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CENTRO DE BIOCIÊNCIAS
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CAROLLINE DE JESÚS PIRES

ANÁLISE ESTRUTURAL E FUNCIONAL DA SUPERCLASSE PR-5 (PATHOGENESIS RELATED 5) NO TRANSCRIPTOMA DO FEIJÃO-CAUPI (*VIGNA UNGUICULATA*)

Recife
2019

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

Área de concentração: Biologia Molecular

Orientadora: Profª. Drª. Ana Maria Benko Iseppon

Coorientador: Dr. José Ribamar Costa Ferreira Neto

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Aprovada em: 20/03/2019

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Aos meus pais, Jorge Luís e Elizabete Maria,

pelo amor, incentivo e confiança,

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Nunca deixe que lhe digam que não vale a pena
acreditar no sonho que se tem
ou que os seus planos nunca vão dar certo
ou que você nunca vai ser alguém....”

(Renato Russo)

RESUMO

O feijão-caupi compreende uma leguminosa de significativa importância social e econômica no Brasil e no mundo, especialmente em regiões semiáridas. No entanto, sua produtividade é frequentemente afetada por estresses bióticos e abióticos. As TLPs (*Thaumatin-Like Proteins*) pertencem à superfamília PR-5 (*Patogenesis-Related 5*), que compreende uma significativa diversidade molecular e funcional, estando associada a estresses bióticos (defesa contra patógenos) bem como à tolerância a estresses ambientais (abióticos). O presente trabalho teve como objetivo caracterizar e analisar o transcriptoma do feijão-caupi inferindo sobre a diversidade estrutural e a expressão de TLPs sob diferentes estresses (inoculação com vírus CABMV ou CPSMV e desidratação de raízes). A análise fenética das VuTLPs utilizando o método *Neighbor-Joining* revelou grupamentos de VuTLPs funcionalmente especializadas. Além disso, a ancoragem de VuTLPs no genoma do feijão-caupi revelou a existência de 34 locos codificantes de TLPs, uma quantidade comparável com aqueles relatados para outras angiospermas. A análise de promotores associados às TLPs em bibliotecas de RNA-Seq de feijão-caupi sob diferentes estresses (biótico e abiótico) indicou uma pluralidade funcional. Por outro lado, a análise da expressão gênica de bibliotecas de RNA-Seq mostrou uma maior modulação de expressão de VuTLPs sob estresse abiótico (desidratação radicular) do que pela inoculação com CABMV ou CPSMV. Além disso, verificou-se que a maioria das VuTLPs apresentou especialização funcional específica a um determinado tipo de estresse, considerando as diferentes condições analisadas.

Palavras-chave: CABMV. CPSMV. Desidratação radicular. TLPs.

ABSTRACT

Cowpea is a legume of significant social and economic importance in Brazil and worldwide, especially in semiarid regions. However, their productivity is often affected by biotic and abiotic stresses. TLPs (Thaumatin-Like Proteins) belong to PR-5 (Pathogenesis-Related 5) protein superfamily that comprises a significant molecular and functional diversity, being associated with biotic stress (pathogen defense) besides tolerance to environmental (abiotic) stresses. The present work aimed to characterize and analyze the cowpea transcriptome inferring on TLP structural diversity and expression under different stresses (inoculation with CABMV or CPSMV viruses and root dehydration). Phenetic analysis of the VuTLPs using the method Neighbor-Joining revealed clusters of functionally specialized VuTLPs. Additionally the anchoring of VuTLPs in the cowpea genome revealed the existence of 34 encoding loci, an amount comparable with those reported for other angiosperms. The analysis of TLP-associated promoters in cowpea RNA-Seq libraries under different stresses (biotic and abiotic) indicated a functional plurality. On the other hand, gene expression analysis of RNA-Seq libraries showed a higher expression modulation of VuTLPs under abiotic stress (root dehydration) than by CABMV or CPSMV inoculation. In addition, it was verified that most VuTLPs presented functional specificity to a given stress type, considering the different conditions analyzed.

Keywords: CABMV. CPSMV. Root dehydration. TLPs.

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LISTA DE ABREVIATURAS E SIGLAS

ABA	Abscisic Acid (Ácido abscísico)
AGO	Proteínas Argonauta
ATP	Adenosina Trifosfato
Avr	Avirulence (Avirulência)
BAK1	BRI1-Associated receptor Kinase 1 (Receptor Quinase 1 Associado ao BRI1)
BCMV	Vírus do Mosaico Comum do Feijoeiro
BiCMV	Vírus do Mosaico do Feijão-caupi, estirpe “blackeye”
BKK1	Quinase 1 do tipo BAK1
BLAST	Basic Local Alignment Search Tool (Ferramenta de Busca por Alinhamento Local)
bZIP	Basic Leucine Zipper (Zíper de Leucina Básica)
CABMV	Cowpea Aphid-Born Mosaic Virus (Vírus do Mosaico do Feijão-caupi Transmitido por Afídeos)
CaLCuV	Geminivírus da Folha de Repolho
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CC	Coiled-Coil (Bobina Espiralada)
cDNA	Complementary DNA (DNA complementar)
CGMV	Vírus do Mosaico Dourado do Feijão-caupi
CGVBV	Vírus da Faixa Verde do Feijão-caupi
CMV	Vírus do Mosaico do Pepino
CpGC	Cowpea Genomics Consortium (Consórcio de Genômica do Feijão-Caupi)
CPRMV	Vírus do Mosaico Rugoso do Feijão-caupi
CPSMV	Cowpea Severe Mosaic Virus (Vírus do Mosaico Severo do Feijão-caupi)
CTD	Conserved Thaumatin Domain (Domínio Conservado da Taumatina)
DAMPs	Damage-Associated Molecular Patterns (Padrões Moleculares

	Associados a Danos)
DCL	Dicer-like ribonuclease
DNA	Deoxyribonucleic Acid (Ácido Desoxirribonucleico)
dsRNA	double-stranded RNA (RNA de fita dupla)
eIFs	Fatores de Iniciação Eucarióticos
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
ERF	Ethylene Response Factor (Fator de Resposta ao Etileno)
EST	Expressed Sequence Tag (Etiqueta de Sequência Expressa)
ET	Ethylene (Etileno)
ETI	Effector-Triggered Immunity (Imunidade Desencadeada por Efetores)
ETS	Suscetibilidade Desencadeada por Efetores
FPKM	Fragments Per Kilobase Million (Fragmentos por Milhões de Quilobases)
HR	Hypersensitive Response (Resposta Hipersensitiva)
JA	Jasmonic acid (Ácido Jasmônico)
LEA	Late Embryogenesis Abundant (Proteínas da Embriogênese Tardia)
LGO	Loss of Giant cells from Organs (Perda de células gigantes de órgãos)
LGOoe	overexpression of LGO throughout the epidermis (Superexpressão de LGO em toda a epiderme)
LMV	Lettuce Mosaic Virus (Potyvirus do Mosaico da Alface)
LTP	Lipid-transfer protein (Proteína de Transferência de Lipídios)
MAMPs	Microbe-Associated Molecular Patterns (Padrões Moleculares Associados a Micróbios)
MAP	Mitogen-Activated Protein (Proteína Ativada por Mitógenos)
MAPK	Mitogen-Activated Protein Kinases (Proteíno-Quinases Ativadas por Mitógenos)
MEGA	Molecular Evolutionary Genetic Analysis (Análises Genéticas da Evolução Molecular)
MethylJA	Methyl Jasmonic Acid (Ácido Metil Jasmônico)

MYB	Myeloblastosis (Mieloblastose)
MYC	Myelocytomatosis (Mielocitomastose)
NAC	NAM/ATAF1/CUC2
NBS-LRR	Sítio de Ligação ao Nucleotídeo - Repetição Nucleotídica rica em Leucina
NCBI	National Center for Biotechnology Information (Centro Nacional para Informação Biotecnológica)
NIK1	NSP-Interacting Kinase I
NRIP1	Nuclear Receptor-Interacting Protein 1
NSP	Nuclear Shuttle Protein
OXO	Oxalase oxidase
PAMPs	Pathogen-Associated Molecular Patterns (Padrão Molecular Associado a Patógenos)
PMeV	Vírus da Papaia Meleira
PPV	Plum Pox Virus (Potyvirus da Pústula da Ameixa)
PR	Pathogenesis-related (Proteínas Relacionadas à Patogênese)
PR5K	PR5-like receptor kinases
PRRs	Pattern Recognition Receptors (Receptores de Reconhecimento de Padrões)
PTGS	Post-Transcriptional Gene Silencing (Silenciamento Gênico Pós-Transcricional)
PTI	PAMP-Triggered Immunity (Imunidade Desencadeada por PAMP)
RISC	RNA-Induced Silencing Complex (Complexo Silenciador Citoplasmático Induzido por RNA)
RLKs	Receptor-Like Kinases (Receptores Quinases)
RLPs	Receptor-Like Proteins (Proteínas Semelhantes a Receptores)
RNA	Ribonucleic Acid (Ácido Ribonucleico)
RNAi	RNA de interferência
RNA-Seq	RNA Sequencing (Sequenciamento de RNA)
ROS	Reactive Oxygen Species (Espécies Reativas de Oxigênio)
RTM	Restricted TEV Movement (Movimento Restrito de TEV)
RT-qPCR	Real Time Quantitative PCR (PCR quantitativa em Tempo Real)

SA	Salicylic Acid (Ácido Salicílico)
SAR	Systemic Acquired Resistance (Resistência Sistêmica Adquirida)
SERK1	Somatic Embryogenesis Receptor Kinase 1 (Receptor Quinase de Embriogênese Somática 1)
SicysPI	Cysteine Protease Inhibitor (Inibidor da Protease de Cisteína)
SiGST	Glutathione-S-Transferase (Glutationa-S-Transferase)
SiSOD	Superoxide Dismutase (Superóxido Dismutase)
sRNA	small RNA (Pequenos RNAs)
siRNA	small interfering RNA (Pequenos RNA de Interferência)
sTLPs	small TLPs (Pequenas Thaumatin-like Proteins)
SuperSAGE	Serial Analysis of Gene Expression (Análise Serial da Expressão Gênica)
T-DNA	Transfer DNA (DNA de Transferência)
TEV	Tobacco Etch Virus (Potyvirus do Tabaco)
TFs	Transcription Factors (Fatores de Transcrição)
TGS	Transcriptional Gene Silencing (Silenciamento Gênico Transcricional)
TIR	Toll-interleucin-1 (Receptor de Interleucina 1)
TLPs	Thaumatin-like proteins
TMV	Tobacco Mosaic Tobamovirus (Tobamovirus de Tabaco)
VPg	Proteína Viral associada ao Genoma
VuTLPs	Thaumatin-like proteins de Vigna unguiculata

SUMÁRIO

1 INTRODUÇÃO

O feijão-caupi [*Vigna unguiculata* (L.) Walp.] é uma leguminosa de grande importância social e econômica no Brasil, principalmente nas regiões Norte e Nordeste. É comumente usada na dieta básica da população mais carente dessas regiões, destacando-se por sua riqueza em proteínas e minerais. Além da importância nutricional, essa espécie apresenta relevante participação na geração de emprego e renda de muitas famílias menos favorecidas. O Brasil, que já foi o maior produtor mundial, atualmente, ocupa terceira posição. Um dos principais fatores causadores da redução da produção nacional se deve ao ataque de patógenos, principalmente vírus do gênero Potyvirus [com ênfase para o *Cowpea aphid-borne mosaic virus* (CABMV)] e *Cowpea severe mosaic virus* (CPSMV), além de fenômenos edafoclimáticos como a seca e a alta salinidade dos solos. Assim, a obtenção de cultivares resistentes / tolerantes é um dos principais objetivos dos programas de melhoramento da referida cultura.

Ao longo de sua evolução, as plantas desenvolveram diversos mecanismos de combate a agentes patogênicos. Dentre esses, destacam-se a ação de proteínas PR (*Pathogenesis-related*). Tais proteínas formam um grupo heterogêneo e são codificadas por genes que são rapidamente induzidos por infecções patogênicas e por hormônios associados à defesa vegetal como ácido salicílico (SA), ácido jasmônico e etileno.

Dentre as 17 famílias que compõem as proteínas PR, destaca-se a classe PR-5 que inclui as proteínas do tipo taumatina (TLPs - *Thaumatin-Like Proteins*). Além de plantas, as TLPs podem ser encontradas em vários organismos, tais como fungos, insetos e nematoides.

O referido grupo proteico tem mostrado atividade antifúngica, sendo às vezes intimamente associado a características de resistência em mapas genéticos, como identificado em grão-de-bico (*Cicer arietinum*), onde marcadores moleculares intimamente ligados aos genes de resistência de *Fusarium oxysporum* fsp. *cicero* apresentaram alinhamentos significativos aos genes codificantes de TLPs localizados nos cromossomos 1 e 5 de *Arabidopsis thaliana*. Essa atividade antifúngica provavelmente ocorre através da permeabilização da membrana desses organismos. Adicionalmente, alguns representantes de TLPs

atuam sobre a invasão de fungos através de suas atividades de ligação ou hidrólise de β-1,3-glucanos ou inibição de enzimas, como xilanases.

Tais proteínas, além de serem responsivas a infecções por patógenos, podem ser reguladas também em resposta a alguns fatores abióticos como seca, alta salinidade e temperatura de congelamento. De outra forma, em algumas espécies, as TLPs são constitutivamente expressas em flores e frutos, possivelmente atuando na defesa pré-formada contra infecções. Vale ressaltar ainda que genes codificadores de TLPs podem ser induzidos por hormônios associados à imunidade vegetal. Além dessas funções, algumas TLPs de frutas destacam-se por possuir propriedades alergênicas, como Mal d 2, uma TLP alergênica da maçã, que foi a primeira TLP descrita apresentando essa propriedade.

Dessa forma, o interesse por genes pertencentes à família das TLPs (ou PR-5) vem tomando proporções cada vez maiores nos programas de melhoramento vegetal, principalmente pelo seu grande potencial biotecnológico para geração de plantas transgênicas com maior resistência / tolerância a estresses. Diversos trabalhos têm relatado o impacto positivo da expressão de TLPs na fisiologia vegetal sob condições não favoráveis.

Dada à importância, multifuncionalidade e impacto positivo desse grupo proteico em plantas sob condições desfavoráveis, o presente trabalho visou identificar, caracterizar e analisar TLPs em feijão-caupi submetido a estresses bióticos e abióticos, além de estudar a modulação de sua expressão gênica, a fim de obter candidatos promissores ao melhoramento genético desta espécie.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

Identificar e avaliar a expressão de TLPs de feijão-caupi envolvidas nos mecanismos moleculares que governam a resistência ao ataque de CABMV ou CPSMV, além daquelas participantes de processos de tolerância à desidratação radicular ou alta salinidade, inferindo sobre sua diversidade e função no feijão-caupi.

1.1.2 Objetivos Específicos

a) Identificar, analisar e caracterizar transcritos codificadores de TLPs expressos em resposta à infecção por CABMV ou CPSMV, além daqueles responsivos à desidratação radicular, nas bibliotecas de expressão gênica do CpGC (*Cowpea Genomics Consortium*);
b) Localizar os domínios e motivos conservados ancorados nos transcritos codificadores de TLPs para a realização de estudos comparativos com sequências de genes previamente caracterizadas;
c) Estabelecer um perfil da expressão *in silico* de no mínimo 20 transcritos candidatos (a partir da análise da presença ou ausência, nos diferentes tratamentos) e validar sua expressão via qPCR;
d) Realizar uma análise de genômica comparativa por meio da ancoragem de transcritos codificadores de TLPs no genoma de *Phaseolus vulgaris* e do feijão-caupi;
e) Realizar análise fenética para comparar os candidatos a TLPs preditos em feijão-caupi com TLPs de outras espécies disponíveis em bancos públicos, inferindo sobre sua estrutura e função.

2 REVISÃO DA LITERATURA

2.1 A IMPORTÂNCIA ESTRATÉGICA DO FEIJÃO-CAUPI: SOCIOECONOMIA, NUTRIÇÃO E GENÉTICA

Globalmente, o feijão-caupi é uma das 23 espécies cultivadas mais importantes (COWPEA GENOMICS, 2008), apresentando produção mundial estimada em 5,3 milhões de toneladas (FAO, 2017). No que se refere ao Brasil, esta leguminosa apresenta produção e consumo destacados nas regiões Norte e Nordeste do país, com expansão para regiões de cerrado. Isso se deve, principalmente, à precocidade de seu desenvolvimento e tolerância ao déficit hídrico, além de seu porte ereto e cultivo mecanizado (BASTOS, 2016; FREIRE-FILHO *et al*., 2011). Para essas regiões, tal leguminosa apresenta grande importância socioeconômica (FREIRE-FILHO *et al*., 2011). Os últimos dados disponíveis do Instituto Brasileiro de Geografia e Estatística (IBGE) sobre indicadores de emprego e faturamento associados à cultura apontaram que mais de um milhão de empregos foram gerados em sua cadeia produtiva, com valor da produção superando R$ 700 milhões de reais (IBGE, 2005-2009).

Em 2011, a produção de feijão-caupi no Brasil foi de aproximadamente 800 mil toneladas, colhidas em 1,7 milhões de hectares, com produtividade média de 464 $kg.ha^{-1}$. Esse índice esteve associado a boas condições pluviométricas, especialmente no Nordeste brasileiro (BASTOS, 2016). Em 2016, a produção da referida cultura foi de aproximadamente 345 mil toneladas, colhidas em 1,1 milhão de hectares, com uma produtividade média de 323 $kg.ha^{-1}$. Essa redução drástica na produção foi devido, principalmente, às adversidades climáticas. Já em 2017, o índice voltou a subir novamente, apresentando produção de cerca de 648 mil toneladas, colhidas em 1,4 milhão de hectares, com uma produtividade média de 466 $kg.ha^{-1}$ (EMBRAPA ARROZ E FEIJÃO, 2017).

No que se refere à conquista do mercado internacional, o Brasil é o terceiro maior exportador mundial de feijão-caupi, atrás somente de Estados Unidos da América e Peru, respectivamente (SOCIEDADE NACIONAL DE AGRICULTURA, 2017). Em 2013, nosso país exportou 24 mil toneladas de feijão-caupi,

movimentando cerca de US$ 16,5 milhões, segundo o Ministério do Desenvolvimento, Indústria e Comércio Exterior (MDIC, 2017).

Quanto ao aspecto nutricional, essa espécie representa cerca de 80 % dos grãos utilizados na alimentação humana das regiões Norte e Nordeste do Brasil (DANTAS *et al*., 2005). Constitui importante fonte de proteínas (23 a 25 % em média) e carboidratos, destacando-se pelo alto teor de fibras alimentares, vitaminas e minerais, além de possuir baixos teores de lipídios que se restringem, em média, a 2 % (EMBRAPA MEIO NORTE, 2003).

Além do exposto, o feijão-caupi é bem adaptado às condições de clima e solo das regiões citadas, sendo ao mesmo tempo possuidora de uma grande variabilidade genética, a qual a torna versátil, podendo ser usada em diferentes sistemas de produção, tradicionais ou modernos. Por isso, é considerada uma espécie de grande valor atual e estratégico (FREIRE-FILHO *et al*., 2005).

2.2 IMPACTO DE ESTRESSES ABIÓTICOS E BIÓTICOS NA CULTURA DO FEIJÃO-CAUPI

O crescimento e desenvolvimento do feijão-caupi pode tornar-se limitado diante de alguns fatores de natureza biótica e abiótica que causam estresses à cultura (BOUKAR *et al*., 2016). Os fatores bióticos compreendem as relações diretas entre a planta e outros organismos, que podem lhes trazer benefícios ou resultar em danos lesivos, neste último caso tornando-se um fator de estresse (HARTLEY, 2001). Os fatores abióticos compreendem parâmetros e recursos da natureza necessários para o desenvolvimento do vegetal, porém quando disponíveis em níveis extremos ou insuficientes tornam-se elementos estressores que prejudicam sua sobrevivência (SCHULZE, BECK e MULLER-HOHENSTEIN, 2005).

Dentre os estresses bióticos de importância econômica que afetam negativamente o feijão-caupi, destacam-se os insetos de maneira geral, as ervas daninhas, fungos, bactérias, nematoides e viroses (BASTOS, 2016; BOUKAR *et al*., 2016). Por sua vez, os elementos estressores de natureza abiótica que mais afetam a produção do feijão-caupi incluem a seca, o calor, a baixa fertilidade do

solo (BOUKAR *et al*., 2016), altos níveis de radiação solar (SINGH *et al*., 2010), entre outros.

2.2.1 Seca

As mudanças previstas no clima podem levar a eventos extremos, como o aumento da precipitação e longos períodos de seca (IPCC, 2007). Dessa forma, a seca destaca-se como um dos fatores mais deletérios para a produção agrícola, uma vez que está diretamente relacionada com um dos recursos mais importantes para o desenvolvimento da planta (PINHEIRO e CHAVES, 2011).

No Nordeste brasileiro, o feijão-caupi é a principal leguminosa cultivada devido à sua plasticidade morfológica e fisiológica sob estresse hídrico (ANYIA e HERZOG, 2004; SOUZA *et al*., 2004; HAMIDOU, ZOMBRE e BRACONNIER, 2007; BASTOS *et al*., 2011). Plantas de feijão-caupi podem produzir mais de 1.000 kg de grãos.ha^{-1}, mas o estresse hídrico reduz esse potencial para aproximadamente 360 kg.ha^{-1} (BASTOS *et al*., 2011; NASCIMENTO *et al*., 2011), especialmente quando ocorre durante a floração. Apesar de ser adaptada às condições ambientais de cultivo, essa espécie pode apresentar baixa produtividade decorrente de vários outros fatores, como o uso de sementes não melhoradas, o cultivo em solos de baixa fertilidade e, principalmente, a ocorrência de precipitações pluviométricas irregulares (NASCIMENTO *et al*., 2011).

2.2.2 Viroses

No contexto das interações bióticas, incluem-se doenças causadas por agentes patogênicos (incluindo fungos, bactérias e nematoides), além de predação por insetos, especialmente na fase pós-colheita. Porém, as viroses são consideradas os fatores mais limitantes ao cultivo do feijão-caupi, podendo ocasionar perda total da produção (BARROS, 2010).

Os vírus são agentes infecciosos cuja incidência e severidade variam dependendo do hospedeiro, do vetor e da fonte de inóculo (CAMARÇO *et al*., 2009). Como parasitas intracelulares obrigatórios, os vírus de plantas dependem da maquinaria do hospedeiro para se multiplicarem e invadirem seus hospedeiros.

