. 2012).

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 from the gymnosperm tree *Taxos brevifolia* (Stierle, Strobel, and Stierle 1993).

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 (Thevissen et al. 2007). 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 (Gerwick 2013). Different applications have been reported on the action of these peptides, demonstrating broad-spectrum activity, such as (i) action against cancer cells (Hoskin and Ramamoorthy 2008; Felício et al. 2017), (ii) production and accumulation of immune cells, (iii) wound healing and (iv) angiogenesis stimulation (Elsbach 2003; Bowdish et al. 2005).

In general, the AMPs exhibit low cytotoxicity to mammalian cells and have a particular mode of action (Holaskova et al. 2015). 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 micellisation, depolarization of membranes, leakage of cytoplasmic material, internalization of biocidal peptides or damage to intracellular macromolecules (Straus and Hancock 2006; Melo, Ferre and Castanho 2009). 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 (Holaskova et al. 2015).

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 (Holaskova et al. 2015). Laverty, Gorman, and Gilmore (2011) 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 (Silva et al. 2011; Tacconelli et al. 2017) 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 (Hancock 2001; Galdino da Rocha Pitta, Galdino da Rocha Pitta and Lins Galdino 2010; Peters, Shirtliff and Jabra-Rizk 2010).

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 (Nadal et al. 2012; Cabanos et al. 2013). 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 (Fukuzawa et al. 2010; Sathish et al. 2011; Rubio-Infante et al. 2012; Cabanos et al. 2013).

Concluding remarks and perspectives

As emphasized in this chapter, 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 also 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 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 greatest 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 a coadjuvant in synergism with traditional antibiotic treatment.

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Anexo IV - Artigo submetido a revista DATABASES – B1

LAITOR4HPC: A text mining pipeline based on HPC for building interaction networks

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Background: The number of published full-text articles have increased dramatically over the last years, with the number of available articles trespassing 31 million available abstracts deposited on the MEDLINE database until June 2017. As primary information distribution source, scientific publication aggregates essential data for System Biology with the potential to improve the understanding about structure and dynamics of biological systems from cellular to organismal level. Thus, text mining tools configure as an important approach to build biological networks, updating databases and providing annotation for new pathways. PESCADOR is an online web server based on the text-mining tools LAITOR and NLProt, which retrieves protein-protein co-occurrences in a tabular-based format and adds a network schema to it.

Results: We present here a HPC-oriented version of the PESCADOR's native text-mining tool re-named to LAITOR4HPC. The priority was to take advantage of a parallel computing infrastructure available at an HPC facility in order to analyze the complete collection of MEDLINE abstracts, in a much shorter period of time when compared to the original LAITOR implementation. The presented use case illustrates how LAITOR4HPC retrieved more than two times the number of interactions available in Soycyc database, and how it allowed the construction of an exemplary pathway for *Arabidopsis thaliana*.

Conclusion: LAITOR4HPC can be used for efficient text-mining based construction of biological networks derived from big data sources such as MEDLINE. Time consumption and data input limitation will be dependent to the available resources at the HPC facility. Text-mining based analysis using the LAITOR4HPC has retrieved more protein-protein co-occurrences in Soybean than those deposited at specific databases for this crop, making evident that pathways enrichment can be achieved with this tool. In terms of identification of terms related to noncoding elements such as transposons, LAITOR4HPC tool is not promptly ready yet. This still demands the manual construction of specific dictionaries containing the names that should be tagged during the named-entity recognition step of the text-mining analysis.

Keywords: Soybean. Bioinformatics. System Biology.

Background

Scientific information used to be shared in the past via letters between peers. This evolved to printed journals and magazines, and, during the early days of computation, diskettes became a fashion way to exchange articles prior to the invention of the World Wide Web. Nowadays, in the digital era, the access to information has gained a paradigm and, in parallel, new challenges [1]. Likewise, keeping updated with the "state-of-the-art" on most, if not all, the subjects of study by using conventional search and retrieve methods have become obsolete, and a challenge since the twenty-first century information boom in the scientific publishing. According to NCBI resource coordinators (2017) [2], the number of full-text articles increased at a rate of 11.9% a year! Until September 2016, there were about 26 million abstracts indexed at the PubMed/MEDLINE database

Additionally, to understand Biology in all its complexity, it is necessary to understand the structure and dynamics of organisms from cellular to organismal levels. Thus, the focus shall change from one element (protein, gene, phenotype), to a multidimensional point of view. Systems Biology aims to access multi-OMICS data in a variety of experimental conditions and time series to exhaustively generate networks, that may offer an overview of mechanismal responses of organisms to different conditions [3].

Research information is mainly widespread on scientific journals, likewise important data useful to system biology studies [4]. The need for a more efficient way to explore the plethora of information buried in the various literature silos, motivated the application of Information Retrieval and Extraction techniques in Biology. The area of Text- or Literature-mining has emerged to fill this gap between published information and useful information contained in scientific journals. Text-mining is now capable of identify and extract biological entities co-occurrences in different levels such as cellular, tissue and organism-specific contexts [4-6].

Text mining tools follow three fundamental processes described by Krallinger & Valencia (2005) [7]: (i) information retrieval (finding relevant literature to be analyzed), (ii) biological entities identification (protein and gene names tagging) and (iii) biological interaction terms to relate / associate the tagged entities.

PESCADOR [5], a web server based on the text-mining tools LAITOR [6] and NLProt [8], uses a list of articles identifiers (PubMed IDs – PMIDS) as query to search and retrieve relevant abstracts, further, it tags bioentities or biointeractions terms mentioned in the text collection (*corpus*) and finally identifies biological concepts and

their co-occurrences along with bioentities. These co-occurrences are classified according to its reliability in four types: (type 1) – bioentities names co-occur in the same sentence with biointeraction term(s) in between them; (type 2) – bioentities names in the same sentence with biointeraction term in any position; (type 3) – bioentities names in the same sentence, permissive identification of biointeraction terms; and (type 4) – all biological entities of the abstract are retained, not being mandatory their co-occurrence in the same sentence. Co-occurrences of biological concepts are taken in consideration and reported for co-occurrences of types 1-3. Other online tools like iHOP [9] and STRING [10-12] have different approaches. The former is based on keyword search and the latter looks for co-occurrences based on a protein query with two text mining steps added after the update, respectively.

Thus, text mining tools have been a valuable approach to support Systems Biology, not only for updating databases but also for providing annotation of new pathways *ab initio*, by using automated processing of texts [13,14]. In this study, we aimed to improve LAITOR text-mining tool by adding customized programming functions suitable for HPC (High-performance computing) environments, making it possible to analyze a higher amount of text in a shorter period of time.

HPC parallelization

Abstracts preparation

In this new version, the abstracts must be provided as NCBI-PubMed XML format, the files can be downloaded from the PubMed server by doing a search using key-words or accessed on the MEDLINE FTP servers. A python 2.7 script was written to parse the XML tree structure to recover the PMID, title and abstract of each record. The script provides an output which is used as NLProt input [8]. We used the head node to run the parser, which is composed by: Bull B500, 2 * Intel Xeon L5640 @ 2,26 GHz, 12 cores and 2880 Gb of RAM.

Parallelization

To parallelize the analysis the GNU Parallel software [16] (https://www.gnu.org/software/parallel/) was used, with the flag "-j N", where **N** represents the number of cores to be used, where each core is running the i_{th} input file at

a time. To do that, a file containing the list of paths for all the input files was generated and shared across the cores to be used.

Implementation

The approach described here uses all the papers from our selected *corpus* (i.e. MEDLINE) retrieved as described on the above session. The XML files for the corpus is then parsed, and the parsed output is used as input for the NLProt software to highlight all bioentities (i.e. genes, proteins, species names, tissues and cell types). We used NLProt 1.0.2 described at [15] made available by Rostlab.

The final step is to run LAITOR4HPC. This tool was originally developed using PHP (http://www.php.net/) and had its database designed using MySQL as database management system (https://www.mysql.com/) [6]. The LAITOR4HPC implementation is intent to be used as a stand-alone application, differently to the version integrated to PESCADOR, mainly due to the fact that jobs originated from web servers are normally executed in a dedicated machine (or virtual machine) rather than in an HPC environment. Nevertheless, some of the new implemented features can also run in a single core, such as the in-memory database query and the name tagging recovery.