Em sua forma mais simples, os vírus consistem em um segmento genômico de DNA ou RNA que codifica apenas alguns genes e é encapsulado em um invólucro de proteína, denominado capsídeo. Por causa das barreiras físicas naturais (cutícula, parede celular), os vírus só conseguem entrar nas células das plantas através de feridas ou através da ação de vetores (insetos, nematóides, fungos), que se alimentam ou infectam as plantas. Após a entrada em uma célula hospedeira e decapsidação do genoma, o ciclo infeccioso inclui tradução e replicação do genoma viral, montagem de partículas virais da progênie, invasão generalizada do hospedeiro através de movimentos célula a célula (por meio dos plasmodesmos), de longa distância (sistêmico, ou seja, por meio dos tecidos vasculares do floema), de partículas virais ou complexos de ribonucleoproteínas, e finalmente, transmissão para novos hospedeiros por meio de vetores. Em alguns casos, a transmissão para a geração seguinte da planta hospedeira também é observada como resultado da infecção de sementes (NICAISE, 2014).

No Brasil, os principais vírus que infectam o feijão-caupi são: o Vírus do Mosaico do Pepino (*Cucumis mosaic virus* - CMV), o Vírus do Mosaico Severo do Feijão-caupi (*Cowpea severe mosaic virus* - CPSMV), o Vírus do Mosaico Dourado do Feijão-caupi (*Cowpea golden mosaic virus* - CGMV), o Vírus do Mosaico Comum do Feijoeiro (*Bean common mosaic virus* - BCMV), o Vírus do Mosaico do Feijão-caupi Transmitido por Afídeos (*Cowpea aphid-borne mosaic virus* - CABMV), o Vírus da Faixa Verde do Feijão-caupi (*Cowpea green vein banding virus* - CGVBV), o Vírus do Mosaico Rugoso do Feijão-caupi (*Cowpea rugose mosaic virus* - CPRMV) (KITAJIMA, 1995; LIMA *et al*., 1998; BASTOS, 2016), e o Vírus do Mosaico do Feijão-caupi, estirpe "*blackeye*" (*Blackeye cowpea mosaic virus* - BiCMV) (LIN, KITAJIMA e RIOS, 1981). Dentre esses, será dado destaque aos dois mais importantes (CPSMV e CABMV), devido à severidade com que acometem a cultura (BASTOS, 2016).

2.2.2.1 Vírus do Mosaico do Feijão-caupi Transmitido por Afídeos (CABMV)

O CABMV pertence à família Potyviridae e ao gênero *Potyvirus*. Este gênero reúne mais de 100 espécies virais que infectam plantas, correspondendo a 16 % de todas as viroses que acometem esses organismos, o que justifica a

grande importância econômica do referido grupo (LIMA, SITTOLIN e LIMA, 2005). Sua transmissão ocorre em segundos, por meio de várias espécies de pulgões (como *Aphis craccivora* e *Myzus persicae*), através de “picadas de prova”, transmissão não persistente (PIRONE, 1991; GRAY, 1996; BASTOS, 2016). Adicionalmente, tal vírus pode ser transmitido também pelas sementes (BASTOS, 2016).

O CABMV apresenta partículas filamentosas e flexuosas, com dimensões de cerca de 690 a 760 nm. Seu genoma é constituído de RNA de fita simples, senso positivo, e um único tipo de proteína capsidial de aproximadamente 38 kDa (VAN REGENMORTEL *et al*., 2000).

Estudos mostraram que há relatos dessa doença no Brasil desde 1974, tendo sido observada também nos Estados Unidos, Itália, Holanda, Austrália, Japão, Filipinas, China, Marrocos e Zâmbia (PIO-RIBEIRO, ANDRADE e ASSIS FILHO, 2005).

Os sintomas são caracterizados pelo aparecimento, nos folíolos, de um mosaico forte marcado por áreas amareladas alternadas por outras de verde normal (**Figura 1**). A presença de faixas verdes nas nervuras pode estar associada ou não à distorção foliar. Quando as plantas são infectadas no início de seu desenvolvimento, elas podem apresentar redução no porte, podendo levar ao nanismo (BASTOS, 2016).

Entre as medidas de controle para o CABMV, pode-se destacar: emprego de cultivares resistentes, uso de sementes sadias, eliminação de plantas hospedeiras do vírus, controle da população de vetores, bem como plantio em época de baixa população dos insetos transmissores. Recomenda-se, ainda, o uso de fileiras adensadas de milho ou sorgo, para proteger a cultura, plantadas 15 dias antes do plantio de feijão-caupi (BASTOS, 2016).

Figura 1. Morfologia de uma planta de feijão-caupi inoculada com CABMV.

Fonte: BARROS (2010).

2.2.2.2 Vírus do Mosaico Severo do Feijão-caupi (CPSMV)

Esse vírus pertence à família Comoviridae, gênero *Comovirus* e apresenta forma isomérica de aproximadamente 28 nm de diâmetro, possuindo um genoma bipartido com um total de 9,73 kb. É constituído de duas moléculas de RNA de fita simples (uma de 6,0 kb – RNA 1 e outra de 3,73 kb – RNA 2), com senso positivo (VAN REGENMORTEL *et al*., 2000; LIMA, SITTOLIN e LIMA, 2005). Os RNAs genômicos possuem uma pequena proteína básica [proteína viral associada ao genoma (VPg)] covalentemente ligada às suas extremidades 5', além de serem poliadenilados na extremidade 3' (CHEN e BRUENING, 1992).

No Brasil, o seu primeiro registro foi datado de 1947 (PIO-RIBEIRO, ANDRADE e ASSIS FILHO, 2005). Atualmente, encontra-se disseminado em praticamente todas as regiões produtoras do país, tendo sua ocorrência registrada nos seguintes estados brasileiros: Acre, Amapá, Amazonas, Bahia, Ceará, Goiás, Maranhão, Pará, Paraíba, Paraná, Pernambuco, Piauí, Rio Grande do Norte, Rio Grande do Sul, Rio de Janeiro, São Paulo e no Distrito Federal (BRIOSO *et al*., 1994). Além do Brasil, a ocorrência do vírus do CPSMV abrange

também Estados Unidos, Trinidad e Tobago, Porto Rico, El Salvador, Venezuela, Costa Rica, Suriname e Peru (PIO-RIBEIRO, ANDRADE e ASSIS FILHO, 2005).

O CPSMV é de fácil transmissão mecânica e em condições naturais é transmitido por espécies de besouros dos gêneros *Diabrotica* e *Cerotoma* (COSTA *et al*., 1978), havendo também registros de sua transmissão pelo manhoso (*Chalcodermus bimaculatus*; ATHAYDE SOBRINHO, VIANA e SANTOS, 2000).

Os principais sintomas apresentados por essa virose são: intenso encrespamento do limbo foliar (em virtude das bolhosidades) e a presença de mosqueado, ou seja, alternância de zonas de coloração verde-clara com outras de cor verde-escura nos folíolos (**Figura 2**). Também pode ser observado subdesenvolvimento das nervuras principais, resultando em franzimento e redução do limbo e distorção foliar (BASTOS, 2016).

Quando infectadas no início do ciclo, as plantas apresentam intenso nanismo, o que pode ocasionar grandes perdas na produção. Essa virose pode diminuir a produção em até 80 %, dependendo da idade em que a planta foi infectada. As sementes obtidas de plantas doentes apresentam-se deformadas, murchadas e manchadas, com redução de seu poder germinativo (BASTOS, 2016).

Figura 2. Morfologia de uma planta de feijão-caupi inoculada com CPSMV.

Fonte: BARROS (2010).

A melhor forma de controle a ser adotada é por meio da utilização de cultivares comerciais altamente resistentes, como a BR 10 - Piauí, BR 14 - Mulato e BR 17 - Gurguéia. Outras medidas podem ser tomadas, como controle dos vetores, plantio em época de baixa população dos vetores e eliminação das plantas hospedeiras silvestres (BASTOS, 2016).

2.3 MECANISMOS VEGETAIS DE RESPOSTA A ESTRESSES

As plantas devem produzir uma resposta adequada às várias condições específicas de estresse biótico e abiótico, tendo em vista que em muitos casos os diferentes tipos de pressões ambientais provocam reações diferenciadas. Assim, as plantas devem equilibrar a alocação de recursos entre crescimento e defesa frente aos estresses, já que a resposta ao estresse pode exigir da planta um maior esforço, em detrimento do crescimento e da produção vegetal (BECHTOLD *et al*., 2010).

Dessa forma, compreender os mecanismos pelos quais as plantas transmitem os sinais para a maquinaria celular para ativar respostas adaptativas é de fundamental importância para desenvolver culturas mais tolerantes e resistentes, melhorando a eficiência da produção.

2.3.1 Seca

De forma geral, os estresses abióticos iniciam com a percepção do estresse, que desencadeia uma cascata de eventos moleculares, levando à transmissão e ao processamento de sinais para geração de respostas mediadas por fitormônios, mensageiros secundários, fatores de transcrição, genes e ou proteínas sinalizadoras (**Figura 3**) (PANDEY, 2008).

Além disso, várias respostas bioquímicas e fisiológicas são induzidas nas plantas, de maneira a proporcionar tolerância ou aumentar as chances de sobrevivência às condições adversas. Por exemplo, plantas mais adaptadas à seca, mesmo ocorrendo em diferentes continentes, apresentam frequentemente estratégias semelhantes de sobrevivência. Entre essas estratégias, pode-se citar

suculência, dormência, folhas com camadas serosas ou ainda a capacidade de armazenar água e nutrientes em estruturas específicas das raízes, entre outras adaptações, as quais dificilmente podem ser transferidas para as plantas cultivadas por meio do melhoramento genético ou mesmo da biotecnologia (BENKO-ISEPPON *et al*., 2011).

Segundo Seki *et al*. (2002), os mecanismos genéticos de percepção que estão relacionados às respostas à seca em plantas incluem diversos genes, os quais têm sido tradicionalmente divididos em duas categorias: (I) os envolvidos na cascata de sinalização e no controle transcricional, como MYC, quinases MAP e quinase SOS2, fosfolipases e fatores de transcrição, como MYB, NAC, bZIP, ERF (**Figura 3A**); (II) aqueles que funcionam diretamente na proteção de membranas e proteínas, como proteínas de choque térmico e chaperonas, proteínas LEA, osmoprotetores e removedores de radicais livres, bem como aqueles envolvidos na captura e transporte de água e íons, como as aquaporinas e os transportadores de íons (**Figura 3B**).

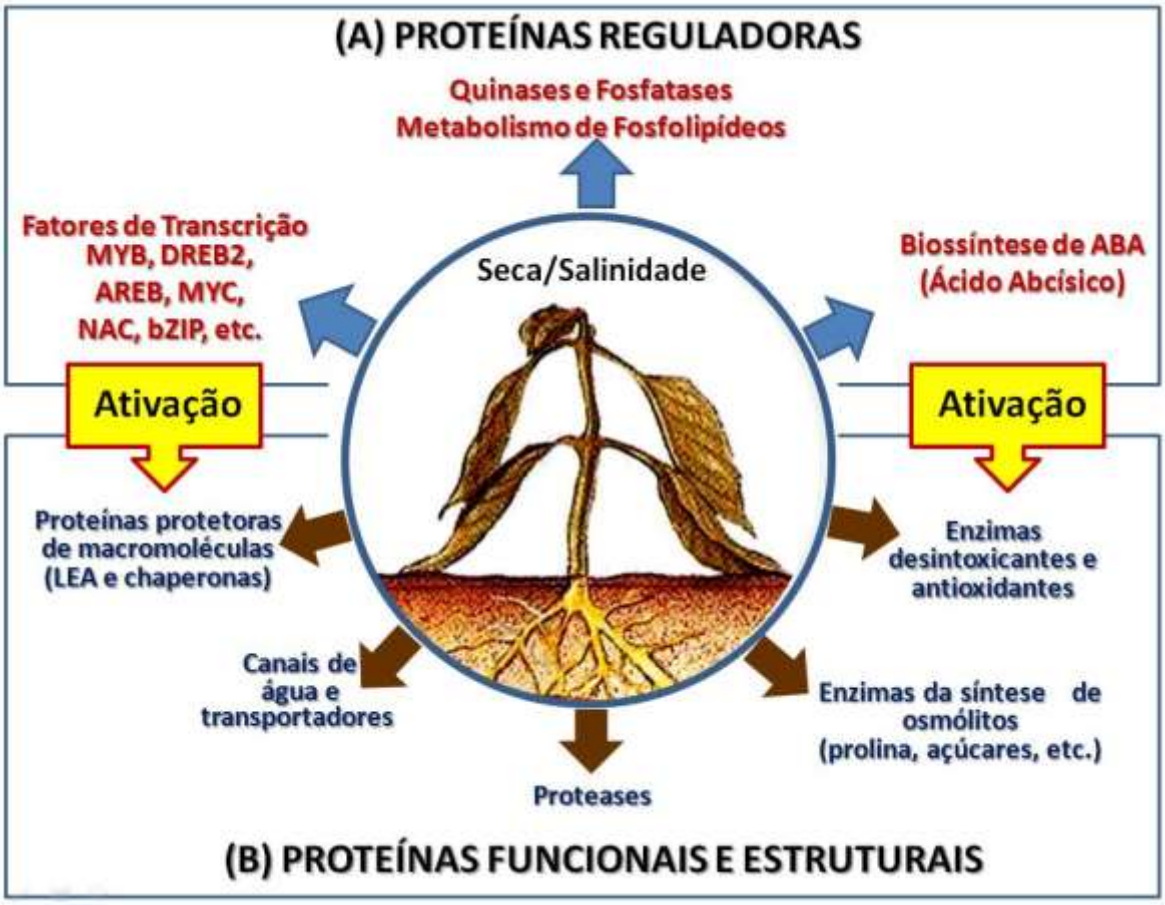
Nesse contexto, alguns mecanismos de adaptação à seca já foram descritos para alguns genótipos do feijão-caupi, considerada uma cultura tolerante por sua capacidade de crescer em solos com baixa disponibilidade hídrica (AYALA *et al*., 2014). A tolerância à seca do feijão-caupi afeta praticamente todos os estágios de crescimento, e as respostas ao estresse hídrico têm um componente genético (MUCHERO, EHLERS e ROBERTS, 2008), o que torna a espécie um modelo interessante para investigar as bases da adaptação à seca.

Algumas variedades de feijão-caupi, por exemplo, escapam da seca terminal, florescendo 12 dias mais cedo, em média, enquanto outras podem permanecer verdes por semanas sem irrigação e só florescem quando condições climáticas favoráveis são restabelecidas (FATOKUN, BOUKAR e MURANAKA, 2012). Sendo assim, o feijão-caupi é bastante incomum entre as plantas cultivadas, pois exibe mudanças discretas no teor de água das folhas em secas extremas (AGBICODO *et al*., 2009).

Nesse aspecto, alguns fatores são responsáveis por reduzir a transpiração no feijão-caupi sob estresse hídrico (HALL e SCHULZE, 1980; EKOW, 1985). Dentre eles, pode-se citar: movimentos foliares extensos para minimizar a interceptação da radiação (SHACKEL e HALL, 1979) e a redução na condutância

estomática (EKOW, 1985). Entre os mecanismos associados em feijão-caupi para manter o status da água destacam-se: (a) aumento da densidade e profundidade das raízes durante o estresse para explorar um volume maior de solo; e (b) mecanismos que resultam apenas na lenta extração da água do solo, à medida que o solo começa a secar (TURK e HALL, 1980), sendo que este último permite a sobrevivência da cultura durante a seca.

Figura 3. Principais categorias de genes envolvidos na resposta de plantas a estresses abióticos como seca. O grupo A inclui as proteínas reguladoras que estão envolvidas na regulação e transdução de sinais e são ativadas logo após o estresse. Tais proteínas induzem cascatas de sinais envolvendo algumas proteínas do grupo B, consideradas estruturais e funcionais, envolvidas nos mecanismos de tolerância ao estresse citado.



Fonte: BENKO-ISEPPON *et al*., 2011.

Rivas *et al*. (2016) estudaram o comportamento fotossintético *in vivo* de duas cultivares contrastantes de feijão-caupi sob condições de disponibilidade de

água, estresse hídrico e recuperação a fim de explorar os mecanismos fisiológicos que contribuem para a tolerância à seca. Eles observaram que após 10 dias de déficit hídrico (imposto pela suspensão de rega), o potencial de água na folha da cultivar tolerante (Pingo de Ouro) foi maior que na cultivar sensível (Santo Inácio), sugerindo um mecanismo de tolerância à seca relacionado à manutenção do *status* hídrico da parte aérea. Além disso, durante o estresse hídrico, os parâmetros de troca gasosa e clorofila diminuíram mais rapidamente na cultivar sensível quando comparada à tolerante. Após 48 h de reidratação, as plantas estressadas de ambas as cultivares não recuperaram as taxas máximas de carboxilação. No entanto, a cultivar tolerante recuperou todos os parâmetros fotossintéticos mais rapidamente que a cultivar sensível após 60 h de reidratação. Assim, a cultivar tolerante foi capaz de manter maior atividade fotoquímica e troca gasosa foliar durante o déficit hídrico por um período mais longo que a cultivar sensível, o que poderia aliviar os efeitos do estresse sobre o mecanismo fotossintético e melhorar sua capacidade de recuperação. Trabalhos como esse, abordando o metabolismo fotossintético de cultivares de feijão-caupi sob estresse hídrico e sua posterior recuperação, ainda são limitados (SINGH e REDDY, 2011; SOUZA *et al*., 2004).

Por outro lado, vale ressaltar que a base genética da resposta à seca é bastante complexa e envolve modificações anatômicas, morfológicas, fisiológicas e bioquímicas, afetando vários aspectos do crescimento vegetal (MENDES *et al*., 2007), o que torna o feijão-caupi um modelo interessante para investigar os mecanismos genéticos da adaptação à seca.

2.3.2 Viroses

2.3.2.1 Sistema imune em plantas

A imunidade inata é um mecanismo evolutivo antigo que protege plantas e animais de uma ampla gama de patógenos (ZVEREVA e POOGGIN, 2012). As plantas reconhecem os patógenos principalmente através de duas classes distintas de receptores imunes (SCHWESSINGER e RONALD, 2012; SPOEL e DONG, 2012; ZVEREVA e POOGGIN, 2012; DANGL, HORVATH e

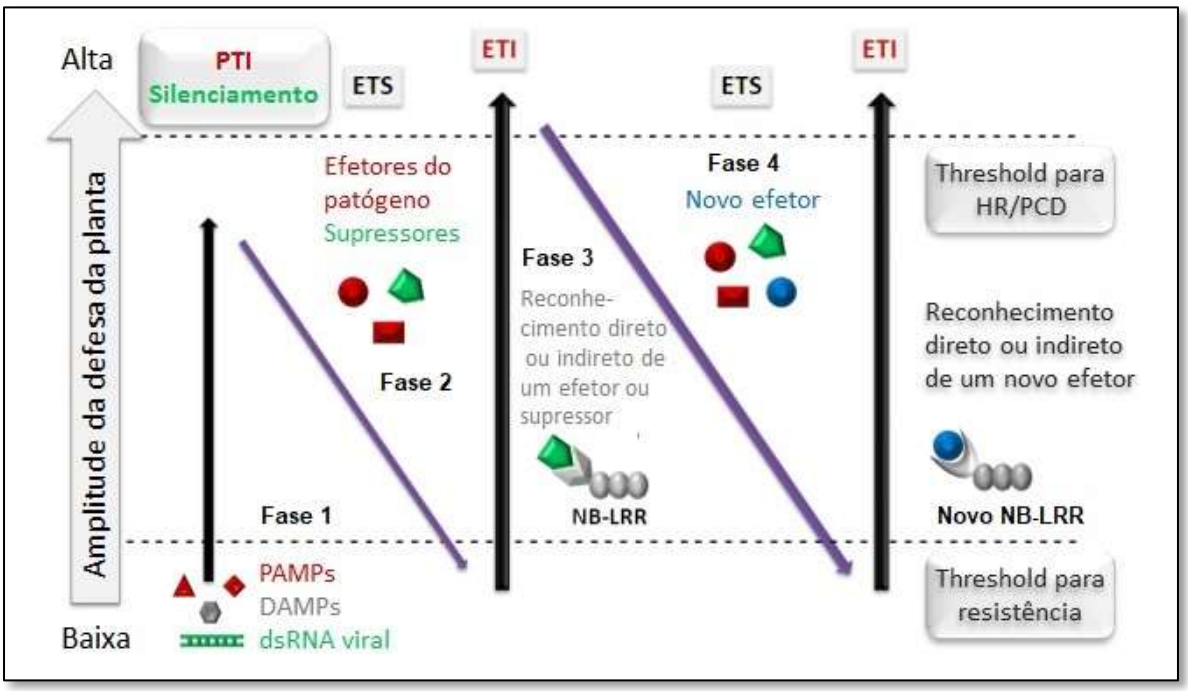
STASKAWICZ, 2013). A primeira classe consiste em receptores de reconhecimento de padrões (PRRs - *Pattern Recognition Receptors*) associados à superfície celular, que são frequentemente representados por receptores quinases (RLKs - *Receptor-Like Kinases*) e proteínas semelhantes a receptores (RLPs - *Receptor-Like Proteins*). Os PRRs reconhecem motivos estruturais conservados presentes em micróbios, que são conhecidos como padrões moleculares associados a micróbios ou a patógenos (MAMPs ou PAMPs - *Microbe- or pathogen-Associated Molecular Patterns*) (MACHO e ZIPFEL, 2014). Dessa forma, a percepção de PAMPs por PRRs ativa a imunidade desencadeada por PAMP (PTI - *PAMP-Triggered Immunity*), que consiste em uma cascata de transdução de sinais que culmina com a reprogramação transcricional e biossíntese de moléculas de defesa específicas (HOGENHOUT *et al*., 2009; BIGEARD, COLCOMBET e HIRT, 2015). Assim, a ativação dessa resposta imune permite que as plantas respondam rápida e eficientemente a uma grande variedade de patógenos (ROUX *et al*., 2014).

Já a segunda classe de receptores imunes inclui receptores intracelulares chamados proteínas R (JONES e DANGL, 2006; TSUDA e KATAGIRI, 2010). Esses receptores reconhecem direta ou indiretamente efetores secretados por patógenos para o ambiente intracelular do hospedeiro e ativam a imunidade desencadeada por efetores (ETI - *Effector-Triggered Immunity*; HOWDEN e HUITEMA, 2012), que é frequentemente manifestada na resposta hipersensitiva (HR, *Hypersensitive Response*), associada à morte celular rápida, produção de espécies reativas de oxigênio (ROS) e ácido salicílico (SA), bem como a expressão de genes relacionados à defesa (WIN *et al*., 2012).

O modelo amplamente aceito para evolução do sistema imune em plantas foi proposto originalmente por Jones e Dangl (2006), sendo conhecido como modelo Zig-Zag. Porém, tal modelo levava em consideração apenas as interações entre plantas e patógenos não virais. Uma vez que vários estudos têm mostrado a atuação do sistema imune de plantas em resposta às infecções virais, um novo modelo Zig-Zag foi proposto por Zvereva e Pooggin (2012). Esse novo modelo agrega informações relacionadas às interações planta-vírus ao modelo proposto por Jones e Dangl (2006), sendo, portanto, uma extensão desse modelo.