In order to distribute the LAITOR program as a parallel process, it was necessary to make sure that processes running on different nodes could query the bioentities and biointeraction dictionaries seamlessly. However, by using MySQL it would be necessary to install it in every node, which is possible but against the user practices in most HPC systems, including ours. Therefore, we chose to switch the original disk-stored LAITOR databases (MySQL) by an in-memory database approach. For that purpose, we used SQLite (version 3.0): a self-contained, high-reliability, embedded, full-featured, public-domain, SQL database engine (https://www.sqlite.org). Consequently, we needed to adapt the queries from the former system to the latter (Figure 1).

```
###OLD MySQL QUERIES
(A)    #Connect to database
       $conn=mysql_connect($server,$user,$pass);
       $sele=mysql_select_db($db);

(B)    #Preparing and executing query
       $query=mysql_query("select name_txt from ".$table_genes.
                          "where tax_id=\"".$tax_id."\"");
       if(mysql_num_rows($query)>0){
(C)    #Fetching the results
              $result=mysql_fetch_array($query);
              return($result['name_txt']);
       }
       else{
              return(FALSE);
       }


###NEW SQLite QUERIES
(D)    #Creating the in-memory SQL database
       $pdo = new PDO('sqlite::memory:');
       $pdo->setAttribute(PDO::ATTR_ERRMODE, PDO::ERRMODE_EXCEPTION);
       $pdo->exec('ATTACH "./laitor_nocase.db" as laitor_db');

(E)    #Preparing the SQL query
       $sql = $pdo->prepare("select * from ".$table_genes.
                          "where tax_id=\"".$tax_id."\"");
       $sql->setFetchMode(PDO::FETCH_ASSOC);

(F)    #Executing the SQL query
       $sql->execute();

(G)    #Fetching the results
       $result = $sql->fetchAll();
```

Figure 1: LAITOR4HPC database management system updates. The principles are the same, however MySQL connects to a server where the database is stored in the disk (A) whereas SQLite loads the database file in the RAM memory of the node executing the query (D). The remaining processes are similar when using both technologies: (B, E and F) preparing and executing the query; and (C and G) retrieving the results.

HPC execution

The NLProt step (bioentity tagging) analysis was launched as four distinct jobs, with 15 cores each (60 core in total). LAITOR was run as a single job, using a total of with 20 cores.

Study case

For this purpose, the taxonomy identifier (*taxid*) filter option of LAITOR was used to check all soybean (*Glycine max* – Taxonomy ID: 3847) interactions. The analyses were performed using the 1,034 XML files downloaded from MEDLINE and submitted to the pipeline. The second test was performed by using keywords, in order to build a small pathway of the regulation of plant defensin (PDF) in *Arabidopsis thaliana*. Two keywords were chosen with the purpose to build the respective pathways, "Plant AND Defensin" (PDF) and "Plant AND Transposon" (PTP).

Results and Discussion

All analyses were performed on the Gaia Cluster maintained by the University of Luxembourg High Performance Computing Department. System configuration and cluster organization can be accessed online [19]. The Python parser script has been run on the interactive node against the 1,034 XML files during about 5 min. Whereas, the NLProt and LAITOR runs took approximately 6 days, in total. A rate of approximately 0,017 s per abstract for processing. This is a speed-up of approximately 117 times faster in comparison to the original implementation per abstract, which only the NLProt tagging took nearly 2 s to complete [5]. Running time should vary depending on node configuration and cores available on the HPC, but it is obviously faster than using a single core approach.

Figure 2 represents the general pipeline obtained for the preparation of the MEDLINE abstracts as input for the LAITOR4HPC text-mining process. After download of the full MEDLINE collection we obtained a dataset of 1,034 XML files each containing approximately 30,000 PMIDS (Figure 2A). These files were transferred to the HPC environment via SCP (secure copy protocol) over a SSH (secure shell) protocol (Figure 2B). The Python parser converted these records into NLProt readable MEDLINE input files (Figure 2C), this step took approximately 5 min in the interactive (head) node. Next, the NLProt job was launched (Figure 2D) where 60 computing nodes where used to run i NLProt processes (where: $\{i \in Z \mid \{0 < i < 1{,}305\}$) in order to tag the bioentities names present on those 1,304 input files (Figure 2E). Upon conclusion, those 1,304 NLProt output files were made available on the head node (Figure 2F) ready for the LAITOR4HPC step.