A interação segue quatro fases (**Figura 4**). Na fase 1 as plantas detectam PAMPs e padrões moleculares associados a danos (DAMPs - *Damage-Associated Molecular Patterns*) via PRRs para induzir a PTI e, no caso de patógenos virais de RNA, as plantas detectam RNA viral de cadeia dupla (dsRNA) para ativar o silenciamento de RNA. Na fase 2 os agentes patogênicos virais e não virais bem-sucedidos apresentam efetores / supressores que interferem tanto no PTI quanto no silenciamento, resultando em susceptibilidade desencadeada por efetores (ETS). Na fase 3 um efetor ou supressor é reconhecido direta ou indiretamente por uma proteína NB-LRR, ativando a ETI, que poderá levar a uma resposta hipersensível e morte celular programada (PCD - *Programmed Cell Death*). Já na fase 4, os patógenos desenvolveram mecanismos (perderam ou modificaram o efetor/supressor especificamente reconhecido) para evitar a ETI. Em contrapartida, as plantas desenvolveram novas especificidades de proteínas R com a finalidade de acionar novamente a ETI. Assim, o resultado da interação planta-patógeno é proporcional à [(PTI + Silenciamento – ETS) + ETI] (ZVEREVA e POOGGIN, 2012).

Figura 4. Modelo Zig-Zag para evolução da imunidade inata e silenciamento de plantas baseado na defesa contra patógenos virais e não virais [extensão do modelo proposto por Jones e Dangl (2006)].



dsRNA: RNA de cadeia dupla; DAMPs: Padrões Moleculares Associados a Danos; PAMPs: Padrões Moleculares Associados a Patógenos; PTI: Imunidade Desencadeada por PAMP; ETS: Susceptibilidade Desencadeada por Efetores; ETI: Imunidade Desencadeada por Efetores; HR: Resposta Hipersensitiva; PCD: Morte Celular Programada. Adaptado de Zvereva e Pooggin (2012).

2.3.2.2 PTI em resposta a vírus

A existência de PRRs específicos de animais para os PAMPs virais levanta a questão da existência de PRRs que percebem os vírus de plantas (NICAISE, 2014). Sendo assim, dados atuais sugerem que os componentes virais podem atuar como PAMPs, mas não eliminam a possibilidade de que os DAMPs (Padrões Moleculares Associados a Danos), produzidos pela planta durante ferimentos ou ataque patogênico, possam potencialmente provocar respostas antivirais baseadas em PTI nas plantas. Recentemente, RNA de fita dupla (dsRNA) funcionou como PAMPs virais em *Arabidopsis* ao induzir a via de PTI (NIEHL *et al*., 2016). De fato, a aplicação de dsRNA em discos foliares de *Arabidopsis* resultou na indução de respostas típicas de PTI, incluindo ativação de MAPK, síntese de etileno e expressão gênica de defesa. Curiosamente, a PTI mediada por dsRNA parece ser independente da via de silenciamento de RNA, mas envolve o co-receptor SERK1 quinase. Esses achados relacionam eventos de sinalização associados à membrana com PTI mediados por dsRNA em plantas (NIEHL *et al*., 2016). Embora tenha sido demonstrado que os co-receptores de PRRs localizados na membrana plasmática, como BAK1, BKK1 e SERK1, estão envolvidos em PTIs virais. Resta determinar como os patógenos intracelulares, que entregam PAMPs intracelularmente, são percebidos extracelularmente.

Embora não tenham sido formalmente caracterizadas as vias do PTI contra vírus de plantas, são observadas respostas celulares típicas de PTI durante interações planta-vírus, tais como fluxos iônicos (SHABALA *et al*., 2010 , 2011; OTULAK e GARBACZEWSKA, 2011), produção de ROS (ALLAN *et al*., 2001; LOVE *et al*., 2005; DÍAZ-VIVANCOS *et al*., 2008), sinalização de etileno (DE LAAT e VAN LOON, 1982; LOVE *et al*., 2005) e deposição de calosidades (IGLESIAS e MEINS, 2000; LI *et al*., 2012; ZAVALIEV *et al*., 2013). A MAPK4, que regula negativamente os mecanismos de PTI da planta (GAO *et al*., 2008; KONG *et al*., 2012), reprime a defesa da soja contra o comovírus de grãos de feijão (LIU

et al., 2011). Receptores do tipo quinases (RLKs) BAK1 (receptor quinase 1 associado ao BRI1) e BKK1 (quinase 1 do tipo BAK1), ativadores chave dos PRRs da planta após a percepção do PAMP (KIM, KIM e NAM, 2013), são necessários para resistência de *Arabidopsis* a vírus de plantas (YANG *et al*., 2010; KØRNER *et al*., 2013). Além disso, a proteína NIK1, outro RLK altamente similar ao BAK1 e BKK1, tem sido fortemente associada à defesa de *Arabidopsis* contra o geminivírus da folha de repolho (CaLCuV). Surpreendentemente, a NIK1 é clivada pela proteína viral NSP durante a infecção, suprimindo sua atividade quinase, desta forma aumentando a suscetibilidade à infecção por CaLCuV (FONTES *et al*., 2004; CARVALHO *et al*., 2008). Esses dados sugerem a existência de um mecanismo de sinalização de PTI visando vírus de plantas. Considera-se que a identificação das vias mediadas por PRR, bem como a caracterização de PRRs com detecção de ácido nucléico devem lançar luz sobre o mecanismo pelo qual o PTI é induzido em plantas e seu papel na resistência antiviral.

Diante de ataques virais, as plantas se defendem através de vários mecanismos de resistência, que são complementares em termos de tempo de defesa (nas fases iniciais ou tardias da infecção), localização (na primeira folha infectada ou em tecidos sistêmicos) e direcionamento das moléculas derivadas do vírus (o genoma viral ou as proteínas virais).

Dessa forma, grandes avanços foram feitos para compreensão da imunidade de plantas contra vírus. Os principais mecanismos imunológicos antivirais conhecidos em plantas são: resistência dominante, resistência recessiva, resistência mediada por RNA de interferência e resistência mediada por hormônios.

2.3.2.3 Resistência dominante

A maioria dos genes de resistência dominante (genes *R*) identificados nas interações planta-vírus pertence à classe NBS-LRR (sítio de ligação ao nucleotídeo - repetição nucleotídica rica em leucina), que reconhecem especificamente os produtos gênicos de fatores de avirulência viral (*avr*), através do estabelecimento da chamada interação “gene-a-gene”. Embora uma interação

física direta entre os produtos dos genes *avr* e *R* tenha sido originalmente sugerida, o entendimento atual favorece o modelo mais sofisticado de “hipótese de guarda” (SOOSAAR, BURCH-SMITH e DINESH-KUMAR, 2005). Tal hipótese postula que os efetores do patógeno são reconhecidos indiretamente pelas proteínas R (*guard protein*) através do monitoramento de proteínas guardadas (*guardee protein*) (DANGL e JONES, 2001; GŁOWACKI, MACIOSZEK e KONONOWICZ, 2011).

Muitas proteínas NBS-LRR que conferem resistência contra vírus foram identificadas até o momento, e são classificadas com base em sua estrutura N-terminal, que possui um domínio de receptor de interleucina 1 denominado TIR (*Toll-interleucin-1*) ou um domínio de bobina espiralada – CC (*coiled-coil*) (MOFFETT, 2009 ; DE RONDE, BUTTERBACH e KORMELINK, 2014).

Um dos exemplos mais bem caracterizados é o produto do gene *N* de tabaco, o qual exibe uma estrutura de TIR-NBS-LRR, que interage diretamente com o domínio helicase da replicase de *Tobamovirus* de tabaco (TMV - *Tobacco mosaic tobamovirus*), de uma forma dependente de ATP (UEDA, YAMAGUCHI e SANO, 2006). A resistência total ao TMV requer a proteína 1 interagindo com o receptor N (NRIP1), que é recrutado do cloroplasto para o citoplasma e o núcleo, interagindo tanto com a replicase viral quanto com o fator N (CAPLAN *et al*., 2008).

Eventos imunológicos à jusante da ativação da proteína R vem sendo frequentemente associados ao influxo de íons de cálcio, sinalização mediada por MAPK, produção de espécies reativas de oxigênio (ROS), acúmulo de ácido salicílico (SA) e extensa reprogramação transcricional. Além disso, a ativação de genes *R* está, na maioria das vezes, associada a uma resposta hipersensível (HR), fenômeno que envolve a morte programada das células infectadas e circunvizinhas, confinando o agente patogênico na lesão hipersensível e desta forma prevenindo seu espalhamento na planta.

Em contraste com a estrutura dos genes *R* clássicos, os genes *RTM* (*Restricted TEV Movement* - Movimento restrito de TEV - *Tobacco Etch Virus*) foram os primeiros genes de resistência dominante não NBS-LRR clonados contra vírus. A caracterização genética de acessos naturais e mutantes de *Arabidopsis* mostrou que pelo menos cinco genes *RTM* dominantes estão

envolvidos na resistência ao potyvirus do tabaco (TEV), potyvirus do mosaico da alface (LMV - *Lettuce Mosaic Virus*) e da pústula da ameixa (PPV - *Plum Pox Virus*) (COSSON *et al*., 2012). Recentemente, foi proposto que membros da RTM possam formar um complexo multiproteico residente no floema, envolvido nos mecanismos de resistência para bloquear o movimento de longa distância de potyvirus (COSSON *et al*., 2010a,b).

2.3.2.4 Resistência recessiva

A resistência recessiva é frequentemente alcançada através da ausência de fatores apropriados do hospedeiro que são necessários para o vírus completar seu ciclo biológico. Quase metade dos genes de resistência conhecidos que atacam vírus de plantas são herdados recessivamente (BOITEUX *et al*., 2012). Dessa forma, o uso de tais genes é uma ferramenta muito importante em programas de melhoramento genético para controlar doenças de plantas causadas por vírus patogênicos.

Ao longo da última década, um grande número de genes de resistência recessiva foram clonados (em patossistemas envolvendo exclusivamente vírus de RNA). Em geral representam mutações em genes que codificam fatores de iniciação eucarióticos (eIFs), pertencentes às famílias eIF4E e eIF4G (TRUNIGER e ARANDA, 2009; BOITEUX *et al*., 2012; WANG e KRISHNASWAMY, 2012; JULIO *et al*., 2014; REVERS e NICAISE, 2014). Esses fatores pertencem ao complexo celular 43S, que recrutam mRNAs e ribossomos antes do processo de tradução (PESTOVA *et al*., 2001), supondo-se que atuem como fatores de susceptibilidade recrutados durante as etapas de replicação / tradução do genoma viral. Assim, a presença de genes recessivos indica que o fenótipo resistente é resultante da perda de função de um gene do hospedeiro que conferia susceptibilidade (BOITEUX *et al*., 2012).

O papel de eIF4E e eIF4G, primeiramente destacado para a infecção bem sucedida por potyvirus, foi rapidamente estendido a outras famílias de vírus vegetais (REVERS e NICAISE, 2014), sugerindo que eles contribuem para um amplo mecanismo de susceptibilidade das plantas aos vírus. Um aspecto

intrigante das resistências mediadas por eIF4E e eIF4G é que elas cobrem uma gama diversificada de fenótipos de resistência (NICAISE, 2014).

No entanto, outros mutantes recessivos de plantas não apresentam mutações em eIF4E e eIF4G, indicando que esta classe não seria a única responsável pela resistência monogênica recessiva a vírus em plantas (BOITEUX *et al*., 2012). Uma estratégia de clonagem posicional que explora a variabilidade natural da cevada revelou recentemente o papel fundamental da proteína PDI5-1 (proteína dissulfeto isomerase 5-1) na resistência recessiva aos bymovírus (YANG *et al*., 2014). Outro gene de resistência recessiva denominado *ra* foi geneticamente caracterizado em batata, bloqueando o transporte vascular do *potyvírus A* (PVA) (HÄMÄLÄINEN *et al*., 2000).

2.3.2.5 Resistência mediada por RNA de interferência

Nas últimas décadas, a interferência de RNA (RNAi; também chamado de silenciamento de genes) foi reconhecida como um processo evolutivamente conservado na maioria dos eucariotos, o qual é desencadeado por RNAs de cadeia dupla (dsRNAs). Esses dsRNAs são processados por enzimas do tipo DICER, ribonuclease do tipo III (DCL), em pequenos RNAs (sRNAs), com 21-24 nucleotídeos de comprimento, que são incorporados em um complexo silenciador citoplasmático induzido por RNA (RISC), cujo principal componente catalítico corresponde a um membro da família de proteínas Argonauta (AGO). Uma vez integrados no RISC, sRNAs ligam-se ao seu mRNA-alvo e induzem a sua clivagem (BOLOGNA e VOINNET, 2014). Nas plantas, pelo menos dois processos básicos de silenciamento gênico podem ser distinguidos: silenciamento de RNA citoplasmático (ou silenciamento gênico pós-transcricional; PTGS - *Post-Transcriptional Gene Silencing*) mediado por pequenos RNAs de interferência (siRNAs), microRNAs e silenciamento gênico transcricional (TGS - *Transcriptional Gene Silencing*) mediado por metilação de DNA e proteínas histonas dirigidas por siRNA (BOLOGNA e VOINNET, 2014).

Diversos trabalhos têm demonstrado a importância do silenciamento por RNAi na defesa vegetal contra infecções virais (YU *et al*., 2003; HAMERA *et al*., 2012). Além disso, outros estudos também demonstraram que o silenciamento

mediado por RNAi é um mecanismo de defesa vegetal muito importante em resposta a infecções por CPSMV. Por exemplo, Aragão *et al*. (2013), por meio de linhagens transgênicas de feijão-caupi, testaram a ação do silenciamento por RNAi como mecanismo de defesa frente à infecção por CAMBV e CPSMV. Foi utilizado um constructo de RNAi para silenciar conjuntamente o gene do cofator da proteinase de CPSMV e o gene da proteína de revestimento de CABMV. Assim, as plantas transgênicas apresentaram resistência aos dois vírus simultaneamente inoculados, quando comparadas às plantas não transformadas.

2.3.2.6 Resistência mediada por hormônios vegetais

Os hormônios vegetais desempenham papéis importantes na regulação das redes de sinalização envolvidas nas defesas das plantas (ROBERT-SEILANIANTZ, GRANT e JONES, 2011).

Durante a ativação da resistência mediada por genes R, as respostas celulares elicitadas no local da infecção são emitidas para tecidos distantes não infectados, resultando em uma resistência ou em um estado de susceptibilidade reduzida que pode permanecer eficiente durante várias semanas (FU e DONG, 2013). Este fenômeno é conhecido como SAR (Resistência Sistêmica Adquirida). Acredita-se que modificações epigenéticas, como a metilação do DNA e remodelação da cromatina, sejam críticas para manter um sinal de SAR por um determinado tempo (SPOEL e DONG, 2012).

Além disso, durante uma infecção viral (de forma semelhante a infecções não virais), esta resistência duradoura e de amplo espectro de doenças requer o acúmulo endógeno de SA, resultando na reprogramação transcricional de uma bateria de genes que codificam proteínas relacionadas à patogênese (PR) (TSUDA *et al*., 2008; YI *et al*., 2014). O sinal emitido pelo ponto de infecção para proteger os tecidos não infectados contra a invasão de patógenos pode circular como um heterocomplexo, onde o metil-SA se liga a derivados lipídicos e proteínas de transporte de lipídios e se movem através do floema para o resto da planta. Além disso, os vírus de plantas evoluíram para as vias de sinalização de hormônios, muitas vezes explorando as interações antagônicas entre as vias SA e JA (ácido jasmônico) (KAZAN e LYONS, 2014).

Embora sua função nas interações planta-vírus permaneça pouco compreendida, alguns estudos indicam que outros hormônios vegetais modulam os mecanismos de resistência antiviral, especialmente o ácido abscísico (CHEN *et al*., 2013; ALAZEM, LIN e LIN, 2014; SEO *et al*., 2014), o etileno (FISCHER e DRÖGE-LASER, 2004; LOVE *et al*., 2007; ZHANG *et al*., 2009; CHEN *et al*., 2013) e os brassinosteróides (ALI *et al*., 2014).

2.4 GENES PR E FAMÍLIA GÊNICA PR-5: CONTEXTUALIZAÇÃO DO TEMA

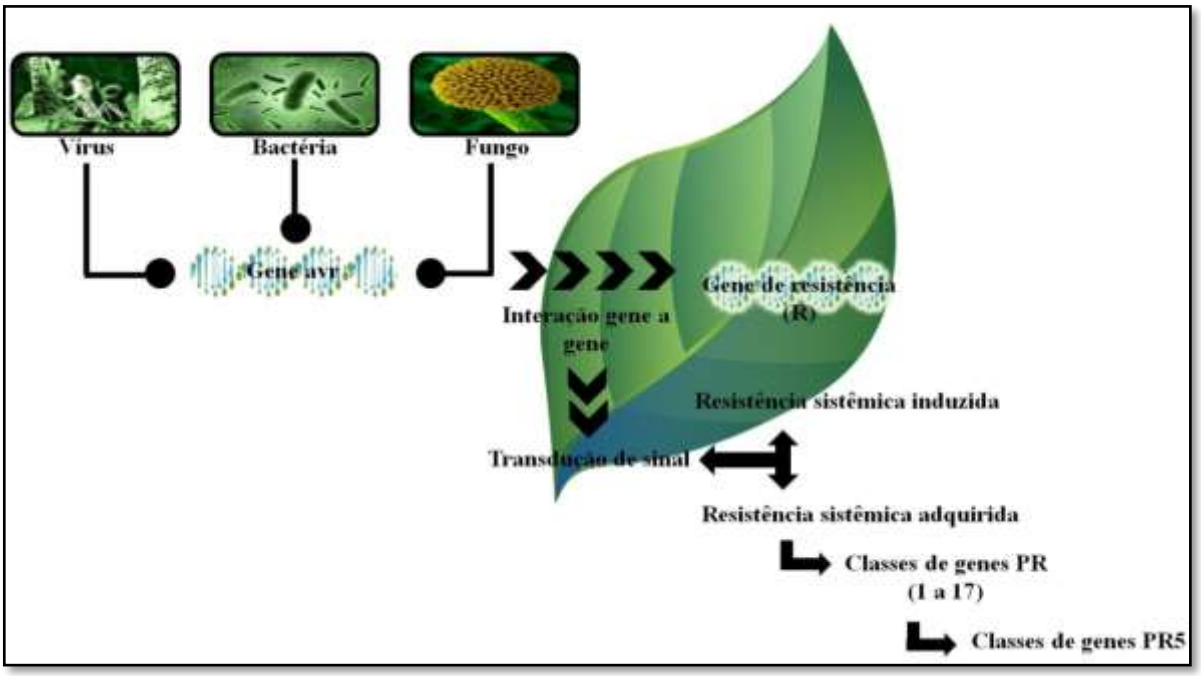
Ao longo de sua evolução, as plantas desenvolveram diversos mecanismos (**Figura 5**), tanto constitutivos quanto regulados, para reconhecer e responder a infecções causadas por patógenos. Dentre vários, podem-se citar barreiras morfológicas externas (espessamento da cutícula foliar e fechamento de estômatos, por exemplo) e mecanismos moleculares (produção de metabólitos secundários, síntese de proteínas antimicrobianas, etc.; BENKO-ISEPPON *et al*., 2010).

A primeira barreira de defesa do vegetal depende de receptores transmembranares que reconhecem padrões moleculares conservados relacionados a patógenos (PAMPs) ou microrganismos (MAMPs; ZIPFEL e FELIX, 2005). A segunda barreira age em grande parte dentro da célula, onde produtos de genes *R* (*Resistance*) da planta podem reconhecer produtos de genes de avirulência (proteínas avr) correspondentes, sintetizados pelo patógeno (**Figura 5**). A planta será resistente e a invasão pelo patógeno será impedida quando ambos (proteínas R e avr) estiverem presentes e forem compatíveis, levando à resposta hipersensitiva (HR). Esta, por sua vez, induz morte celular local, prevenindo a colonização da planta pelo patógeno (BONAS e ANCKERVEKEN, 1999; STASKAWICZ, 2009). Adicionalmente, a HR ativa uma cascata de sinalização (**Figura 5**) que induz a defesa sistêmica contra patógenos (SLUSARENKO, FRASER e VAN LOON, 2000).

Assim, após o contato com o patógeno e ativação do sistema de defesa específico, uma série de respostas secundárias (sistêmicas) é iniciada. Essas incluem o reforço da parede celular, a produção de antibióticos (fitoalexinas) e a

síntese de proteínas relacionadas à defesa, também conhecidas como proteínas PR (*Pathogenesis-Related*; **Figura 5**; VAN LOON, REP e PIETERSE, 2006).

Figura 5. Principais mecanismos de reconhecimento e defesa de patógenos nas plantas. Os organismos patogênicos (principalmente vírus, bactérias e fungos) secretam produtos de genes *avr* (avirulência) que podem ser compatíveis com produtos de genes *R* secretados pelas plantas. As interações compatíveis levam à ativação de cascatas de sinalização induzindo fatores de resistência sistêmica e resistência adquirida. Esta última está associada à ação de 17 famílias de genes *PR*, dentre as quais a *PR-5* se destaca.



Fonte: Autor.

As proteínas PR foram originalmente descobertas em plantas de tabaco infectadas por vírus (VAN LOON e VAN KAMMEN, 1970). Elas são codificadas por genes que são rapidamente induzidos por infecções patogênicas e pelo acúmulo de hormônios associados à defesa vegetal (NARUSAKA *et al*., 2009). Em plantas sadias, a indução das proteínas PR pode ser feita artificialmente por tratamento com produtos químicos, desordens fisiológicas ou filtrados tóxicos de fungos fitopatogênicos ou bactérias (FREITAS e BERED, 2003).

Proteínas PR apresentam duas subclasses, uma básica e outra acídica (VAN LOON e VAN KAMMEN, 1970; KITAJIMA e SATO, 1999), sendo classificadas em 17 famílias gênicas (**Tabela 1**) considerando-se os seguintes

fatores: (i) similaridade entre sequências; (ii) relações sorológicas ou imunológicas e (iii) propriedades enzimáticas (VAN LOON, REP e PIETERSE, 2006).

Dentre as 17 famílias PR, a PR-5 se distingue por abranger proteínas do tipo Taumatina ou *Thaumatin-Like Proteins* (TLPs). Este grupo recebeu essa denominação devido à sua similaridade tridimensional com uma proteína purificada do fruto da planta *Thaumatococcus daniellii* (oriunda da África Ocidental) que recebeu o nome de Taumatina (do inglês, *Thaumatin*; LIU, STURROCK e EKRAMODDOULLAH, 2010). Essa molécula chamou atenção por apresentar uma doçura 100 mil vezes maior que do que a encontrada na sacarose (VAN DER WEL e LOEVE, 1972). Vale ressaltar, entretanto, que a maioria das TLPs não apresenta sabor adocicado (VELAZHAHAN, DATTA e MUTHUKRISHNAN, 1999). Uma vez que todos os membros da família gênica PR-5 abrangem TLPs, no presente texto adotaremos esse epíteto, abrangendo os grupos proteicos Taumatina e *Thaumatin-like protein* (TLP).

Tabela 1. Classificação das proteínas PR e suas principais características.

Família	Peso Molecular (kDa)	Exemplo	Alvo Microbiano	Referência
PR-1	15	PR-1a de tabaco	Desconhecido	ANTONIW *et al*. (1980)
PR-2 (β-1,3-glucanases)	30	PR-2 de tabaco	β-1,3-Glucano	ANTONIW *et al*. (1980)
PR-3 (Quitinases I, II, IV, V, VI e VII)	25–30	P, Q de tabaco	Quitina	VAN LOON (1982)
PR-4 (Quitinases I e II)	15–20	R de tabaco	Quitina	VAN LOON (1982)
PR-5 (Thaumatin-like proteins - TLPs)	25	S de tabaco	Membrana	VAN LOON (1982)
PR-6 (Inibidor de proteinase)	8	Inibidor I de tomate	–	GREEN e RYAN (1972)
PR-7 (Endoproteinase)	75	P69 de tomate	–	VERA e CONEJERO (1988)
PR-8 (Quitinase classe III)	28	Quitinase de pepino	Quitina	MÉTRAUX, STREIT e STAUB (1988)
PR-9 (Peroxidase)	35	Peroxidase formadora de lignina de tabaco	–	LAGRIMINI *et al*. (1987)
PR-10 (Ribonuclease-like proteins - RLP)	17	PR-1 de salsinha	–	SOMSSICH *et al*. (1986)

Família	Peso Molecular (kDa)	Exemplo	Alvo Microbiano	Referência
PR-11 (Quitinase classe I)	40	Quitinase classe V de tabaco	Quitina	MELCHERS *et al*. (1994)
PR-12 (Defensina)	5	RsAFP3 de rabanete	Membrana	TERRAS *et al*. (1995)
PR-13 (Thionina)	5	Thi2.1 de *Arabidopsis*	Membrana	EPPLE, APEL e BOHLMANN (1995)
PR-14 (Lipid-transfer protein - LTP)	9	LTP4 de Cevada	Membrana	GARCÍA-OLMEDO *et al*. (1995)
PR-15 (Oxalase oxidase - OXO)	20	OxOa (germin) de Cevada	–	ZHANG, COLLINGE e THORDAL-CHRISTENSEN (1995)
PR-16 (Oxalate oxidase-like - OXO-like)	20	OxOLP de Cevada	–	WEI *et al*. (1998)
PR-17	27	PRp27 de Tabaco	–	OKUSHIMA *et al*. (2000)

(–) Nenhuma atividade antimicrobiana *in vitro* reportada. Adaptado de Sels *et al*. (2008).

2.5 THAUMATIN-LIKE PROTEINS (TLPS) NO REINO VEGETAL: PADRÕES DE NOMENCLATURA, ABUNDÂNCIA E CARACTERÍSTICAS ESTRUTURAIS

Algumas TLPs têm recebido nomenclatura específica em virtude da função que desempenham. Por exemplo, TLPs que são capazes de criar poros transmembranares são conhecidas como permatinas (ABAD *et al*., 1996; ANŽLOVAR e DERMASTIA, 2003). Essas ocorrem em altas concentrações nas sementes de cereais e ainda recebem denominação especial a depender da espécie que ocorrem: zeamatinas (em milho; *Zea mays*), hordomatina (em cevada; *Hordeum vulgare*) e avematina (em aveia; *Avena sativa*; ROBERTS e SELITRENNIKOFF, 1990; SKADSEN, SATHISH e KAEPPLER, 2000). Além delas, as osmotinas são TLPs assim denominadas por terem sido induzidas sob estresse osmótico (SINGH *et al*., 1989). Essa diversidade nomenclatural ocasiona certa confusão em análises moleculares ou ainda durante análise *in silico* usando informações associadas a esse grupo.

TLPs são abundantes no reino vegetal, sendo encontradas em angiospermas, gimnospermas e briófitas, como o musgo *Physcomitrella patens* ssp. *Patens* (LIU, STURROCK e EKRAMODDOULLAH, 2010). Com base em

sequências genômicas e bancos de dados de ESTs, Liu, Sturrock e Ekramoddoullah (2010) mineraram cerca de 31 genes codificadores de TLPs em arroz (*Oryza sativa*), 28 em *Arabidopsis thaliana*, 13 em *Picea glauca*, 10 em *Pinus monticola* e seis em *P. patens*. Petre *et al*. (2011), por sua vez, encontraram 42 genes de TLPs no genoma de *Populus trichocarpa*.

Quanto à estrutura, esse grupo proteico apresenta assinatura característica das Taumatinas (PS00316): G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)-[QG]-x(2,3)-C (JAMI *et al*., 2007; TACHI *et al*., 2009). A maioria das TLPs tem massa molecular variando de 21 a 26 kD (LIU, STURROCK e EKRAMODDOULLAH, 2010), possuindo 16 resíduos conservados de cisteína envolvidos na formação de oito ligações dissulfeto (BREITENEDER, 2004). Essas ligações ajudam na estabilidade da molécula, permitindo um dobramento correto mesmo sob condições extremas de temperatura e pH (FIERENS *et al*., 2009). As TLPs possuem ainda um peptídeo sinal em sua extremidade N-terminal que é responsável por direcionar a proteína madura para uma determinada via secretória (LIU, STURROCK e EKRAMODDOULLAH, 2010).

A estrutura terciária, por sua vez, apresenta três domínios distintos, os quais são conservados e formam a *cleft* central, responsável pela atividade enzimática da proteína, estando localizada entre os domínios I e II (GHOSH e CHAKRABARTI, 2005; LEONE *et al*., 2006). Essa *cleft* central pode ser de natureza ácida, neutra ou básica dependendo da ligação dos diferentes ligantes / receptores. Todas as TLPs de plantas com atividade antifúngica possuem uma *cleft* acídica, devido à presença de cinco aminoácidos altamente conservados (arginina, ácido glutâmico e três resíduos de ácido aspártico), sendo bastante relevantes para a ligação do receptor específico da atividade antifúngica (BATALIA *et al*., 1996; KOIWA *et al*., 1999; MIN *et al*., 2004).

Algumas TLPs, também conhecidas como sTLPs (*Small* TLPs), apresentam deleções de peptídeos em um de seus domínios, culminando na ausência de uma típica *cleft* central. Essas apresentam apenas dez resíduos conservados de cisteínas, formando cinco ligações dissulfeto, resultando em um peso molecular de aproximadamente 16-17 kDa. Tais sTLPs são encontradas em monocotiledôneas e coníferas (LIU, STURROCK e EKRAMODDOULLAH, 2010).

Adicionalmente, foram identificados primeiramente em *Arabidopsis* e arroz, TLPs que apresentam um domínio TLP extracelular e um domínio quinase intracelular (PR5K – *PR5-like receptor kinases*) (WANG *et al*., 1996). Tais genes PR5K estão presentes tanto em monocotiledôneas quanto em dicotiledôneas. Por exemplo, *Arabidopsis* contêm três genes PR5K enquanto o arroz possui somente um (LIU, STURROCK e EKRAMODDOULLAH, 2010).

2.5.1 Propriedades biológicas de proteínas da família TLP: doçura extrema, alergenicidade e ação antimicrobiana

2.5.1.1 Doçura

A Taumatina inicialmente descrita é uma proteína de sabor doce (VAN DER WEL e LOEVE, 1972) e consiste em uma cadeia simples de 207 resíduos de aminoácidos, sendo traduzida como uma forma pre-sequência com uma extensão N-terminal hidrofóbica com 22 aminoácidos e outra forma pro-sequência, com uma extensão carboxi-terminal com seis aminoácidos (IYENGAR *et al*., 1979; IDE, MASUDA e KITABATAKE, 2007). Além disso, ela consiste em pelo menos cinco formas doces: dois componentes maiores (Taumatina I e II) e três componentes menores (Taumatina a, b e c; MASUDA *et al*., 2011). Apresenta baixo teor calórico e tem potencial para substituir os açúcares e edulcorantes artificiais. Dessa forma, entender os mecanismos moleculares responsáveis pelo sabor doce da Taumatina pode elucidar sua ação em sistemas biológicos e suas implicações médicas no que se refere a doenças relacionadas ao estilo de vida, como hipertensão, diabetes e obesidade (MASUDA *et al*., 2011). Estudos de mutagênese sítio-dirigida bem como modificações químicas têm mostrado que a basicidade no local contendo a *cleft* da referida proteína é requerida para a doçura e dois resíduos de carga positiva, Lys67 e Arg82, desempenham um papel importante na elicitação dessa propriedade (KANEKO e KITABATAKE, 2001; OHTA *et al*., 2008).

MASUDA *et al*. (2016) realizaram um estudo a fim de entender os efeitos dos resíduos de aminoácidos ácidos na relação estrutura-atividade da Taumatina. Para isso, foram construídos seis mutantes para os aminoácidos ácidos (D21,

E42, D55, D59, D60 e E89), localizados próximos aos dois resíduos básicos (K67 e R82) importantes para a doçura. Os resultados mostraram que a maioria dos resíduos ácidos não desempenharam um papel importante na obtenção de doçura. No entanto, a remoção da carga negativa do resíduo D21 produziu um mutante de Taumatina ainda mais doce, mostrando a importância dos potenciais eletrostáticos na interação dessa proteína com o receptor doce.

Estudos biotecnológicos de Taumatina foram implementados em diversas áreas e aplicações. No melhoramento genético, o gene codificante dessa proteína tem sido expresso com sucesso em plantas (BARTOSZEWSKI *et al*., 2003), especialmente para aumentar a doçura e qualidade da fruta (MASUDA e KITABATAKE, 2006). Bartoszewski *et al*. (2003) obtiveram tomate (*Lycopersicon esculentum*) com sabor doce através da introdução do gene Taumatina II de *T. daniellii*, por meio da *Agrobacterium tumefaciens*. Além disso, outras plantas transgênicas expressando o gene Taumatina II foram obtidas, tais como o pepino (SZWACKA *et al*., 2002), batata (WITTY, 1990), pêra (LEBEDEV *et al*., 2002) e morango (SCHESTIBRATOV e DOLGOV, 2005). Vale salientar que neste último trabalho todas as linhagens transgênicas que expressaram o gene Taumatina II apresentaram um nível significativamente maior de resistência ao fungo *Botrytis cinerea* (que provoca apodrecimento dos frutos na fase pós-colheita) quando comparadas com as plantas controle (SCHESTIBRATOV e DOLGOV, 2005).

2.5.1.2 Alergenicidade

Em contrapartida, as demais proteínas da família TLP, que não possuem sabor adocicado, apresentam funções diversificadas. TLPs de plantas foram relatadas como alergênicos alimentares de frutas. Têm-se como exemplo a Mal d2, TLP alergênica de maçã que, segundo Hsieh, Moos e Lin (1995), foi a primeira TLP descrita apresentando tal propriedade. As TLPs alergênicas alimentares incluem: a Pru av2, de cereal (*Prunus avium*; INSCHLAG *et al*., 1998); Cap a1, de pimenta (*Capsicum annuum*; FUCHS *et al*., 2002); Ato c2, de kiwi (*Actinidia chinensis*; GAVROVIĆ-JANKULOVIĆ *et al*., 2002); TLP de uva (*Vitis vinifera*; PASTORELLO *et al*., 2003); e TLP de banana (*Musa acuminata*; LEONE *et al*., 2006). Há, também, TLPs que atuam como alergênicas de pólen de

coníferas (HOFFMANN-SOMMERGRUBER, 2002; BREITENEDER, 2004). Esses compreendem a Jun a3, de cedro de montanha (*Juniperus ashei*; MIDORO-HORIUTI *et al*., 2000); Cup a3, do cipreste do Arizona (*Cupressus arizonica*; CORTEGANO *et al*., 2004); e Cry j3, do cedro japonês (*Cryptomeria japonica*; FUJIMURA *et al*., 2007). Entre as TLPs alergênicas, Jun a3, Mal d2 e Pru av2 destacam-se por apresentarem habilidade de serem reconhecidas pelo anticorpo Ig-E de pessoas alérgicas (GHOSH e CHAKRABARTI, 2008).

2.5.1.3 Ação antimicrobiana e resposta a estresses em geral

Algumas TLPs são conhecidas por apresentarem atividade antifúngica, seja permeabilizando as membranas dos fungos (BATALIA *et al*., 1996) ou por ligação e hidrólise de β-1,3-glucanos (GRENIER *et al*., 1999; TRUDEL *et al*., 1998). Adicionalmente, podem atuar na inibição de enzimas fúngicas, como xilanases (FIERENS *et al*., 2007), α-amilases ou tripsina (SCHIMOLER-O'ROURKE, RICHARDSON e SELITRENNIKOFF, 2001).

A expressão de TLPs é regulada em resposta a alguns fatores, como seca (JUNG *et al*., 2005), ferimentos (RUPERTI *et al*., 2002), temperatura de congelamento (HON *et al*., 1995) e infecção por fungos (KUMAR e KIRTI, 2011; RUDD *et al*., 2015), vírus e bactérias (BREITENEDER e RADAUER, 2004). Tachi *et al*. (2009), por exemplo, caracterizaram um novo gene de soja codificante de uma TLP neutra induzida sob estresse de alta salinidade (NaCl, 100 mM) e a comparou com duas isoformas acídicas de soja. Ele verificou que essa nova proteína possuía uma extremidade C-terminal, responsável pelo possível direcionamento vacuolar. Além disso, o gene foi altamente induzido nas folhas da soja, sendo que tal processo começou duas horas após o início do estresse, alcançando seu ápice entre 18-72 horas.

De outra forma, em algumas espécies, as TLPs são constitutivamente expressas em flores e frutos, órgãos reprodutivos importantes que são suscetíveis à infecção por patógenos. Há a hipótese de que essas TLPs funcionem como uma defesa pré-formada contra infecções (CLENDENNEN e MAY, 1997; FILS-LYCAON *et al*., 1996; SALZMAN *et al*., 1998; SASSA e HIRANO, 1998).

Kim *et al*. (2002) observaram uma correlação entre a expressão do gene PepTLP de pimenta (*C. annuum*), resistência à antracnose, amadurecimento do fruto e a acumulação de açúcar nos frutos, sugerindo um importante papel das TLPs na proteção dos órgãos reprodutivos.

Evidências da regulação de TLPs durante a maturação de frutos têm sido relatadas também na banana (CLENDENNEN e MAY, 1997) e no tomate (PRESSEY, 1997). Além disso, foi reportado que TLPs foram acumuladas em flores de tabaco (RICHARD *et al*., 1992) e sementes de milho (ROBERTS e SELITRENNIKOFF, 1990). Dafoe, Gowen e Constabel (2010) ao analisarem duas TLPs de *Populus* híbrido (*Populus trichocarpa* × *P. deltoides*) verificaram que a TLP1 foi expressa predominantemente em tecidos com sistema vascular mais desenvolvido, enquanto a outra TLP foi expressa principalmente em plastídios de armazenamento de amido. Os autores sugerem que esse padrão de localização é consistente com um papel de defesa pré-formada contra insetos e patógenos que se alimentam do floema.

Vale ressaltar ainda que genes codificadores de TLPs podem ser induzidos por hormônios associados à imunidade vegetal. Por exemplo, em *Solanum commersonii* um gene codificador de TLP é induzido por ácido abscísico (ABA) e por ferimento (ZHU, CHEN e LI, 1993); já em mudas de tabaco (outra espécie da mesma família, Solanaceae), constatou-se indução de TLPs em resposta à aplicação de etileno, ácido metil-jasmônico (metil JA) e ácido salicílico (SA; XU *et al*., 1994). Por fim, um estudo realizado com uma TLP de arroz (Rtlp1) revelou que seu respectivo promotor é induzido pelo elicitor do fungo causador da brusone de arroz e por SA e metilJA (HIROYUKI e TERAUCHI, 2008).

Com relação à atividade antiviral desse grupo proteico pouco tem sido relatado. Porém, Kim *et al*. (2005) verificaram por meio de experimentos de interação proteína-proteína, que uma TLP de *Nicotiana tabacum* interage com proteínas do vírus *Cucumber mosaic virus*, o que justifica a avaliação da expressão das TLPs frente aos vírus no presente estudo.

2.5.2 Abordagens de ômicas para TLPs: enfoque em estresses bióticos e abióticos

Uma recente busca (Outubro/2018) realizada no banco de dados PubMed (NCBI) apresentou seis e 11 trabalhos relacionados aos termos "*PR5* AND *transcriptome*" e "*Thaumatin-like* AND *transcriptome*", respectivamente (**Tabela 2**). A grande maioria dos trabalhos não focam em TLPs, especificamente. Esses abordam a resposta transcricional global de espécies de interesse a situações não favoráveis, ressaltando alguns alvos moleculares potencialmente valorosos. Eventualmente, as TLPs são destacadas em tais análises. Exemplos do exposto podem ser vistos a seguir.

Weng *et al*. (2014) estudaram a resistência de uvas selvagens chinesas (*Vitis pseudoreticulata* Baihe 35-1) à infecção de *Erysiphe necator*, a fim de entender a interação entre hospedeiro e patógeno. Eles observaram que dentre os genes diferencialmente expressos, a expressão de transcritos codificadores de proteínas pertencentes à família TLP (ou PR-5, como designaram os autores) foi elevada nas primeiras 36 horas após a inoculação. Os autores sugerem que isso poderia ter iniciado e levado à morte celular (resposta hipersensível), indicando assim um papel importante dessas proteínas na resistência contra o patógeno fúngico *E. necator*.

Tabela 2. Apresentação dos trabalhos disponíveis de análise global da expressão gênica abordando os termos "*PR5* AND *transcriptome*" e "*Thaumatin-like* AND *transcriptome*" na base de dados PubMed (NCBI), em outubro de 2018, evidenciando o tecido ou órgão estudado, a condição analisada e a tecnologia empregada.

Espécie	Tecido/ órgão	Condição	Tecnologia	Autores
***Vitis pseudoreticulata* Baihe 35-1**	Folhas	Inoculação de acesso resistente Baihe 35-1 com *Erysiphe necator*	Sequenciamento Illumina	WENG *et al*.(2014)
Triticum aestivum	Espigas	Inoculação com *Fusarium graminearum*	Sequenciamento Illumina	XIÃO *et al*. (2013)
Musa acuminata	Raiz	Inoculação de *Meloidogyne incognita*	Illumina HiSeq	CASTAÑEDA *et al*. (2017)
Populus trichocarpa* x *Populus deltoides	Folhas e uredinia	Inoculação de *Melampsoralarici-populina*	Microarray	HACQUARD *et al*. (2013)
Gossypium hirsutum* x *Gossypium barbadense	Fibras	10 dias após a antese	Illumina HiSeq 2000	LI *et al*. (2017)
Pinus sylvestris	Raiz	Inoculação com *Heterobasidion annosum*	Biblioteca subtrativa de cDNA	LI e ASIEGBU (2004)
Zea mays	Raiz	Tratamento com 200 mg / L de $CdCl_2$ $2.5H_2O$	Illumina HiSeq 2500	PENG *et al*. (2015)
Lentinula edodes	Corpo de frutificação e micélio	Quatro dias após a colheita	Illumina HiSeq 2500, Super-SAGE	SAKAMOTO *et al*. (2017)
Malus domestica	Fruto	Inoculação com *Yarrowia lipolytica*	RNA-Seq	ZHANG *et al*. (2017)
Humulus lupulus	Folhas	Infecção por viróide e inoculação com *Podospheara macularis*	Illumina HiSeq 2000	KAPPAGANTU *et al*. (2017)
Aspergillus fumigatus*, *Aspergillus niger*, e *Aspergillus oryzae	Hifas, conídios em repouso e após 1 h de crescimento	Cepas de *Aspergillus* sob diferentes condições de crescimento	Illumina HiSeq	HAGIWARA *et al*. (2016)
***Cicer arietinum* L.**	Tecido foliar jovem	Dois genótipos suscetíveis e tolerantes a herbicida expostos à imidazolina (Imazethapyr)	Illumina HiSeq 2000	IQUEBAL *et al*. (2017)
Nicotiana tabacum	Tricomas	Tratamento com $CdCl_2$	Biblioteca EST	HARADA *et al*. (2010)
Arabidopsis thaliana	Folhas	Plantas expostas a ovos depositados, danificadas pela alimentação de larvas e sem ovos, danificadas pela alimentação após 48 h	Microarray	LORTZING *et al*. (2018)
Carica papaya	Folhas	Plantas inoculadas com o complexo PMeV (infecção combinada do vírus da papaia meleira - PMeV e do papaia meleira 2 - PMeV2) pré e pós-floração	Illumina HiSeq 2000	MADROÑERO *et al*. (2018)
Arabidopsis thaliana	Sépalas	RNA-Seq de cinco diferentes genótipos (Columbia-Col_WT tipo selvagem, atml1-3, lgo-2, LGOoe, and LGOoe atml1-3) para determinar em que medida o LGO (*Loss of Giant cells from Organs*) controla/define a morfologia de verticilos florais	Illumina HiSeq 2000	SCHWARZ e ROEDER (2016)

Espécie	Tecido/ órgão	Condição	Tecnologia	Autores
Pinus massoniana	Tecidos do xilema	10 clones de alto rendimento em oleoresina e 10 clones com baixa produção de oleoresina	Illumina HiSeq 2000	LIU *et al*. (2015)

Xião *et al*. (2013) visaram a identificação de genes envolvidos na resistência do trigo ao fungo *Fusarium graminearum*. Para isso, os autores utilizaram um genótipo resistente (Wangshuibai) e um mutante susceptível (NAUH117) com deleção do gene *Fhb1*, que confere resistência ao *Fusarium*. Foi verificado que proteínas responsivas a patógenos, como TLPs (ou PR-5), PR14, transportadores ABC, além das vias de sinalização de ácido jasmônico (JA), foram cruciais para a resistência ao *F. graminearum*, especialmente aquela mediada por *Fhb1*.

Zhang *et al*. (2017) visaram a determinação do mecanismo pelo qual a levedura *Yarrowia lipolytica* provoca resistência a doenças em maçãs. Para isso, cada maçã foi ferida e inoculada com 30 µL de suspensão de *Y. lipolytica*, sendo inoculadas após 2 h com 30 µL de suspensão de *Penicillium expansum*. Eles observaram que *Y. lipolytica* induziu as vias de sinalização de SA e etileno (ET) / JA, estimulando produtos de genes de defesa, como peroxidases e TLPs.