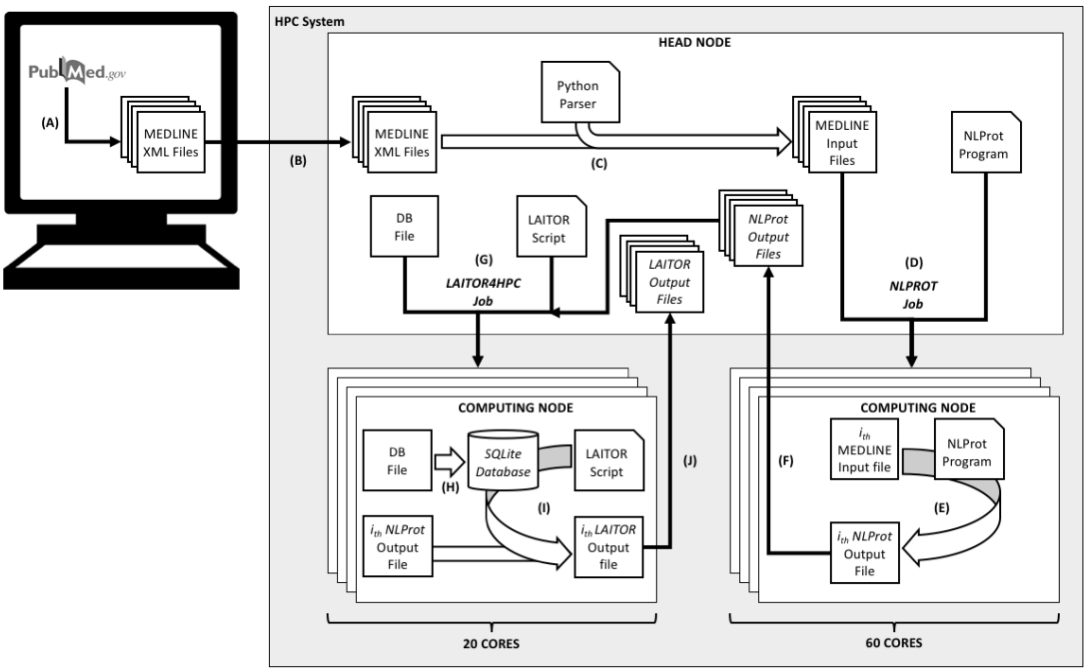

Figure 2: **Complete text-mining pipeline using NLProt and LAITOR4HPC. A)** MEDLINE files are downloaded from NCBI FTP as XML files; **B)** a Python parser is executed to convert the XML files into input files for NLProt which are then **C)** transferred into the interactive (head) node of the HPC system. **D)** A job is then started and *i* different processes are launched in parallel on 60 computing nodes (where: $\{i \in Z \mid \{0 < i < 1{,}305\}\}$). **E)** In each node, the corresponding *i*-th MEDLINE input file is tagged by NLProt which generates **(F)** an *i*-th NLProt output file, which is then placed back to the head node together with the other outputs. **G)** These files are used together with the DB file as input for the LAITOR4HPC job; **H)** which loads an in-memory database prior to the **(I)** tagging of the bioentities and biointeraction present in the corpus. **J)** After completion, the results are placed back to the head node and made available for downstream applications.

The LAITOR4HPC job execution used the DB file and the NLProt output files as inputs (Figure 2G). The jobs were launched from the head node, to be executed in 20 cores. Each *i*-th process is directed to a corresponding computing node together with the DB file, the LAITOR script and the NLProt output. Every computing node loads the DB file as an SQLite in-memory database on that node during execution (Figure 2H). Then the LAITOR4HPC script receives the *i*-th process and analyses it against the loaded in-memory database, which contains the bioentities and biointeraction dictionaries (Figure 2I). Once the results are obtained, they are made available back to the head node (Figure 2J). At the end of the job, all the LAITOR output files are retrieved back to the head node and can be copied by SCP or similar method to user-client computer; from there, users can further explore the text-mining outputs to create co-occurrence networks, for example.