Harada *et al*. (2010), por sua vez, verificaram que osmotinas e TLPs foram induzidas em plantas de tabaco tratadas com cloreto de cádmio ($CdCl_2$), especialmente nos tricomas. Apesar dos resultados promissores, os autores destacam a necessidade de estudos adicionais para entender o papel dessas proteínas na desintoxicação de metais pesados.

O único relato encontrado na literatura abordando genômica estrutural e funcional de TLPs foi executado por Petre *et al*. (2011). Tais autores mineraram 42 genes codificantes de TLPs no genoma de *P. trichocarpa* 'Nisqually-1' (version 2.0). Adicionalmente, foi observado que a grande maioria desses foram responsivos a estresses abióticos (alto teor de ozônio, raios UV-B, seca, alto teor de cobre) e um estresse biótico (infecção pelo fungo *Melampsora laricis-populina*).

2.5.3 Estratégias para resistência a doenças e tolerância a estresses abióticos em culturas vegetais: enfoque no emprego de genes codificadores de TLPs

O interesse por genes pertencentes à família das TLPs (ou PR-5) vem tomando proporções cada vez maiores nos programas de melhoramento vegetal, principalmente pelo seu grande potencial biotecnológico para geração de plantas

transgênicas com maior resistência / tolerância a estresses (RAJAM *et al*., 2007; SINGH *et al*., 2013). Diversos relatos dão conta do impacto positivo da expressão de TLPs na fisiologia vegetal sob condições não favoráveis.

Chowdhury, Basu e Kundu (2017) verificaram que linhagens transgênicas de gergelim superexpressando uma proteína do tipo osmotina (SindOLP), apresentaram tolerância / resistência contra estresses abióticos (seca e alta salinidade – 200 mM NaCl) e biótico (infecção pelo fungo *Macrophomina phaseolina*). Foi observado, adicionalmente, que a superexpressão de SindOLP resultou no aumento da expressão de três genes [*superoxide dismutase* (*SiSOD*)*, cysteine protease inhibitor* (*SiCysPI*)*, glutathione-S-transferase* (*SiGST*)] que codificam enzimas para eliminação de espécies reativas de oxigênio (ROS). Tais resultados sugerem que SindOLP participa da regulação da via de ROS, a qual é comum a ambos os tipos de estresses abordados.

Sripriya, Parameswari e Veluthambi (2017) constataram que linhagens de arroz transgênico expressando uma combinação dos genes quitinase (*chi11*), de arroz, e osmotina (*ap24*), de tabaco, no mesmo T-DNA apresentaram maiores níveis de resistência à ferrugem da bainha do que aquelas que expressam *chi11* ou *ap24* individualmente. Foi observado que três plantas transgênicas (CO1, CO2 e CO3) apresentaram cópias de T-DNAs completos com ambos os genes *chi11* e *ap24*, porém somente as plantas CO1 e CO2 exibiram altos níveis de expressão de ambos os genes.

Misra *et al*. (2016), por sua vez, identificaram e caracterizaram uma TLP (*Ob*TLP1) em manjericão (*Ocimum basilicum*) a partir de bibliotecas de ESTs expressas após tratamento com MeJA. Posteriormente, eles realizaram expressão ectópica de *Ob*TLP1 em *A. thaliana* e observaram que esse gene pode ser útil para proporcionar resistência contra patógenos fúngicos (*Scleretonia sclerotiorum* e *B. cinerea*) e tolerância a estresses abióticos (seca e alta salinidade).

Singh *et al*. (2013) mineraram, em um estudo anterior, uma TLP induzida em *Arachis diogoi* (*Ad*TLP) sob infecção com *Phaeoisariopsis personata*. O cDNA codificante de *Ad*TLP foi clonado usando RACE-PCR e plantas de tabaco foram transformadas com o mesmo. Nesse organismo, foi observado que a superexpressão dessa TLP tem impacto positivo na resistência a fungos

patogênicos e na tolerância a estresses abióticos (alta salinidade e estresse oxidativo). Adicionalmente, as plantas transgênicas também apresentaram maior nível de transcrição dos genes *PR1a*, *PI-I* e *PI-II* em comparação com *WT* (plantas selvagens). Tais genes estão associados a mecanismos vegetais de defesa contra patógenos.

Munis *et al*. (2010) inseriram o gene *GbTLP1*, obtido de bibliotecas de cDNA de fibras de algodão marinho (*Gossypium barbadense*), no vetor pCAMBIA 2300S sob o controle do promotor CaMV 35S e o transferiu para o tabaco, visando determinar seu papel em diferentes condições de estresse biótico e abiótico. Os autores verificaram que o gene *GbTLP1* é útil para induzir resistência sob diferentes condições de estresse, particularmente contra *Verticillium dahliae*. Por fim, a expressão heteróloga do gene TLP de *Camellia sinensis* (*CsTLP*) em batata conferiu resistência aos fungos *M. phaseolina* e *Phytophthora infestans* (ACHARYA *et al*., 2013).

Tais resultados mostram a potencialidade dos genes que codificam TLPs na obtenção de plantas transgênicas com maior tolerância a estresses abióticos e resistência a estresses bióticos, além de estarem envolvidos no processo de *crosstalk* (VELÁZQUEZ, GUERRA e CALDERÓN, 2011).

2.6 ÔMICAS EM FEIJÃO-CAUPI: THE COWPEA GENOMICS CONSORTIUM (CpGC)

O Brasil tem dado sua parcela de contribuição para o avanço científico do feijão-caupi por meio do desenvolvimento de importantes ferramentas para aplicação em seus programas de melhoramento e decifrando as bases moleculares de sua robustez perante a estresses. A UFPE (Universidade Federal de Pernambuco) está no centro de uma rede de pesquisa (CpGC); http://bioinfo03.ibi.unicamp.br/vigna/) que organizou um leque de laboratórios para executar ensaios de:

(a) Genômica Estrutural: desenvolvimento de mapa genético saturado, incluindo QTLs associados à tolerância à seca;
(b) Genômica Funcional: análises de bibliotecas SuperSAGE, RNA-Seq e de ESTs (*Expressed Sequence Tags*) associadas a estresses bióticos (infecção

por CABMV ou CPSMV) e abióticos [alta salinidade (NaCl, 100 mM) e desidratação radicular], visando identificar genes-candidatos úteis para o melhoramento da cultura.

Além das referidas bibliotecas, o CpGC gerou 49.820 ESTs de folhas e raízes obtidas a partir da exposição da cultura a diferentes estresses bióticos e abióticos. Essas foram agrupadas em conjunto com 264.945 ESTs depositados no banco de dados Cowpea HarvEST V.1.33 (http://harvest.ucr.edu/) e GenBank (NCBI), estando disponíveis para ancoragem e anotação de transcritos em análise.

A partir desse esforço, Kido *et al*. (2011) mineraram dados em bibliotecas SuperSAGE para a produção de um catálogo de quinases expressas em feijão-caupi sob diferentes condições de estresses biótico (infecção por CABMV) e abióticos (injúria mecânica foliar e alta salinidade).

Wanderley-Nogueira *et al*. (2012), por sua vez, realizaram avaliação *in silico* de genes PR-2 no banco de dados de ESTs do CpGC, obtido a partir dos mesmos estresses analisados por Kido *et al*. (2011). Araújo (2015) identificou *in silico* genes NBS-LRR nos bancos de dados de RNA-Seq e SuperSAGE do CpGC sob estresses abiótico (desidratação radicular) e biótico (inoculação com CPSMV). Além disso, três genes alvo com indução nos níveis de expressão após 16 horas da inoculação com o patógeno foram validados por RT-qPCR. Matos (2015), por sua vez, identificou e avaliou *in silico* a expressão diferencial de TFs da família MYB no banco de dados SuperSAGE do CpGC sob inoculação com CPSMV. Além disso, determinou genes de referência para normalização dos dados em RT-qPCR sob diferentes condições de estresse (desidratação radicular, alta salinidade e inoculação com CPSMV) e controles. Somado a esses dados, diversos outros abordando temas como estresse hídrico e regulação de aquaporinas, estresse hídrico e orquestração da biossíntese de inositol, estresse hídrico e orquestração de genes associados a enzimas participantes da fosforilação oxidativa, análise da expressão de defensinas sob estresses bióticos, dentre outros, vêm sendo analisados (BENKO-ISEPPON *et al*., 2013).

Logo, observa-se que o CpGC possui significativa disponibilidade de dados moleculares associados a análises globais dos transcriptomas de feijão-caupi

submetidos a diferentes condições. Além disso, os ensaios apresentam desenhos experimentais maximizadores do ganho de informação, utilizando acessos fisiologicamente contrastantes, cuja comparação da expressão dos transcritos expressos capacita a descoberta de isoformas intimamente relacionadas ao processo de tolerância ou resistência. Assim, o conteúdo informacional de tal Rede permite estudar, especificamente, diversos temas ainda pouco analisados pela ótica da transcriptômica, mesmo em espécies cultivadas de importância econômica (BENKO-ISEPPON, 2013).

3 RESULTADOS

3.1 ARTIGO 1 – COWPEA THAUMATIN-LIKE PROTEINS: CHARACTERIZATION AND TRANSCRIPTIONAL MODULATION UNDER ABIOTIC AND BIOTIC STRESSES

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Cowpea thaumatin-like proteins: characterization and transcriptional modulation under abiotic and biotic stresses

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Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important legume, cultivated mainly in areas with limited water availability of the African and American continent. Its productivity is significantly affected by environmental stresses. Thaumatin-like proteins (TLPs) are responsive to biotic and abiotic stresses, being classified under the PR-5 (Pathogenesis-Related 5) protein family. Their role is still controversial, with some TLPs being associated with plant defense (especially against fungal infection) and other to drought response. In the present work, the diversity and expression of TLPs were evaluated in cowpea (VuTLPs) under different stresses, including biotic (inoculation with CABMV or CPSMV virus) and abiotic (radicular dehydration). The anchoring of VuTLPs in the cowpea genome revealed the existence of 34 loci encoding such proteins. Neighbor-joining analysis indicated that clusters of potential VuTLPs are functionally diversified while genome anchoring points out that segmental duplication may have contributed to the current gene diversity and neofunctionalization. VuTLP promoters associated bHLH, Dof-type, and MYB-related TFs with VuTLPs, also justifying their plurality of roles. VuTLP diversity was also observed when analyzing their expression under various conditions. Gene expression data revealed that most VuTLPs are recruited during the first minutes after stress imposition, except for of the root dehydration assay, where most of the transcripts were induced at 150 minutes after stress imposition, a condition also validated by qPCR. Besides, the data suggest that VuTLPs present functional specialization concerning each analyzed assay, reinforcing their diversified role and biotechnological potential.

Keywords: PR-5 gene family, osmotin-like, TLPs, *Vigna unguiculata*, zeamatin.

Abbreviations: BLAST, Basic Local Alignment Search Tool; CABMV, Cowpea Aphid-born Mosaic Virus; CpGC, Cowpea Genomics Consortium; CPSMV, Cowpea Severe Mosaic Virus; CTD, Conserved Thaumatin Domain; ESTs, Expressed Sequence Tags; ET, Ethylene; FPKM, Fragments Per Kilobase Million; IPA, Instituto Agronômico de Pernambuco; JA, Jasmonic acid; MEGA, Molecular Evolutionary Genetic Analysis; MethylJA, Methyl Jasmonic Acid; NCBI, National Center for Biotechnology Information; PR, Pathogenesis-related; RNA-Seq, RNA Sequencing; SA, Salicylic Acid; SuperSAGE, Serial Analysis of Gene Expression; TLPs, Thaumatin-like proteins; VuTLPs, Thaumatin-like proteins of *Vigna unguiculata.*

1. Introduction

In the course of evolution, plants have developed a series of components to fight pathogens. Among several families of polypeptides involved in plant defense, thaumatin-like proteins (TLPs) are members of the Pathogenesis-related 5 (PR-5) gene family, considered a prominent protein group. They received this name because of their structural similarity to proteins called thaumatins, isolated from the fruit of *Thaumatococcus daniellii* Benth. (Maranthaceae family) [1]. They share the Thaumatin conserved domain (Pfam: PF00314) that covers almost 95% of the entire mature peptide [2]. About their potential biological functions, TLPs have shown antifungal activity, acting on fungal membrane permeabilization [3]. There is evidence that some TLP representatives act on invading fungi through their binding activities or hydrolysis of β-1,3-glucans [4,5] or, still, through inhibition of enzymes such as xylanases [6].

Genes encoding TLPs can also be induced by hormones associated with plant immunity. For example, in tobacco seedlings, the induction of TLPs occurred in response to the application of ethylene (ET), methyl jasmonate (methylJA) and salicylic acid (SA) [7]. A similar fact was also reported for rice, where the promoter of a TLP (Rtlp1) was induced by the rice blast fungus elicitor, besides the hormones SA and methylJA [8]. TLP expression was also associated with abiotic responses, as drought [9], wounding [10], and freezing [11]. TLPs were also induced after infection by virus or bacteria [12]; however, their mechanism of action in these situations remains indeterminate.

Due to their importance and biotechnological potential, TLPs have been analyzed in a large number of species, such as *Arabidopsis thaliana*, rice (*Oryza sativa*), *Populus* spp., maize (*Zea mays*), *Physcomitrella patens*, *Chlamydomonas* spp., and wheat (*Triticum aestivum*) [13,14], among others. However, the vast majority of these studies does not explicitly focus on TLPs but comprise their verification among the global transcriptional response of the analyzed plant species under stress.

Concerning their role in pathogen defense, the vast majority of data on TLPs addressed their association with response to fungal agents. Examples can be found in Wang et al. [14] and Misra et al. [15]. About legumes, information on this protein class is still restricted to soybean (*Glycine max*) [16] and wild peanut (*Arachis diogoi*) [17].

Cowpea is an important legume, being widely consumed and cultivated in Africa and the Americas. The Cowpea Genomics Consortium (CpGC) occupies an outstanding position due to the availability of transcriptomics data with Next Generation Sequencing,

that generated expression data including RNA-Seq and HT-SuperSAGE (Super Serial Analysis of Gene Expression) libraries. The available resources (Suppl. Table S1) cover cowpea responses to abiotic (radicular dehydration and high salinity) and biotic [inoculation by *Cowpea Severe Mosaic Virus* (CPSMV) and *Cowpea Aphid-borne Mosaic Virus* (CABMV)] stresses. Besides the mentioned libraries, the NordEST consortium (an earlier initiative of the Brazilian CpGC group) generated 49,820 ESTs (*Expressed Sequence Tags*) from leaves and roots under the cited biotic / abiotic conditions that were clustered together with 264,945 ESTs deposited at Cowpea HarvEST database V.1.33 (http://harvest.ucr.edu/) and GenBank (NCBI; https://www.ncbi.nlm.nih.gov/genbank/). These sequences are available for anchoring and annotation of transcripts under analysis.

The only report found in the literature addressing the structural and functional genomics of TLPs was performed by Petre et al. [2]. These authors identified 42 validated TLP-loci anchored in the genome *Populus trichocarpa* 'Nisqually-1' (version 2.0). In addition, it was observed that most TLPs were responsive to abiotic stresses (high ozone content, UV-B rays, drought, and high copper content) besides being also responsive to biotic stress (infection by the fungus *Melampsora laricis-populina*). Regarding the potential antiviral activity of TLPs only the work of Kim et al. [18] evaluated TLP under this type of stress by protein-protein interaction experiments, observing that tobacco (*Nicotiana tabacum*) TLP interacted with *Cucumber mosaic virus* proteins.

Thus, given the importance, multifunctionality, the responsiveness of plant TLPs under unfavorable conditions and the availability of CpGC data, the present work brings structural characterization and expression data of cowpea TLPs expressed under different stress types (CABMV or CPSMV inoculation and radicular dehydration). The present work uncovered relevant information to the molecular physiology of cowpea under unfavorable conditions and provide data about the performance of TLPs in legumes, an issue still little addressed.

2. Materials and methods

2.1. Biological material and stress conditions

The inoculation experiments with CABMV and CPSMV were carried out under greenhouse conditions at the Instituto Agronômico de Pernambuco (IPA), in Recife, Pernambuco, Brazil. For the CABMV assay, the resistant (IT85F-2687) and susceptible

(BR14-Mulato) genotypes were used for inoculation with the virus. For the CPSMV treatment, the genotypes BR-14 Mulato (resistant) and IT85F-2687 (susceptible) were used. For both trials, genotypes were cultivated for 21 days under natural photoperiod, with temperatures ranging 28 to 32 °C. For the virus inoculation, plants were mechanically injured with Carborundum (silicon carbide) followed by application of each viral inoculum. The experimental design was factorial (cultivar vs. period of infection) with three biological replicates (BRs) for each control and treatment implemented. Each BR was composed of five plants. Each treatment was carried out in an isolated place together with the respective replicates to avoid the impact of volatile plant compounds across treatments. Additionally, each inoculation time presented its respective control, with two sampling time points: 60 minutes and 16 hours after the inoculation with each virus. The radicular dehydration assay was performed as described by Amorim et al. [19]. The collection sampling times and experimental design are shown in Fig. 1; Suppl. Tab. S1. After applying the stress in each respective experiment/treatment, the leaf and root tissues were collected, being immediately frozen in liquid nitrogen and stored in the deep-freezer at –80º C until RNA extraction.

2.2. RNA Extraction and cDNA synthesis

Total cowpea RNA were extracted from leaves (viruses) and roots (dehydration experiment) using the “SV Total RNA Isolation System®” kit (Promega), following manufacturer's recommendations. The concentration and purity of the extracted RNA samples were verified using a fluorimeter Qubit (Invitrogen®), and their integrity verified in 1% agarose gel electrophoresis. cDNA synthesis was carried out with the *Improm-IITM Reverse Transcription Systems* kit (Promega) according to the manufacturer's recommendations.

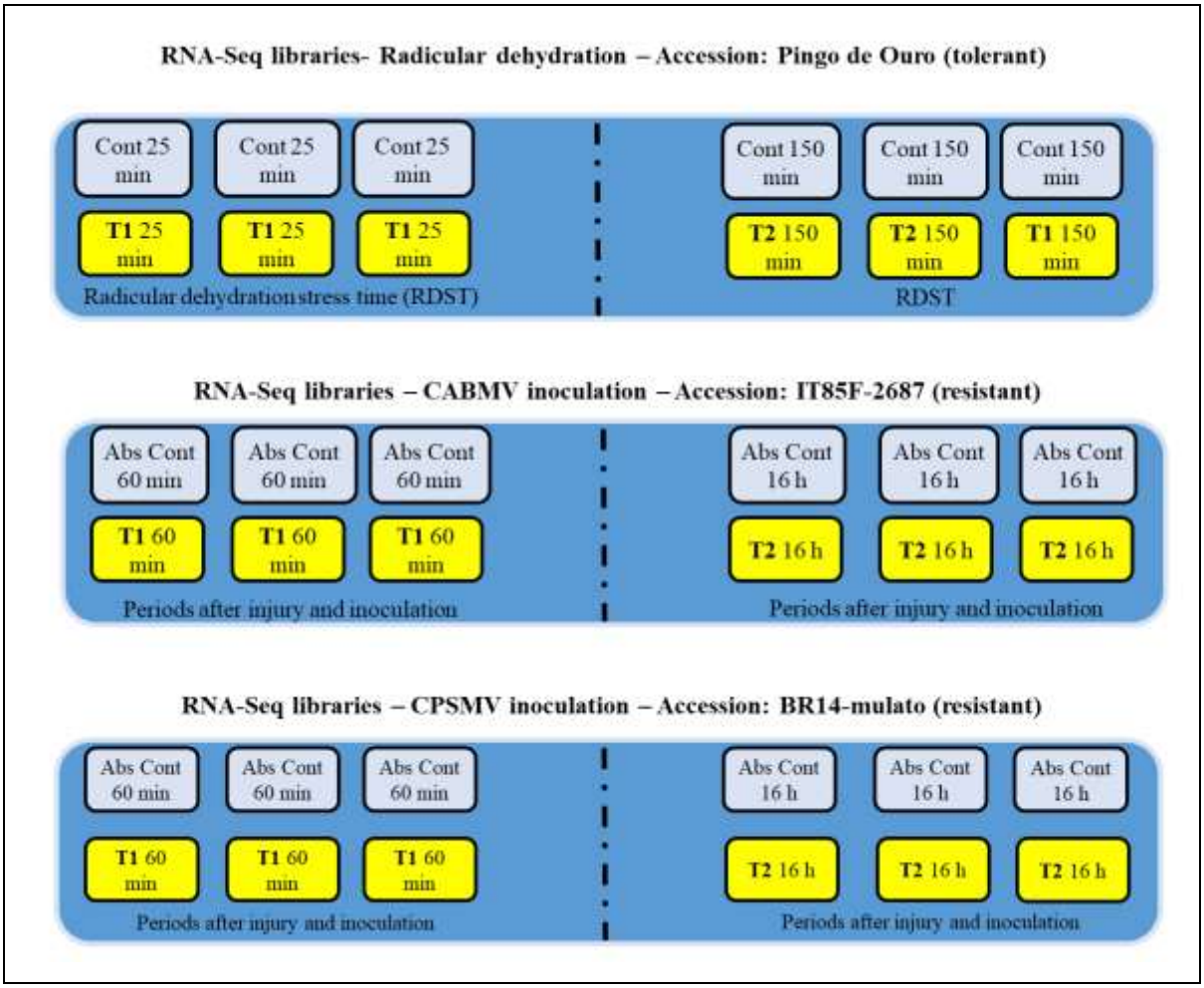

Fig. 1. Experimental design of the RNA-Seq libraries synthesized for each assay performed, presenting the treatment times, stresses analyzed and accessions employed. Dashed and dotted lines separate the different libraries from the same assay. Cont: control; Abs Cont: absolute control; RDST: Radicular Dehydration Stress Time; T1: treatment 1 (60 min); T2: treatment 2 (16 h).

2.3. Construction of RNA-Seq libraries

RNA samples were subjected to Illumina HiSeq 2500 paired-end sequencing (100 bp reads) at the Functional Genomics Center Facility (ESALQ/USP, Piracicaba, São Paulo, Brazil). In order to obtain a more robust transcriptome, the reads from all treatments (virus inoculation, root dehydration, and controls) were assembled together. The assembly was performed by the 'de novo' approach using the Trinity software [20], and the transcriptome was deposited in the CpGC.

2.4. Cowpea TLPs (VuTLPs) *in silico* characterization

The *in silico* analysis was carried out using reads of cowpea RNA-Seq libraries under CABMV or CPSMV inoculation or under radicular dehydration (Suppl. Tab. S1). VuTLPs were predicted based on sequence homology searches, using thaumatin-domain with sequences previously characterized. An outline of the annotation strategy is presented in Fig. 2. For the identification of VuTLPs candidates, the alignments were carried out against CpGC database using tBLASTn [21] (Fig. 2) with a cut-off value of $1e^{-05}$. VuTLP candidates were annotated against NCBI and UniProt and analyzed for their score, e-values, sequence size, and presence of Conserved Thaumatin domains (CTDs), as shown in Suppl. Tab. S2.

All sequences were screened for conserved motifs, with the aid of rps-BLAST on CD-search tool [21] (Fig. 2). Only orthologs presenting the expected features (CTDs and motifs) were translated into proteins and considered for subsequent evaluation (Fig. 2).

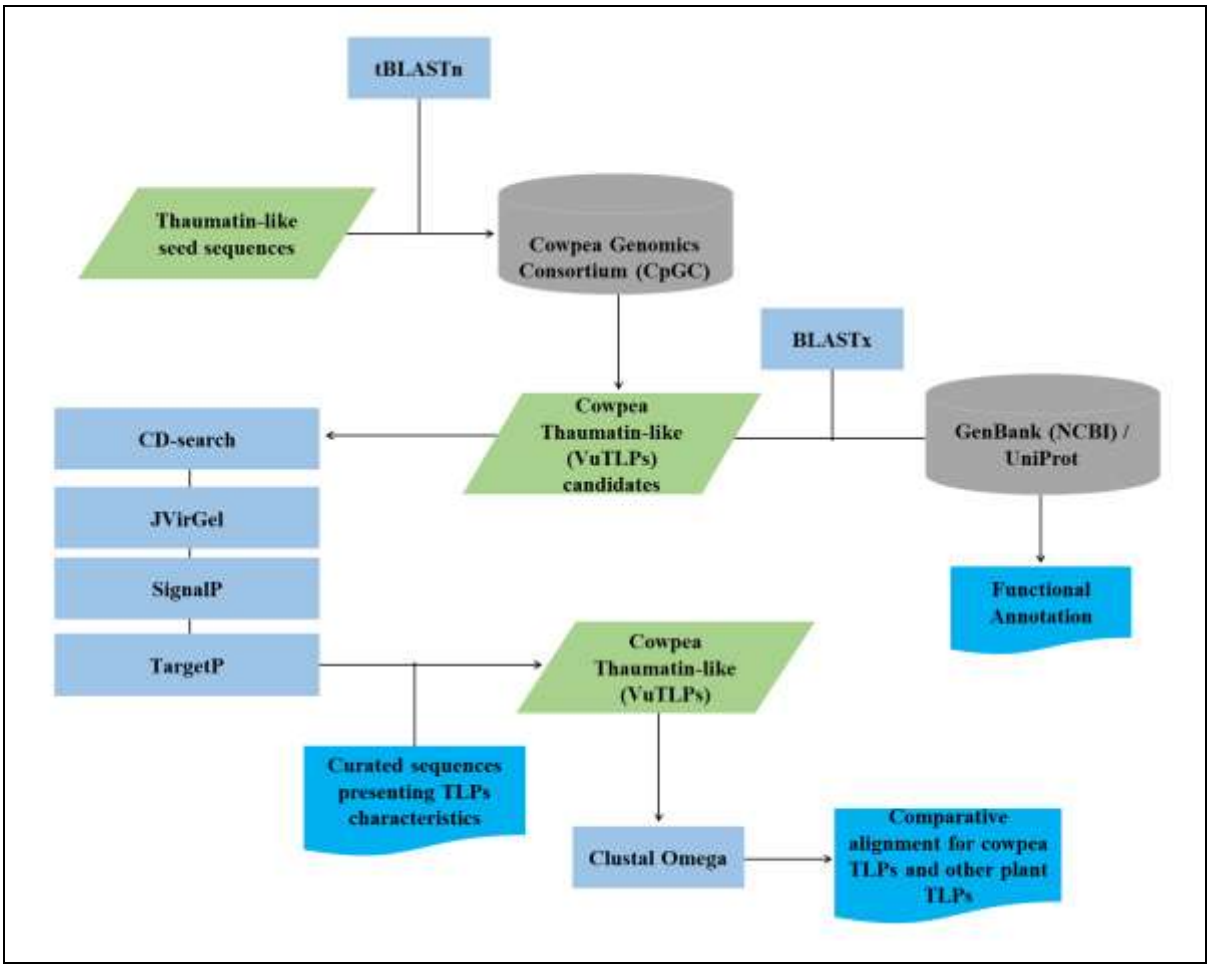

Fig. 2. Workflow used for *in silico* identification and prediction of VuTLPs applied to the cowpea transcriptome.

Aiming to enrich the characterization of predicted TLPs, the identified amino acid sequences were further characterized including the following analyzes (Fig. 2):

- **i.** Determination of the putative isoelectric point and molecular weight using ProtParam (https://web.expasy.org/protparam/; [22]; Fig. 2);
- **ii.** Prediction of signal peptide for each cowpea TLP candidate with SignalP 4.1 Server ([23]; http://www.cbs.dtu.dk/services/ SignalP/);
- **iii.** Prediction of the subcellular localization with TargetP 1.1 Server ([24]; http://www.cbs.dtu.dk/services/TargetP).

Additionally, we used Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/; [25]) to generate a multiple sequence alignment of full-length VuTLPs compared with previously described TLPs from other plant species, aiming at the localization and comparison of CTDs and residues among TLP sequences. For this step, some homolog proteins denominated “osmotin” and “zeamatin” were also employed (Suppl. Fig. 1; Suppl. Mat. SM1), considering literature evidences [26-28] that such proteins present similarities with TLPs.

2.5. VuTLPs expression in cowpea RNA-Seq libraries

For CABMV, CPSMV inoculation and radicular dehydration assays, the transcriptional modulation of VuTLPs candidates was carried out by direct correlation of RNA-Seq reads frequencies (regarding each putative ortholog in different libraries), based on FPKM (Fragments Per Kilobase Million) values in each treatment available. For this purpose, the data were analyzed by edgeR, a Bioconductor software package for examining differential expression of replicated count data [29]. Descriptions of all RNA-Seq libraries, accessions and treatment times employed in this work are available in Suppl. Tab. S1. For analysis of the differential expression in each treatment, VuTLPs candidates from each treatment/library in a given time interval were compared with their respective control. Thus, the identification of induced or repressed transcripts was performed based on $\log_2$ fold change ($\log_2$FC) and *p-value*. VuTLPs were considered as induced or repressed when $\log_2$FC values were higher than +1 or less than -1, respectively, associated with a *p-value* < 0,05. Hierarchical clustering of VuTLPs was carried out with CLUSTER 3.0 [30], and the *heatmap* was visualized using TreeView [31]. In addition, Venn diagrams were also generated using software Venny [32].

2.6. Neighbor-Joining (NJ) analysis

For the NJ analysis, selected VuTLPs from cowpea RNA-Seq libraries under radicular dehydration or after viral (CABMV or CPSMV) inoculation were employed. Only VuTLPs presenting a complete CTD were used (see Suppl. Mat. SM2). Sequences from other species were recovered from UniProt (https://www.uniprot.org/) and GenBank (NCBI, https://www.ncbi.nlm.nih.gov/). For this purpose, 17 TLPs from 14 different plant taxa were obtained (Suppl. Mat. SM2, multifasta format). Next, the CTDs of the sequences were recovered using the SMART [33] tool.

After recovering the CTDs, the multiple alignment was submitted to manual edition and elimination of not aligned extremities and uninformative autapomorphies. The NJ analysis was carried out using the MEGA 7 [34]. Statistical support of the branches was evaluated by a bootstrap analysis with 2,000 replicates.

2.7. Distribution of VuTLP-candidates in *V. unguiculata* genome

To determine the genomic distribution of VuTLPs, cowpea candidates were aligned against *V. unguiculata* genome available at Phytozome Database (https://phytozome.jgi.doe.gov/) aiming to anchor these sequences in virtual chromosomes through the BLASTp tool. This step aimed to infer on the distribution, relative position, and abundance of TLPs-coding loci. We considered the best-hit (e-value cut off < $1e^{-10}$) to allow the identification of VuTLPs along the virtual chromosomes. Afterward, the identified anchoring positions were used to build a virtual ideogram (Fig. 3) regarding the TLPs distribution on *V. unguiculata* pseudochromosomes (n = 11).

2.8. Promoter analysis

Cowpea promoter regions (up to 1.0 kb upstream of TLP-coding genes) were downloaded from Phytozome database v8.0 (https://phytozome.jgi.doe.gov). The motifs (candidate cis-regulatory elements) in each promoter were revealed through the MEME v5.0.3 software [35] (http://meme-suite.org/tools/meme). The software reports an e-value for each identified motif and gives an estimate of the number of expected motifs found by chance if the input sequences were shuffled. In the present simulation, an e-value < 10^{-2} was adopted as the cut-off for characterization of *bona fide* cis-regulatory element candidates. The maximum number of motifs searched in the present analysis was 10. The

motifs analyzed exhibited between six and 50 nucleotides in length. The command line used was: meme (file name) -dna -oc . -nostatus -time 18000 -mod anr -nmotifs 10 -minw 6 -maxw 50 -objfun classic -revcomp -markov_order 0.

After MEME software analysis, the Tomtom v4.11.2 software [36] (http://meme-suite.org/tools/tomtom) was used with the JASPAR database (file: JASPAR2018_CORE_plants_non-redundant) to annotate putative cis-regulatory elements. JASPAR searches one or more queries (candidate cis-regulatory element) against annotated motifs ranked by p-value (cut-off < 10^{-2}). The q-value (false discovery rate; cut-off < 10^{-2}) of each match is also reported. In this work, the presented identities of the cis-regulatory element candidates were associated with the target motif that exhibited the most significant p-value. The command line used was:
tomtom -no-ssc -oc . -verbosity 1 -min-overlap 5 -mi 1 -dist pearson -evalue -thresh 10.0 query_motifs db/JASPAR/JASPAR2018_CORE_plants_non-redundant.meme

2.9. RT-qPCR Analyses

For validation of the RNA-Seq data of the different experiments, RT-qPCR analyses were performed with treatments and control for each time sampled. For each time point three biological replicates, each with three technical replicates, were analyzed to ensure statistical reliability. The qPCR reactions were performed in 96-well plates and performed on the LineGene 9660 (Bioer), using SYBR Green detection. The PCR program was adjusted to 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 15 s. After amplification, dissociation curves were produced (65–95 °C at a heating rate of 0.5 °C / sec and acquiring fluorescence data every 0.3 °C) to confirm the specificity of the PCR products.

The amplification efficiency ($E = 10^{(-1/\text{slope of the standard curve})}$ for all primer pairs was determined from a 4-point standard curve generated by serial dilutions of cDNA (10-fold each) in technical triplicates. *β-tubulin-2*, *F-box* and *UBQ10* were used as reference genes for normalization of CABMV and CPSMV assays while *Actin* and *UE21D* were used as reference genes for normalization of radicular dehydration assay [19]. Primers for VuTLPs candidates were designed based on the *V. unguiculata* transcriptome using the Primer3 tool (http://bioinfo.ut.ee/primer3-0.4.0/) under program´s default settings.

The Rest2009 [37] software package (standard mode) was used for relative expression analysis of target transcripts. Hypothesis testing ($p < 0.05$) was used to

determine whether the differences in target transcripts expression between the control and treatment conditions were significant.

3. Results

3.1. *In silico* identification and characterization of VuTLPs

The tBLASTn search allowed the identification of 126 putative VuTLPs (Suppl. Tab. S2) under biotic and abiotic stresses. From these 33 presented no ORF associated with TLP, while 32 presented complete TLP conserved domain (CD) and 61 presented incomplete CDs. Among candidate VuTLPs with complete CD, 30 were expressed in cowpea RNA-Seq libraries generated under radicular dehydration, while 28 and 27 VuTLPs were expressed under CABMV and CPSMV inoculation, respectively. Considering the total amount (abiotic + biotic stresses), 30 non-redundant VuTLPs were recognized (data not shown).

All 30 predicted VuTLPs presented CTD in at least one searched database (NCBI CDD: cd09218 and cl02511, Pfam: pfam00314, SMART ID: smart00205; Suppl. Tab. S2). Five highly conserved amino acids (REDDD motif), in an acidic cleft, were detected in most VuTLPs. However, the alignment of the referred candidates with other TLPs from *A. thaliana*, *Nicotiana tabacum*, *Zea mays,* and *Cicer arietinum* revealed that some VuTLPs did not contain all expected amino acids in REDDD motif. For this group, some VuTLPs exhibited the following substitutions: the glutamic acid (E) by a glutamine (Q) in the transcript Vu54351|c0_g1_i1 and in the transcripts Vu85277|c0_g1_i1 and Vu28453|c0_g1_i1 the glutamic acid (E) was replaced by aspartic acid (D) of the same functional group; and the first aspartic acid (D) by a glycine (G) in the transcript Vu54351|c0_g1_i1. Other modifications in the mentioned motif were also perceived. For instance, in the transcripts Vu85277|c0_g1_i1 and Vu28453|c0_g1_i1 (Suppl. Fig. S1) the second aspartic acid (D) was replaced by an asparagine (N).

Regarding the 16-cysteine conservation, just three cowpea sequences (Vu58024|c0_g1_i1, Vu49480|c1_g2_i2 and Vu49480|c1_g2_i1; Suppl. Fig. S1) lacked the first cysteine; whereas the sixth cysteine of transcript Vu83475|c0_g1_i1 was replaced by a serine (S). In turn, all other VuTLPs presented the 16 residues. Additionally, expressed VuTLPs exhibited a molecular weight between 18.97 and 34.93 kDa and an isoelectric point varying from 4.23 to 9.05 (Suppl. Tab. S2).

The SignalP prediction, in turn, revealed that 20 VuTLPs present hydrophobic signal peptide sequences.

3.2. NJ tree with VuTLPs

In the present work, an NJ tree (Suppl. Fig. S2) was generated including 28 cowpea sequences containing CTD (Suppl. Mat. SM2**)** besides 17 TLP sequences from other plant species containing CTD (Suppl. Mat. SM2). Cowpea sequences and other Fabaceae and Brassicaceae TLPs (Suppl. Fig. S2) emerged in the dendrogram in a basal position (node IA; Suppl. Fig. S2) with a bootstrap value of 97. In turn, the node IB grouped VuTLPs with Rosaceae and Fagaceae TLPs. From this basal group, a second node (II; Suppl. Fig. S2) emerged, including VuTLPs and a *Cajanus cajan* TLP (all from the Fabaceae family), with a bootstrap value of 83.

The third node included two subgroups, where subgroup A (IIIA; Suppl. Fig. S2) included only VuTLPs and a soybean (*G. max*, Fabaceae), with a high bootstrap value (100), indicating a close structural similarity among both TLPs. In turn, the subgroup IIIB was divided into subgroups IIIBI and IIIBII, where BI included an Osmotin-like TLP (from the Solanaceae family), and a TLP of the Zeamatin-group (from Poaceae family), together with VuTLPs, and TLPs of Fabaceae family (bootstrap 100), indicating a close structural similarity among the involved TLPs.

Furthermore, subgroup BII (Suppl. Fig. S2) included VuTLPs and TLPs of Euphorbiaceae family.

3.3. Anchoring of VuTLPs in *V. unguiculata* genome

The approach revealed the distribution of the VuTLPs in 34 loci located along the chromosomes, with exception chromosome ten (Fig. 3).

A clustering of VuTLPs loci was observed, being more pronounced on chromosomes two and eight (Fig. 3; Suppl. Tab. S4). For example, the long arm of *V. unguiculata* chromosome 2 presents six and two clustered TLP coding genes (Fig. 3). Such a distribution is indicative of evolutionary processes through tandem duplication. Besides the observed clustering in most chromosomes, the position of VuTLP coding genes was mainly subterminal or intercalary, while a pericentric position of VuTLPs is observed only in chromosomes 6 and 7 (Fig. 3).

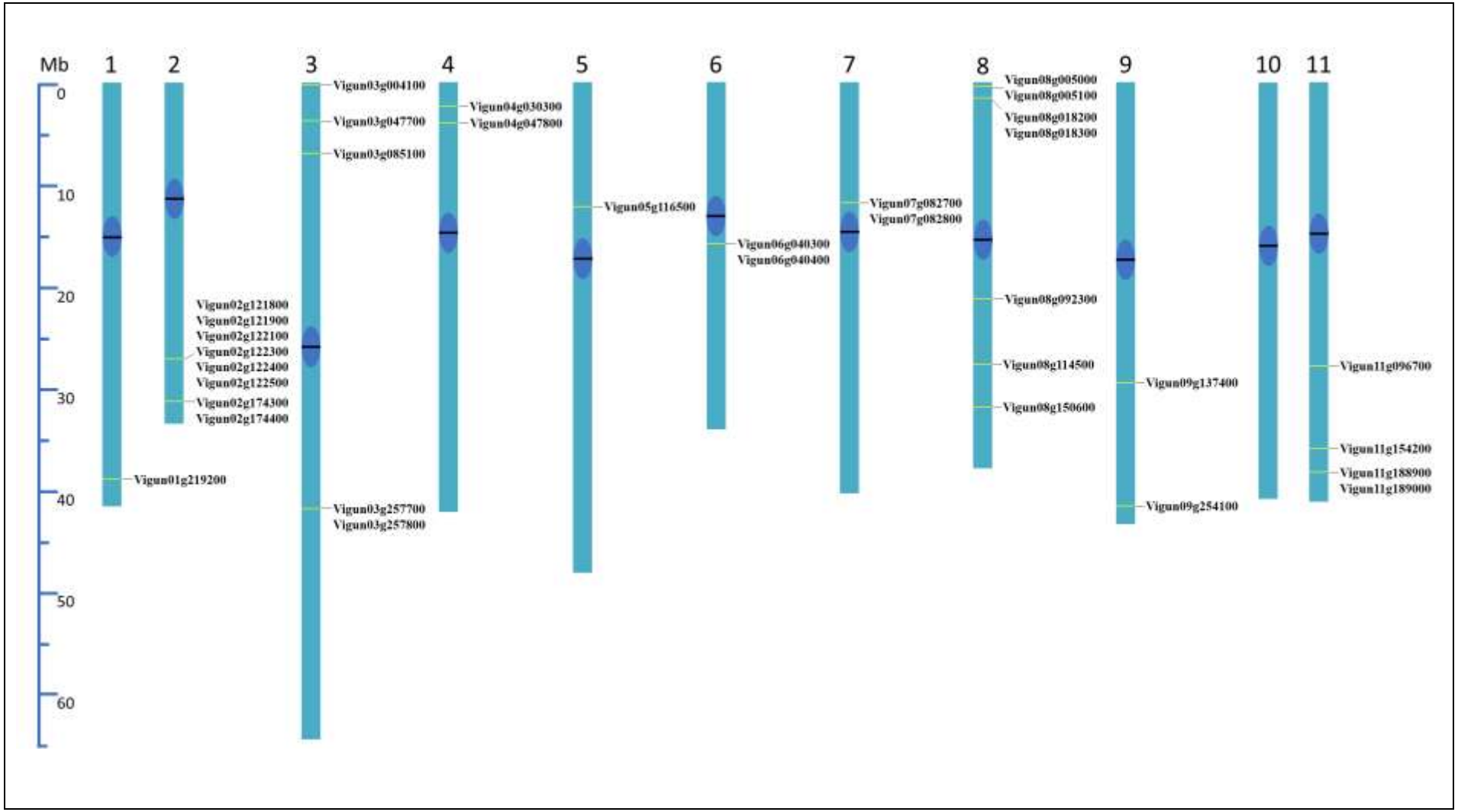

Fig. 3. Distribution of cowpea thaumatin-like genes in *Vigna unguiculata* virtual chromosomes (Chr1 to Chr11). Positions of TLP anchored genes or clusters are indicated in yellow. Centromeres are indicated with black bars and dark blue spheres.

3.4. Promoter VuTLPs

The 34 cowpea TLP coding genes had their predicted promoters (1 kb) analyzed regarding the presence and identity of candidate cis-regulatory elements (CCREs), via the MEME and Tomtom tools. Four of the identified promoter motifs were within the stipulated cut-off (e-value < 10^{-2}, Fig. 4; Suppl. Fig. S3A, S3B, S3C e S3D), being considered as *bona fide* CCREs, (see Suppl. Fig. S3A, S3B, S3C e S3D). The six remaining motifs (Fig. 4) were associated to TFs [Dof-type (dark green and pink boxes), C2H2 (light green), G2-like (orange), and CPP (light blue), and one without specific annotation] but did not achieve the specified parameters, being excluded from the discussion.

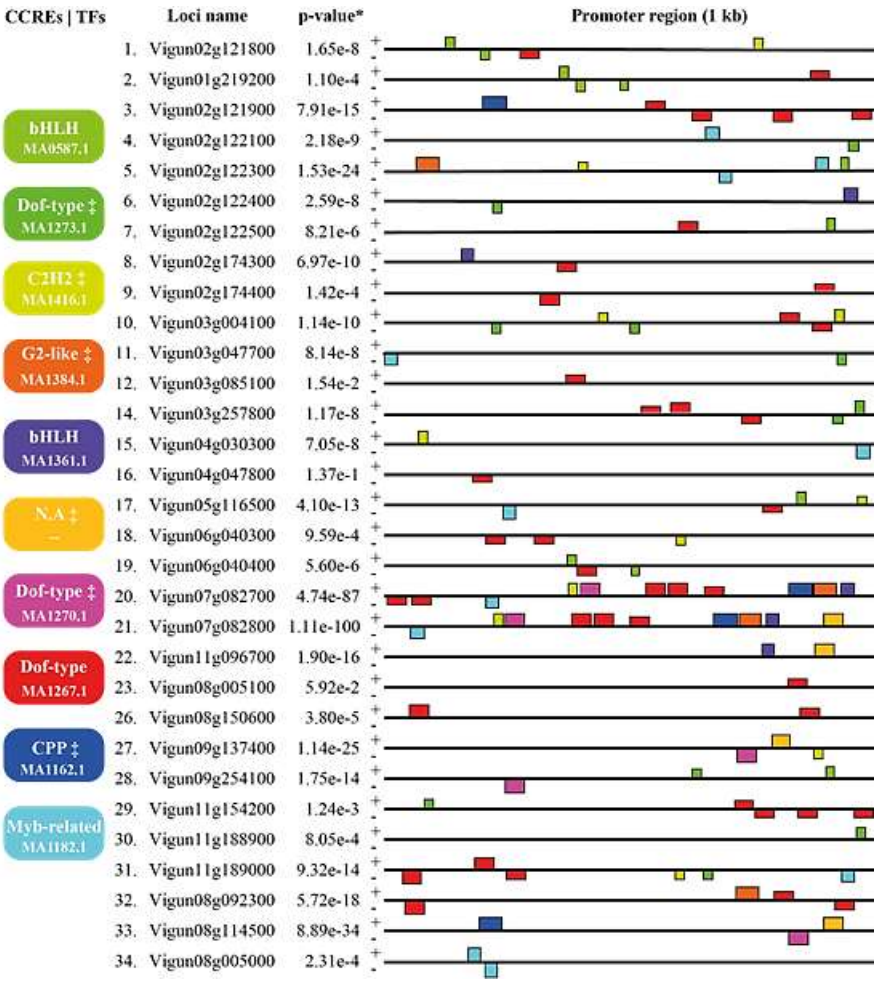

Fig. 4. Motif analysis of cis-regulatory elements (CCREs) detected in promoter regions of thaumatin-like protein coding genes. Promoter content and distribution represented by colored rectangles and squares. Colored boxes (CCREs | TFs section) bring information on transcription factors (TFs) associated with the identified motifs, as well as their respective

JASPAR ID Matrix. "+" and "-" signals represent sense and antisense strands of the promoter regions analyzed. *MEME combined match p-value. ‡ Motifs with statistical significance below of the adopted cut-off (e-value < 10^{-2}; data not shown). N.A (not annotated motif).