By switching from MySQL to SQLite we avoid HPC limitations during the database querying in the HPC architecture as mentioned previously. Using SQLite in-memory, a new database is created purely in the memory of the computing nodes, this database ceases to exist as soon as the database connection is closed. As the database is self-contained in a text file, this file needs to be distributed across the computing nodes together with the input file to be analyzed.

The proposed parallel approach allowed us to query the whole *corpus* and to extract all its bioentities co-occurrences. In this context: the more articles available, the better the result. LAITOR4HPC running time is drastically decreased by the parallelization approach, as in comparison to the original version used by the PESCADOR website, where only a maximum of 1,000 papers could be read on-the-fly. Parallel SQL limitations caused by competitive accesses on the HPC environment were avoided by loading the database in the RAM memory of each computing node.

After soybean analysis, the pipeline tagged 7,894 co-occurrences, more than two times the amount of interactions available for this species in Soycyc (3,539 entries), a specific database that contains pathway data for soybean. This shows the efficiency of the pipeline. LAITOR tags four types of co-occurrences, according to the scope by which they were found in the text, being type 1 more and type 4 less likely to correspond to effective bioentities interactions. Consequently, to build reliable pathways, manual curation is advised [6].

By the keywords (Plant AND Defensin) search on PubMed, 770 papers were retrieved and analyzed. However, only five had interactions related to the regulation of PDF. This specific regulation pathway contains 14 elements among different proteins and hormones, showing that even the genes related to plant responses to biotic stress may use factors that are not directly related to this stress, regarding the known cross-talk response. This mechanism has been recognized, especially for plant antimicrobial peptides like PDF [19-21] where genes related to other functions are induced, like EIN3, related to salt tolerance [22], which can also induce defense against biotic factors (Figure 3).

The query “Plant AND Transposon” (PTP) didn’t retrieve abstracts where NLProt could identify bioentities names with precision. Consequently, it lacked to establish bioentities co-occurrences for this corpus. To succeed with this task, it will be necessary to build specific dictionaries and, likely, to use machine learning methods so that the text mining tools succeed to work with noncoding, repetitive sequences terms. It is an essential

demand to prepare those tools to pursue such a challenge, due to the influence of TEs in gene regulation, especially in eukaryotic organisms [23-28].