The identified *bona fide* CCREs are associated with the three TF families (Fig. 4): bHLH (two motifs; light green and blue boxes, Suppl. Fig. S3A and S3C), MYB-related (light blue, Fig. 4; Suppl. Fig. S3B) and Dof-type (red, Fig. 4; Suppl. Fig. S3D). CEMR associated with a Dof-type transcription factor (JASPAR ID MA1267.1; Fig. 4; Suppl. Fig. S3D) was the most abundant promoter, observed in 42 sites, followed by MYB-related (JASPAR ID MA1182.1; Fig. 4; Suppl. Fig. S3B) and bHLH (JASPAR ID MA0587.1; Fig. 4; Suppl. Fig. S3C) each anchored in 11 sites. Finally, bHLH (JASPAR ID 1361.1; Fig. 4; Suppl. Fig. S3A) anchored in five sites.

3.5. TLP content and expression in cowpea transcriptomes under different stress types

Considering all libraries RNA-Seq available for biotic and abiotic stresses (Suppl. Tab. S5), a total of 30 VuTLPs were identified.

CABMV and CPSMV assays

Both virus inoculation treatments (CABMV and CPSMV) did not include separated injury library (Suppl. Tab. S1; Fig. 1). Thus, the stress component regarded a combination of injury plus viral inoculation compared with the control (not injured and not inoculated), where each treatment is compared with its respective control at the same time point. As a result, twenty-eight VuTLPs-coding transcripts were expressed in the CABMV RNA-Seq libraries (Suppl. Tab. S5; Suppl. Fig. S4A). Of these, six were induced exclusively at 60 min (Suppl. Fig. S4B), indicating that TLPs are preferably recruited during the first hours after the CABMV inoculation / injury. In addition, only one transcript encoding VuTLP (Vu44758|c0_g1_i1) showed up induced at 16 h. Besides, the transcript Vu9781|c2_g1_i3 encoding VuTLP was induced at 60 min and repressed at 16 h treatment, whereas the others presented constitutive expression or were not expressed in the comparisons.

Regarding the CPSMV assay, twenty-seven transcripts encoding VuTLPs were expressed (Suppl. Tab. S5; Suppl. Fig. S5A). Of these, five were induced exclusively at 60

min and three at 16 h (Suppl. Fig S5B). In addition, a transcript encoding VuTLP (Vu83475|c0_g1_i1) was induced in both treatments (60 min and 16 h; Suppl. Fig. S5A). These results also point out that potential VuTLPs were recruited in the first hour after CPSMV inoculation / injury.

When comparing the transcriptional orchestration in response to the two viral types, it was observed that four potential VuTLPs were induced in response to both viruses / injury (Suppl. Fig. S6A). However, four and five transcripts encoding VuTLPs were induced exclusively in response to CABMV and CPSMV, respectively (Suppl. Fig. S6A).

Radicular dehydration assay

Thirty potential transcripts coding for VuTLPs were expressed in cowpea under radicular dehydration (Suppl. Tab. S5; Suppl. Fig. S7A). Of these, Vu73314|c0_g1_i1 and Vu49480|c1_g2_i1 were induced and repressed at 25 min, respectively. Additionally, the transcript Vu94809|c0_g2_i1 encoding VuTLP was induced at 25 min and repressed at 150 min treatment. In addition, the transcript Vu88791|c0_g1_i2 was induced in both treatments (25 min and 150 min). Besides, eight and three transcripts encoding for VuTLPs were induced and repressed exclusively at 150 min treatment, respectively. These results indicate that some TLPs are recruited in the first minutes after stress imposition, even though a higher number of transcripts were detected at 150 min (Suppl. Fig. S7B).

CABMV inoculation vs. CPSMV inoculation vs. Radicular dehydration assays

A comparison between the orchestration in response to both viruses (in leaves) and radicular dehydration (roots) revealed interesting insights. There are 20 potential VuTLPs expressed jointly in both assays (CABMV and CPSMV inoculation and radicular dehydration, Suppl. Tab. S5; Suppl. Fig. S6B), thus indicating a crosstalk role for TLPs under different stress types and tissues.

Qualitative analyses of VuTLPs show a heterogeneous orchestration of VuTLPs. Only two VuTLPs candidates were induced in both biotic and abiotic assays (Suppl. Tab. S5). Exclusive responses were also observed under different treatments. For example, three, four and seven transcripts encoding VuTLPs were induced exclusively under CABMV and CPSMV inoculation and radicular dehydration, respectively. Thus, there is no overall conservation in VuTLPs orchestration towards different viral isolates and

radicular dehydration, indicating specific TLP associated with the response of each virus type and radicular dehydration.

Expression validation by RT-qPCR - CABMV and CPSMV

The selection of target transcripts (Vu44758|c0_g1_i1, Vu95346|c0_g1_i1, Vu83475|c0_g1_i1 and Vu9781|c2_g1_i3) was based on their regulation in the RNA-Seq libraries (Suppl. Tab. S5) for CABMV experiment. Considering the functional primer pairs, all presented acceptable efficiency values (90 to 110% [38]) in the qPCR analysis and ranged between 98.40 and 99.70% (Suppl. Fig. S8). Relative expression analysis showed that Vu95346|c0_g1_i1 and Vu9781|c2_g1_i3 were up-regulated (UR) at 60 min (Suppl. Fig. S9) while Vu44758|c0_g1_i1 was UR at 16 h (Suppl. Tab. S6 and Suppl. Fig. S10), confirming RNA-Seq data. Besides, qPCR results have shown that Vu9781|c2_g1_i3 was down-regulated (DR) at 16 h, while Vu95346|c0_g1_i1 and Vu83475|c0_g1_i1 were not modulated at 16 h (not significant; ns) also confirming RNA-Seq data (Suppl. Tab. S6 and Suppl. Fig. S10).

For CPSMV treatment, three differentially expressed target transcripts (Vu95346|c0_g1_i1, Vu44758|c0_g1_i1 and Vu83475|c0_g1_i1) were evaluated, all primers presented acceptable efficiency values (between 97.3 and 106.30%; Suppl. Fig. S11). Both Vu95346|c0_g1_i1 and Vu83475|c0_g1_i1 were UR at 60 min (Suppl. Tab. S6 and Suppl. Fig. S12). Besides Vu44758|c0_g1_i1 and Vu83475|c0_g1_i1 were also UR at 16 h (Suppl. Tab. S6 and Suppl. Fig. S12), while Vu95346|c0_g1_i1 was ns at the same time point (16 h; Suppl. Tab. S6 and Suppl. Fig. S12). All these results confirm RNA-Seq expression data. The qPCR analysis uncovered an additional induced transcript, showing that Vu44758|c0_g1_i1 was also UR (ca. 12x in the virus inoculated treatment) at 60 min (Suppl. Tab. S6 and Suppl. Fig. S12) differing from RNA-Seq data where it was not modulated (ns).

Radicular dehydration

For radicular dehydration treatment, three differentially expressed target transcripts were validated by qPCR (Vu88791|c0_g1_i1, Vu149510|c0_g2_i1 and Vu49480|c1_g3_i1), all with acceptable efficiency values (91.00 to 95.10%; Suppl. Fig. S13). Relative expression analysis showed that all three target transcripts were up-regulated at 150 min (Suppl. Tab. S6; Suppl. Fig. S15). Besides, transcripts

Vu149510|c0_g2_i1 and Vu49480|c1_g3_i1 presented no modulation (ns) at 25 min (Suppl. Tab. S6 and Suppl. Fig. S14). All these results confirmed RNA-Seq data. In turn, transcript Vu88791|c0_g1_i1 was UR at 25 min (Suppl. Tab. S6 and Suppl. Fig. S14), differing from RNA-Seq results where it was ns.

4. Discussion

Using TLPs as seed sequences, cowpea VuTLP candidates were searched in the RNA-Seq libraries generated under radicular dehydration or CABMV or CPSMV inoculation. Such conditions were chosen to exemplify the participation of TLPs in response to both, biotic and abiotic stresses, since TLP participation in response to abiotic stress is still incipient when compared to its biotic counterpart. Additionally, RNA-Seq reads have up to 400 bp, depending on the sequencing technology used [39], allowing direct VuTLP structural characterization.

Most of the identified VuTLPs presented the REDDD motif, in an acidic cleft. This configuration is responsible for the reported antifungal activity of TLPs in plants [13,40]. However, the alignment of the referred candidates with other TLPs from *A. thaliana*, *N. tabacum*, *Z. mays*, and *C. arietinum* revealed that some VuTLPs did not contain all expected amino acids in REDDD motif. It is still not clear whether these small differences have a significant impact on the substrate selectivity or protein function [2]. Petre et al. [2] found that the acidic cleft is the most conserved region among the eukaryotic TLPs. For example, in sTLPs (small TLPs), the most of REDDD amino acids are conserved, but wheat sTLPs sequences with resolved structure have revealed no acidic clefts nor any particular conserved region which may be linked to the xylanase inhibitor function reported for such proteins [2,6].

Regarding the 16-cysteine conservation, with the exception of most VuTLPs, all other VuTLPs presented the 16 residues that form eight disulfide bonds necessary for correct folding and to ensure a high level of thermostability and pH constancy [41]. Most TLPs presented molecular weights ranging from 21 to 26 kDa [42] and isoelectric point ranging between 3.4 and 12.0 [43], having this last one influence on the electrostatic potential at the molecular surface [44]. Thus, our results show that most of the VuTLPs presented values within the expected standard for both characteristics, except for some VuTLPs that presented specificities regarding their molecular weight.

The SignalP prediction revealed that most VuTLPs present hydrophobic signal peptide sequences, indicating that they are predominantly secreted, a result confirmed by TargetP prediction. TLPs are located mainly in two subcellular compartments; apoplastic space or vacuole because of an N-terminal signal peptide that directs the mature protein to a secretory pathway and in some instances, a C-terminal polypeptide that directs them to a vacuolar compartment [45]. Previous studies have used GFP-gene approach associated with a transient expression system to study the localization of these proteins. Most of them point to a location in extracellular spaces [17,46], corroborating with the *in silico* prediction.

Osmotins are reported as responsive to osmotic stress, while zeamatins (which are more frequent in grasses) possess antifungal activity due to membrane permeabilization. They also present a TCD. However, the topology of the NJ tree confirms that the decomposition of TLPs into different protein families / nomenclatures (i.e., osmotin and zeamatin) is improper since both categories are structurally similar and have been positioned in the middle of other sequences containing the TCD, with no evident particularity or specificity, confirming the proposition done by Petre et al. [2].

Analyzing eukaryotic TLPs, Liu et al. [42] observed the formation of nine distinct groups showing, what he called TLP superfamily, as highly diversified. This presumption may also be valid for the present data, considering the low support of many branches and also of basal nodes. A possible explanation for the formation of these groups can be attributed to the structural diversity presented by TLPs. According to Petre et al. [2], such diversity may influence the biological and biochemical functions, and differences in the topology around the cleft could determine the specificity of TLPs to their target ligands [47]. This may be true also considering the VuTLPs since their diversity and position in different positions of the NJ tree is possibly due to the presence of orthologs and paralogs, with structural and functional specificities. One should have in mind that some cowpea candidates may also regard different transcripts (isoforms) that may be associated with splicing variants of the same gene, what also occurred in the case other species previously studied as *P. trichocarpa* with 42 putative TLP-coding gene candidates [2].

To infer on the distribution, relative position, and abundance of VuTLPs, we anchored candidate transcripts against the cowpea genome, recently available [48]. The amount of VuTLPs is similar to those found in other plant genomes. For instance, *P. trichocarpa* presents 42 putative TLP genes [2]; *O. sativa*, has 24 TLP loci identified [49];

A. thaliana, 28 TLP genes [42]; white spruce (*Picea glauca*) exhibits 13 [42], western white pine (*Pinus monticola*) 10 [42] and the moss *P. patens*, six TLP genes [42]. Since cowpea is a diploid species with 2n = 22 [50] this number similar to rice and *A. thaliana* is justified, being lower than *P. trichocarpa*, considered a tetraploid species [51].

The clustering of VuTLPs loci was observed and it is indicative of evolutionary processes through tandem duplication. Recently, Cao et al. [13] suggested that tandem and segmental duplications are possibly the main drivers of the existing diversity in plants. Wanderley et al. [52] observed a similar feature for *R* (resistance) genes and other *PR* (Pathogenesis-Related) protein-coding genes in soybean and *Medicago truncatula* genomes, a similar feature observed for defense genes in other plant groups [53]. Besides the observed clustering in most chromosomes, the position of VuTLP coding genes was mainly subterminal or intercalary. A similar situation was also observed for *R* and *PR* genes in legumes [52,54] besides genes associated with response to abiotic stresses, as osmoprotectants and heat shock factors [55,56].

Over time plants acquired new weapons to promote resistance over time [57,58] while pathogens also continued to evolve, bringing pressure on the structure and diversity of some associated gene families, increasing the size of some protein families [59], including PR-genes. Such an increase represents a major significance for the functional diversity via sub- or neo-functionalization of paralogs [60]. In this scenario, the natural selection of genes with new functions under environmental pressure probably played a significant role also in the evolution of TLPs [42], as may also be suggested for cowpea.

The 34 cowpea TLP coding genes had their predicted promoters (1 kb) analyzed in regard to the presence and identity of CCREs. Considering CCREs, a MEME motif is a sequence pattern that repeatedly occurs in one or more sequences in the input group. MEME can be used to discover novel patterns because discoveries are based only on the input sequences, not on any prior knowledge, such as databases of known motifs [35]. Discovered *bona fide* CCREs can be used to identify potential physiological or adaptive processes in which TLPs participate, associating transcription factors and their intrinsic biological processes. This action adds value to the molecular dynamics of cowpea TLPs, especially in association with the considered transcriptomic data.

Concerning the promoters analyzed, CCREs associated with bHLH, Dof-type, and MYB-related TFs attended the stipulated statistical parameters. Also considering literature data, these TFs present an intimate association with plant stress response and other

physiological processes. bHLH members can act as transcriptional activator or repressor and play essential roles in metabolic and developmental processes [61]. Some bHLH have also been reported to be modulated under cold, drought, and salt stress [62,63]. Dof-type transcription factors, in turn, participate widely in plant development and abiotic stress response [64,65]. In tobacco, the *Sar8.2b* gene can be activated by the Dof-type TF, which is related to systemic acquired resistance [66]. Finally, there are reports on MYB-related TFs acting as retrotransposon regulators and controlling defense-related genes, besides being induced by wounding and other elicitors, in tobacco plants [67]. Such *in silico* data on promoters associated with TLPs in RNA-Seq libraries under different stresses (biotic and abiotic) indicate a plurality of roles for TLPs, also reinforcing their biotechnological potential.

The RNA-Seq data showed that potential VuTLPs were induced in the first hours in response to the CABMV or CPSMV / injury. There are previous reports in the literature about the interaction between viral proteins and TLPs. Kim et al. [18] observed that a *Nicotiana tabacum* (NtTLP1) TLP specifically interacted with CMV (*Cucumber mosaic virus*) - ‘movement-related protein’, ‘movement protein’ and ‘coat protein’ - during a yeast two-hybrid screen experiment. However, the determination of the possible role of TLP-virus interaction requires deepened studies.

When comparing the transcriptional orchestration in response to the two viral types, the results indicate a functional specialization of VuTLPs in cowpea submitted to CABMV and CPSMV inoculation / injury, since only specific representatives are induced, in detriment of others, in response to inoculation by a given virus / injury.

The results for radicular dehydration assay indicate that some TLPs are recruited in the first minutes after stress imposition, even though a higher number of transcripts were detected at 150 min. The availability of information on the regulation of TLP proteins in plants under drought or water deficit stress has been poorly addressed. This can be verified by a search at PubMed literature data repository using the keywords "thaumatin-like protein and drought stress" that recovered only eight items (December / 2018) from which only four addressed the issue. Additionally, previous studies did not address the global transcriptional orchestration of these proteins and referred to individual case studies. An example is given by Jung et al. [9] that reported a new TLP isoform isolated from carrot, regarding a drought-specific, ABA-independent, non-organ-specific, and non-developmental-stage-specific protein.

The comparison between the orchestration in response to both viruses (in leaves) and radicular dehydration (roots) revealed potential VuTLPs expressed in both assays, indicating a crosstalk role for TLPs under different stress types and tissues. Besides, there is no overall conservation in VuTLPs orchestration towards different viral isolates and radicular dehydration, indicating specific TLP associated with the response of each virus type and radicular dehydration.

Signalization response in plants submitted to abiotic and biotic stressors can induce separate and overlapping sets of genes, leading to the expression of distinct as well as common components [68,69]. These separate pathways show nodal points where they converge and crosstalk to optimize the various defense responses [70], resulting in shared stress mitigation strategy by combined morpho-physiological processes and molecular responses [71]. The identification of crosstalk between biotic and abiotic signaling pathways has been crucial for envisaging and strengthening our understanding of the regulation of plants response against combined stresses. Genes such as those coding for TLPs that impart abiotic as well as biotic stress tolerance are highlighted in some studies in order to understand the mechanism underlying tolerance to dual stresses.

Singh et al. [17] analyzed an *Arachis diogoi* (AdTLP) TLP induced under fungal infection (*Phaeoisariopsis personata*). The cDNA encoding AdTLP was cloned using RACE-PCR and used to transform tobacco plants. Overexpression of AdTLP resulted in increased resistance to pathogenic fungi and tolerance to abiotic stresses (high salinity and oxidative stress). In addition, transgenic plants also exhibited a higher level of transcription of genes *PR1a*, *PI-I* and *PI-II* compared to *WT*. Such genes are associated with plant pathogen defense mechanisms. Chowdhury et al. [72] showed that transgenic lines of sesame overexpressing an osmotin-like protein (SindOLP) presented tolerance against abiotic stresses (drought and high salinity) and resistance against the fungus *Macrophomina phaseolina*. Overexpression of SindOLP resulted in the induction of three genes [*superoxide dismutase* (*SiSOD*), *cysteine protease inhibitor* (*SiCysPI*), and *glutathione-S-transferase* (*SiGST*)] which encode enzymes for the elimination of reactive oxygen species (ROS), indicating that SindOLP participates in the ROS regulation, which is common to both stresses addressed.

Considering the results of qPCR, it is evident that TLPs are recruited in the first minutes after CABMV or CPSMV treatment (inoculation + injury). Even though both

treatments had the injury in common, different TLPs were recruited after each virus inoculation, indicating some specificity that deserves additional evaluations.

Some recently published papers have shown the induction of TLPs after virus inoculation. Madroñero et al. [73] analyzed changes in the papaya transcriptome in response to the inoculation with PMeV complex (Papaya Meleira Virus, PMeV, and Papaya Meleira Virus 2, PMeV2). To evaluate the effects of SA signaling on PMeV complex load, *Carica papaya* seedlings were exposed to this hormone and inoculated with both viruses. The SA-treated plants showed increased *PR1*, *PR5* and *CHIA* levels confirming SA signaling activation. The same plants presented reduced PMeV complex load, suggesting that SA signaling plays a role in *C. papaya* tolerance to PSD before flowering.

Kappagantu et al. [74] studied the effect of the host response of hop plants (*Humulus lupulus*) to Hop stunt viroid (HSVd) on the development of hop powdery mildew (*Podospheara macularis*; HPM). Transcriptome analysis followed by qPCR analysis showed that transcript levels of PR-genes such as PR-1, chitinase, and TLPs were induced in leaves infected with HPM alone. The response of these genes to HPM was significantly down-regulated in leaves with HSVd-HPM mixed infection. These results confirm that HSVd alters host metabolism, physiology, and plant defense responses.

The results of qPCR show that – despite being traditionally responsive to biotic stresses – TLPs have been reported as recruited under abiotic stresses [9, 10, 11]. In the present work, a higher number of VuTLPs were induced at 150 min after root dehydration, even though a transcript was already recruited at 25 min after stress imposition, as observed in RNA-Seq data and validated by qPCR. Jung et al. [9] reported a new TLP isolated from carrot and expressed under drought conditions. In turn, Misra et al. [75] identified and characterized a new basil (*Ocimum basilicum*) TLP (ObTLP1) from ESTs recovered after MeJA treatment. The ectopic expression of ObTLP1 in *A. thaliana* provided fungal resistance (*Scleretonia sclerotiorum* and *B. cinerea*) and tolerance to abiotic stresses (drought and high salinity).

Despite the evidence of the present work and previous studies, the mechanism of action of such proteins against abiotic stress is still unknown. The work of Chowdhury et al. [72] with sesame, indicated a role in the induction of three genes [*superoxide dismutase* (*SiSOD*), *cysteine protease inhibitor* (*SiCysPI*), *glutathione-S-transferase* (*SiGST*)] which encode enzymes for the elimination of reactive oxygen species (ROS). These results

suggest that SindOLP participates in the regulation of the ROS pathway, which is common to both types of stresses.

Highlights

- Cowpea presented 34 members of thaumatin-like proteins.
- Most VuTLPs presented the canonical configuration of TLPs found in other organisms, with some members presenting specificities in the REDDD motif and concerning the number of cysteines.
- VuTLPs sequence diversity reflect functional groups regarding neofunctionalized TLPs.
- Promoters associated with VuTLPs indicate a plurality of roles for TLPs.
- VuTLPs present functional specialization in relation to CABMV and CPSMV inoculation and radicular dehydration.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

As figuras suplementares e as tabelas suplementares S1 e S6 estão apresentadas a seguir. Porém, as demais tabelas suplementares S2-S5 e os materiais suplementares SM1-2 deste artigo estão disponíveis no link "https://drive.google.com/open?id=1cohI2Lwh7_RvDyP4YdZUE6vywdYJSavf" para consulta na pasta intitulada "**Materiais_Suplementares_Artigo**", devido à quantidade significativa de material que foi gerado.