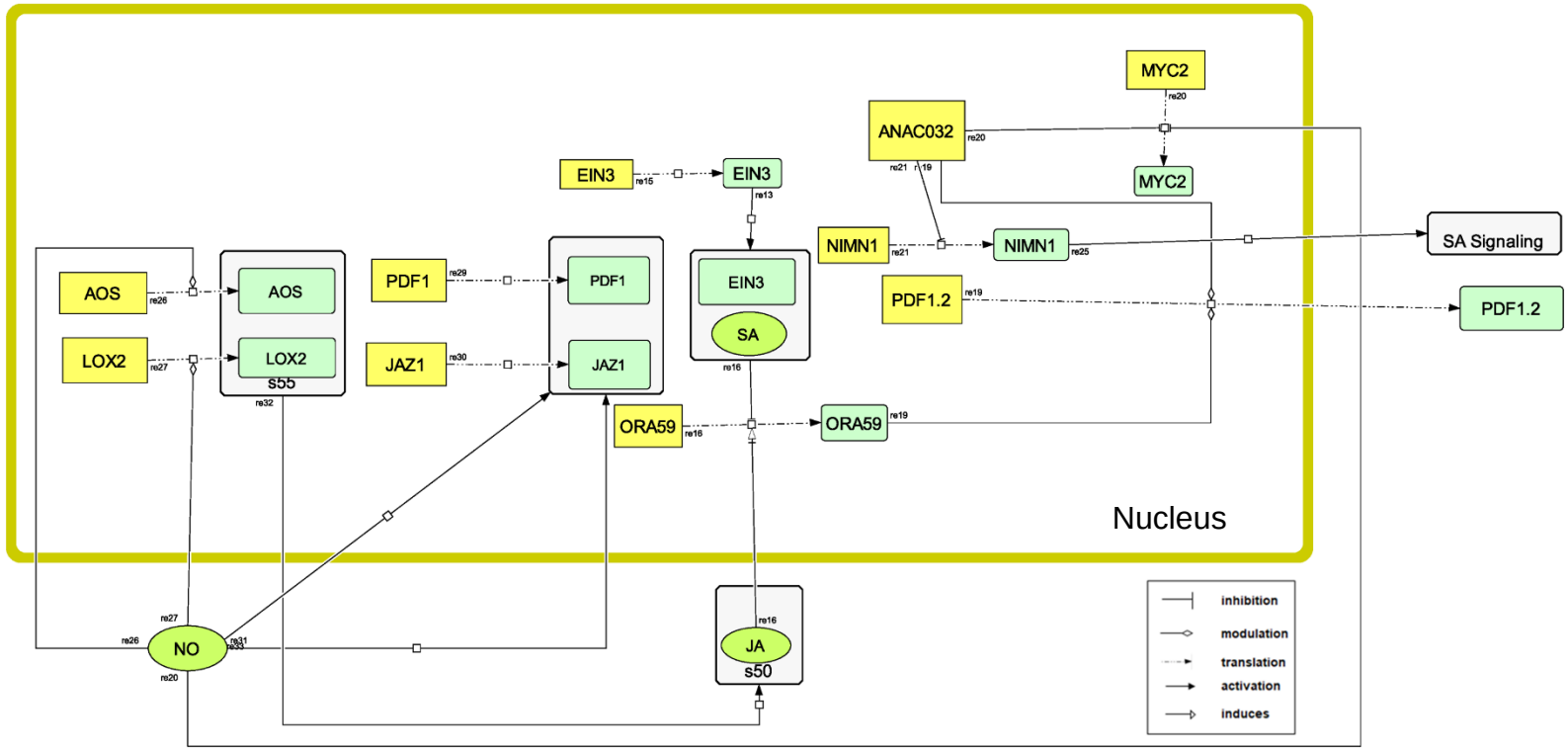

Figure 3: Regulation pathway of the PDF gene recovered by LAITOR4HPC. The pathway evidenced the elements that may induce (like the hormone jasmonic acid - JA), or repress (like the nitric oxide - NO), the expression of the gene in the nucleus. The yellow rectangles are the genes and the light green are the proteins.

Conclusion

The improvement of LAITOR, with the achievement of LAITOR4HPC decreased significantly the computing time, due to the implementation of parallelization. Such an improvement resulted not only in much faster run time, but also maintained the consistence of LAITOR4HPC considering the expected results. Time will vary according to available hardware resources, especially considering memory capacity and the number of available cores. Since this improved online tool include only data from abstracts, it is important to consider manual data curation to confirm predicted protein-protein interactions from term co-occurrences.

Text mining-based analysis using LAITOR4HPC has identified almost two times more putative protein-protein interactions in soybean than the ones deposited in the specific database Soycyc, making it a suitable tool for the enrichment of previously defined pathways, as well as for the establishment new ones. Therefore, it is expected that LAITOR4HPC will be useful not only to search for plant interactions, as in the present work, but also considering many other research questions, including human research and interactions involving multiple taxonomic categories.

Availability and requirements

Project name: BioComp - InterSys

Project home page: Will be available on GitHub

Operating system(s): Linux and MacOSX

Programming language: PHP and Python

Other requirements: SQLite / PHP interpreter

License: Not applicable

Restriction: Not applicable

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Anexo V

Patente submetida a Diretoria de Inovação e Empreendedorismo (DINE)

Novo peptídeo antimicrobiano modificado a partir da espécie *Cajanus cajan* com potencial atividade antimicrobiana

Autores: Ana Maria Benko-Iseppon; Lívia Maria Batista Vilela; Carlos André dos Santos Silva; Sergio Crovella; **Marx Oliveira de Lima**

UFPE POSITIVA DIRETORIA DE INOVAÇÃO

Universidade Federal de Pernambuco
Diretoria de Inovação

COMPROVANTE DE RECEBIMENTO DE DOCUMENTOS

Declaro para os devidos fins que esta diretoria recebeu no dia 17/05/2018, Comunicado de Invenção, Termo de Sigilo e Anexo de Inventores da solicitação para depósito do Pedido de Patente de título: **"Novo peptídeo antimicrobiano modificado a partir da espécie *Cajanus cajan* com potencial atividade antimicrobiana".**

Para:
(X) estudo por esta diretoria;
(X) busca prévia;
(X) análise e emissão de parecer de patenteabilidade;
(X) depósito dos documentos para Pedido de Patente de Invenção.

A presente solicitação pertence a um grupo tendo como inventora vinculada à UFPE Ana Maria Benko Iseppon, Portador do RG: 9.111.082 SSP/SP e CPF: 9.111.083, nascida em São Paulo - SP, estado civil: casada. A referida é professora da UFPE, do Departamento de Genética, do Centro de Biociências.

Os outros membros do Grupo, coinventores da tecnologia são: Lívia Maria Batista Vilela, Carlos André dos Santos Silva, Sergio Crovella, Marx Oliveira de Lima.

Recife, 14 de junho de 2018.

Atenciosamente,

Ana Carolina Borba Fernandes Padrão
Assistente em Administração
SIAPE 2156155
UFPE

Anexo VI

Patente submetida a Diretoria de Inovação e Empreendedorismo (DINE)

Novo peptídeo sintético modificado a partir de uma defensina de *Manihot esculenta*

Autores: Ana Maria Benko-Iseppon; Lívia Maria Batista Vilela; Carlos André dos Santos Silva; Sergio Crovella; **Marx Oliveira de Lima**

UFPE

POSITIVA
DIRETORIA DE INOVAÇÃO

Universidade Federal de Pernambuco
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COMPROVANTE DE RECEBIMENTO DE DOCUMENTOS

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Para:

(X) estudo por esta diretoria;

(X) busca prévia;

(X) análise e emissão de parecer de patenteabilidade;

(X) depósito dos documentos para Pedido de Patente de Invenção.

A presente solicitação pertence a um grupo tendo como inventora vinculada à UFPE Ana Maria Benko Iseppon, Portador do RG: 9.111.082 SSP/SP e CPF: 9.111.083, nascida em São Paulo - SP, estado civil: casada. A referida é professora da UFPE, do Departamento de Genética, do Centro de Biociências.

Os outros membros do Grupo, coinventores da tecnologia são: Carlos André dos Santos Silva, Lívia Maria Batista Vilela, Sergio Crovella, Marx Oliveira de Lima.

Recife, 14 de junho de 2018.

Atenciosamente,

Ana Carolina Borba Fernandes Padrão
Assistente em Administração
SIAPE 2156155
UFPE

Anexo VII – Resultados parciais

Trabalho em fase de conclusão

Prospecção, expressão e análise genômica: Uma busca por esnaquinas no transcriptoma do feijão caupi (*Vigna unguiculata*).

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- Foram utilizadas 30 sequencias *seed* para a prospecção no transcriptoma, onde foram recuperados 24 homólogos não redundantes que foram anotados para: presença e composição do domínio, peso molecular, pI, localização subcelular, provável atividade antimicrobiana e presença do peptídeo sinal.
- Os transcritos recuperados foram avaliados para o seu perfil de expressão baseado no log do *fold change* nas situações: infectado com Poty vírus 60 minutos e 16 horas, infectado com vírus do mosaico severo 60 minutos e 16 horas, Défict hídrico 25 minutos e 150 minutos. Onde foram gerados *heat maps* que serviram a seleção de 10 representantes para validação via RT-qPCR a qual encontra-se em andamento.
- Na análise genômica avaliamos a estrutura dos 24 trancritos mapeados, onde observamos a conservação das estruturas gênicas com dois, três ou quatro éxons. Esta análise encontra-se em andamento para a comparação com outras leguminosas modelo e/ou de interesse econômico.
- Por fim pretendemos observar a estrutura dos peptídeos recuperados via modelagem molecular, bem como avaliar sua estabilidade.