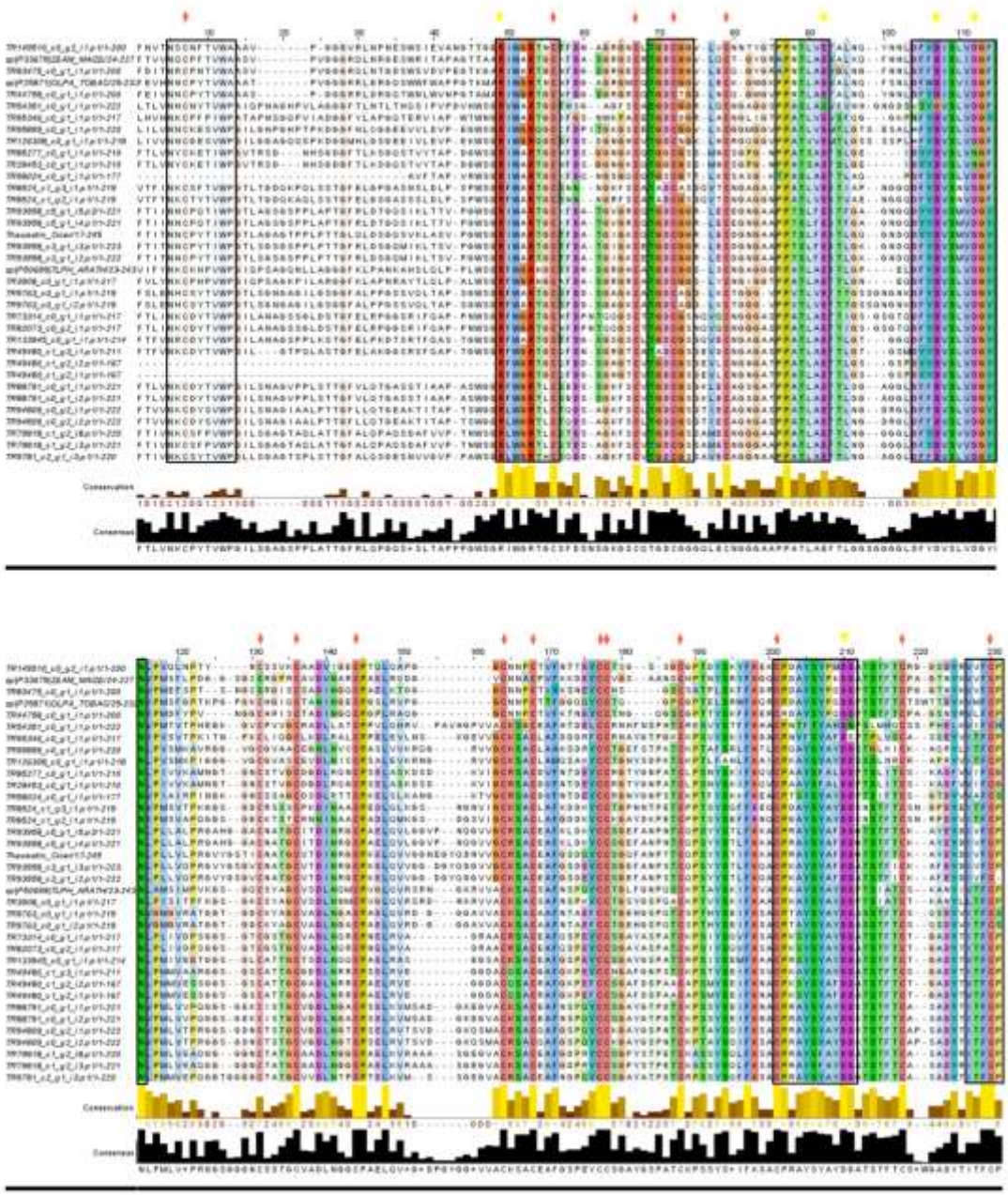
Suppl. Tab. S1. Cowpea libraries generated by the Cowpea Genomics Consortium (CpGC) including treatments, stress types, number of sequenced transcripts and sequencing methods used.

Stress Classification	Stressor Agent	Nº of Sequences / Methodology	Tissue	# of Libraries	(Libraries) Treatments	Tolerant / Resistant Genotype (type of libraries)	Sensitive / Susceptible Genotype (type of libraries)	Sequence Annotation and Libraries Synthesis Reference
Biotic	CABMV	~9,5 million / SuperSAGE‡	Leaves	4	(a) Negative (not stressed) control (time 0); (b) Injured control; (c) Bulked library of leaves injured and exposed to CABMV at 30, 60 and 90 min after inoculation (Early Response to CABMV); (d) Library of leaves collected at 16 h after virus inoculation (Late Response to CABMV).	IT85F-2687	–	Kido et al. [18]
		~149,2 thousand / RNA-Seq		4	(a) Negative control (60 min); (b) Library of leaves collected at 60 min after injury and inoculation with CABMV; (c) Negative control (16 h); (d) Library of leaves collected at 16 h after injury and inoculation with CABMV.			
	CPSMV	~7,2 million / SuperSAGE‡	Leaves	3	(a) Negative control (time 0); (b) Bulked library of leaves injured and exposed to CPSMV at 30, 60 and 90 min after inoculation (Early Response to CPSMV); (c) Library of leaves collected at 16 h after virus inoculation (Late Response to CPSMV).	BR14 Mulato (a; b; c)	–	
		~149,2 thousand / RNA-Seq		4	(a) Negative control (60 min); (b) Library of leaves collected at 60 min after injury and inoculation with CPSMV; (c) Negative control (16 h); (d) Library of leaves collected at 16 h after injury and inoculation with CPSMV.			
Abiotic	High Salt	~4,7 million / SuperSAGE**	Leaves	4	(a) Negative control (time 0); (b) Bulked library of roots collected at 30, 60 and 90 min after salt stress (NaCl 100 mM) exposition.	Pitiúba (a; b)	BR14 Mulato (a; b)	Kido et al. [18]
	Radicular Dehydration	2,5 million (SuperSAGE‡)	Roots	4* / 6†	SuperSAGE: (a) Negative control (time 00; (b) Bulked library of roots collected at 25, 50, 75, 100, 125, and 150 min after radicular dehydration;	Pingo de Ouro (a; b; c; d; e)	Santo Inácio (a; b; c; d; e)	Kido et al. [18] and present work
		~455 million (RNA-Seq***)			RNA-Seq: (c) Negative control (time 0); (d) Library of roots collected at 75 min after radicular dehydration; (e) library of roots collected at 150 min after radicular dehydration.			

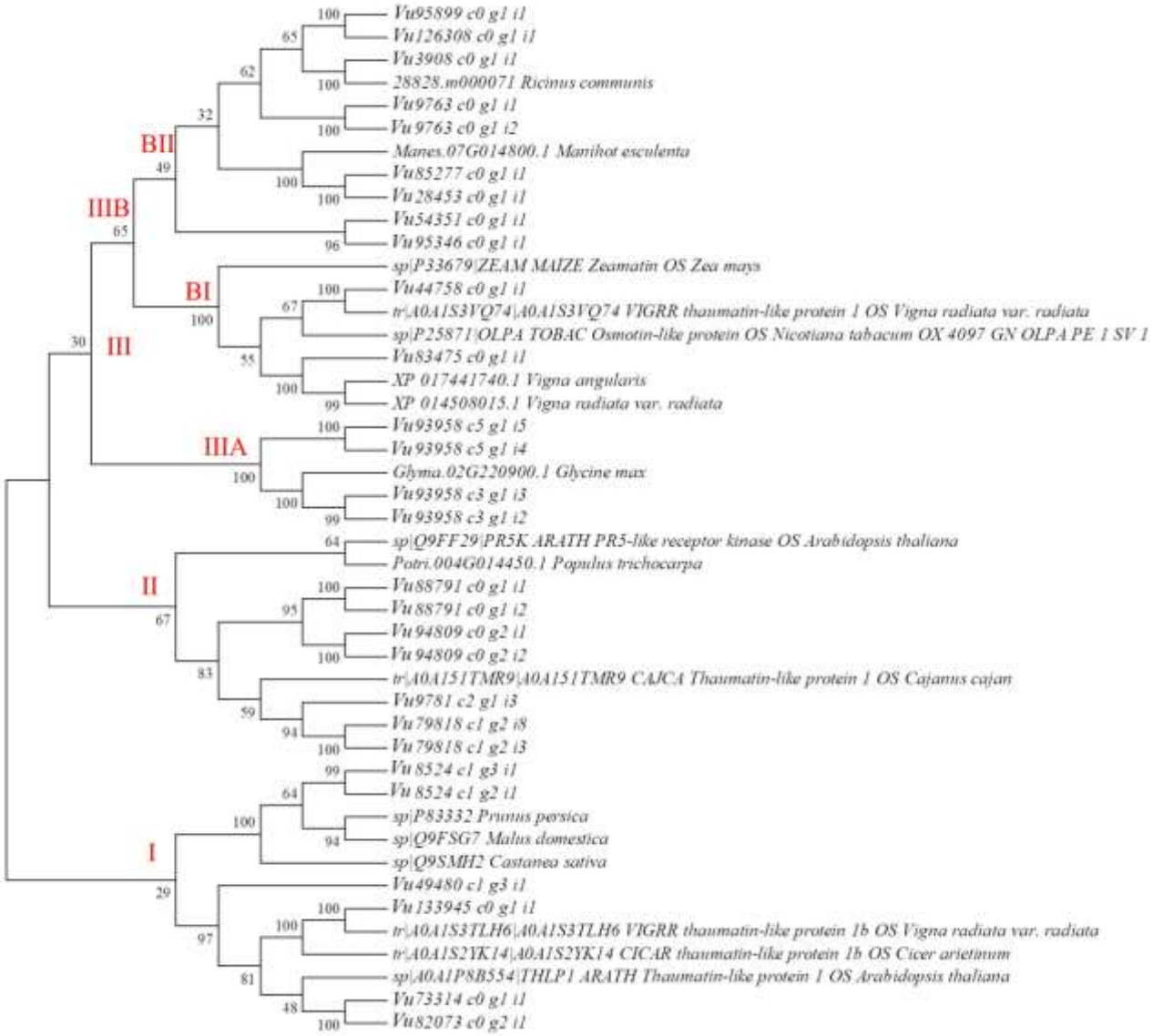
Legend for abbreviations: CABMV: *Cowpea Aphid-borne Mosaic Virus*; CPSMV: *Cowpea Severe Mosaic Virus*. SuperSAGE: Super Serial Analysis of Gene Expression; HT-SuperSAGE: High Throughput Super Serial Analysis of Gene Expression. Sequencing methodologies: ‡Solexa-Illumina; **454-Roche; ***Illumina Hi-Seq Paired End. *Refer to SuperSAGE; † Refer to RNA-Seq.

Suppl. Tab. S6. Data explored by REST software to analyze the relative expression of target transcripts in cowpea under CABMV and CPSMV inoculation and under radicular dehydration. REF (reference gene); TRG (target gene); Std. Error (standard error); 95% C.I. (95% Confidence Intervals); P(H1) (Hypothesis Test); UR (up-regulated at $p < 0.05$); DR (down-regulated at $p < 0.05$); ns (not significant at the level of $p \leq 0.05$).

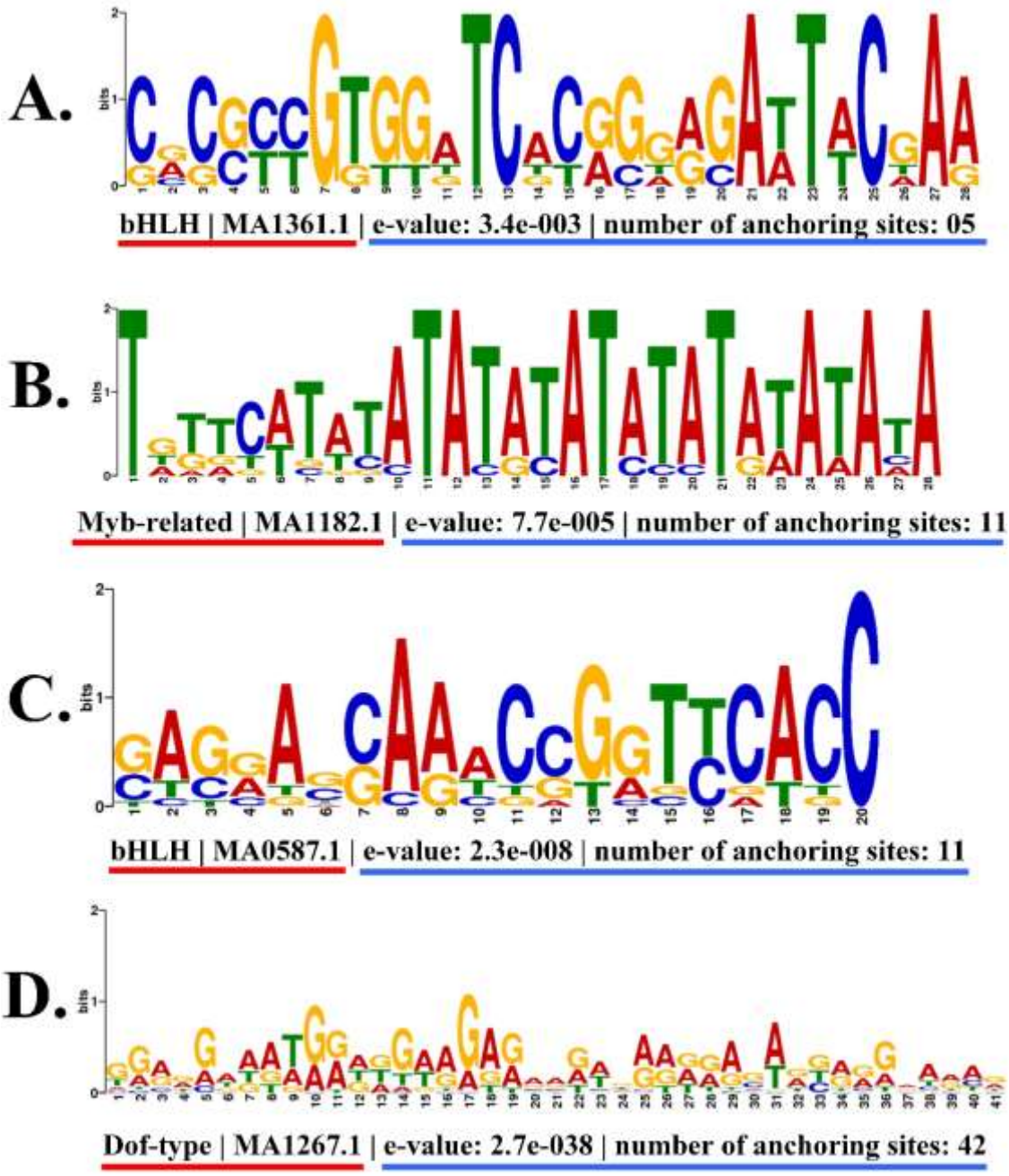
CABMV assay - IT85F-2687 60 minutes							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
B-tubulin	REF	1	1,351				
F-box	REF	1	0,896				
UBQ10	REF	0,97	0,826				
P1	TRG	0,98	0,826	0,100 - 12,640	0,050 - 24,521	0,79	ns
P15	TRG	0,99	10,526	1,220 - 39,503	0,739 - 62,107	0,002	UP
CABMV assay - IT85F-2687 60 minutes							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
B-tubulin	REF	1	1,098				
F-box	REF	1	0,822				
UBQ10	REF	0,97	1,108				
P5	TRG	0,99	3,452	1,257 - 11,147	0,787 - 17,533	0,002	UP
P14	TRG	0,98	1,493	0,793 - 3,185	0,376 - 4,463	0,144	ns
CABMV assay - IT85F-2687 16 hours							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
B-tubulin	REF	1	1,03				
F-box	REF	1	0,777				
UBQ10	REF	0,97	1,25				
P1	TRG	0,98	4,077	1,094 - 12,952	0,538 - 29,875	0,004	UP
P15	TRG	0,99	0,616	0,381 - 0,991	0,249 - 1,349	0,007	DOWN
CABMV assay - IT85F-2687 16 hours							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
B-tubulin	REF	1	0,973				
F-box	REF	1	0,755				
UBQ10	REF	0,97	1,36				
P5	TRG	0,99	1,184	0,285 - 1,951	0,190 - 793,921	0,975	ns
P14	TRG	0,98	0,956	0,242 - 5,147	0,149 - 11,461	0,928	ns
CPSMV assay - BR-14 Mulato 60 minutes							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
B-tubulin	REF	1	1,314				
F-box	REF	1	1,113				
UBQ10	REF	0,97	0,684				
P18	TRG	1	4,184	1,530 - 10,287	0,716 - 28,504	0	UP
P19	TRG	1	3,127	0,753 - 13,038	0,256 - 111,980	0,033	UP
P23	TRG	0,97	5,254	2,371 - 8,151	1,149 - 48,685	0	UP
CPSMV assay - BR-14 Mulato 16 hours							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
B-tubulin	REF	1	0,767				
F-box	REF	1	1,271				
UBQ10	REF	0,97	1,026				
P18	TRG	1	0,862	0,268 - 2,321	0,167 - 8,722	0,727	ns
P19	TRG	1	12,034	6,304 - 24,723	4,458 - 49,095	0	UP
P23	TRG	0,97	9,275	3,910 - 24,708	2,064 - 45,663	0	UP
Radicular dehydration assay - Pingo de Ouro 25 minutes							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
Actina	REF	1	0,971				
UE21D	REF	1	1,03				
P4	TRG	0,95	1,628	1,352 - 2,049	1,162 - 2,391	0	UP
P6	TRG	0,91	1,446	0,823 - 2,478	0,577 - 4,032	0,08	ns
Radicular dehydration assay - Pingo de Ouro 25 minutes							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
Actina	REF	1	0,911				
UE21D	REF	1	1,097				
P8	TRG	0,91	1,106	0,760 - 1,590	0,665 - 3,557	0,598	ns
Radicular dehydration assay - Pingo de Ouro 150 minutes							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
Actina	REF	1	0,92				
UE21D	REF	1	1,087				
P4	TRG	0,95	3,066	2,024 - 4,808	1,500 - 6,473	0	UP
P6	TRG	0,91	5,773	3,329 - 8,874	2,517 - 14,635	0	UP
Radicular dehydration assay - Pingo de Ouro 150 minutes							
Gene	**Type**	**Reaction Efficiency**	**Expression**	**Std. Error**	**95% C.I.**	**P(H1)**	**Result**
Actina	REF	1	0,825				
UE21D	REF	1	1,212				
P8	TRG	0,91	2,701	1,577 - 4,763	1,133 - 7,800	0	UP



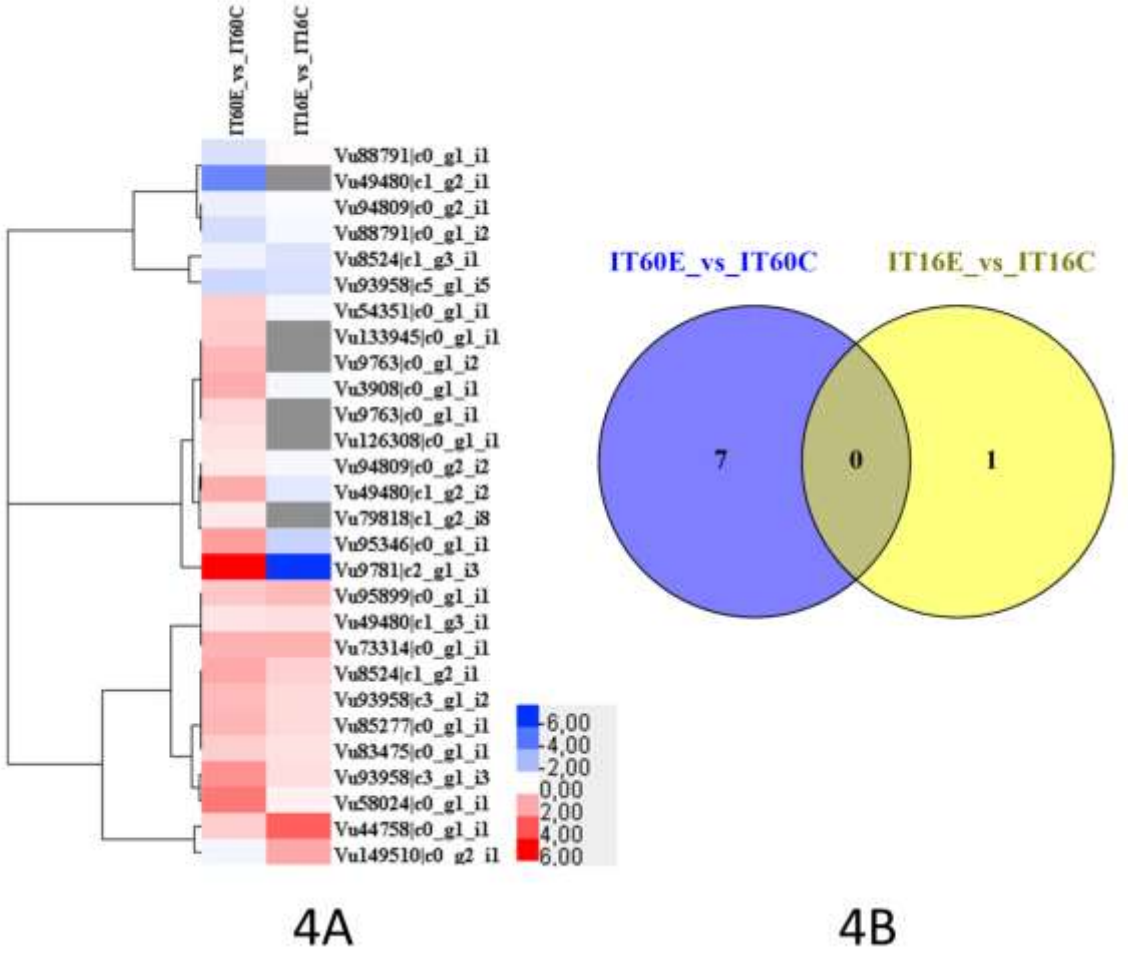
Suppl. Fig. S1. Alignment of amino-acid sequences of *Vigna unguiculata* thaumatin-like proteins (TLPs) together with TLPs from other species. Sequence comparison was carried out with Clustal Omega software with standard parameters. Colored bars in the sequence show conserved residues and conserved motifs. Black boxes indicate conserved domain regions; Pink diamonds indicate the cysteine residues that form the eight disulfide bonds; Yellow triangles indicate the position of REDDD motif and yellow bars in the base show the level of conservation for each position.



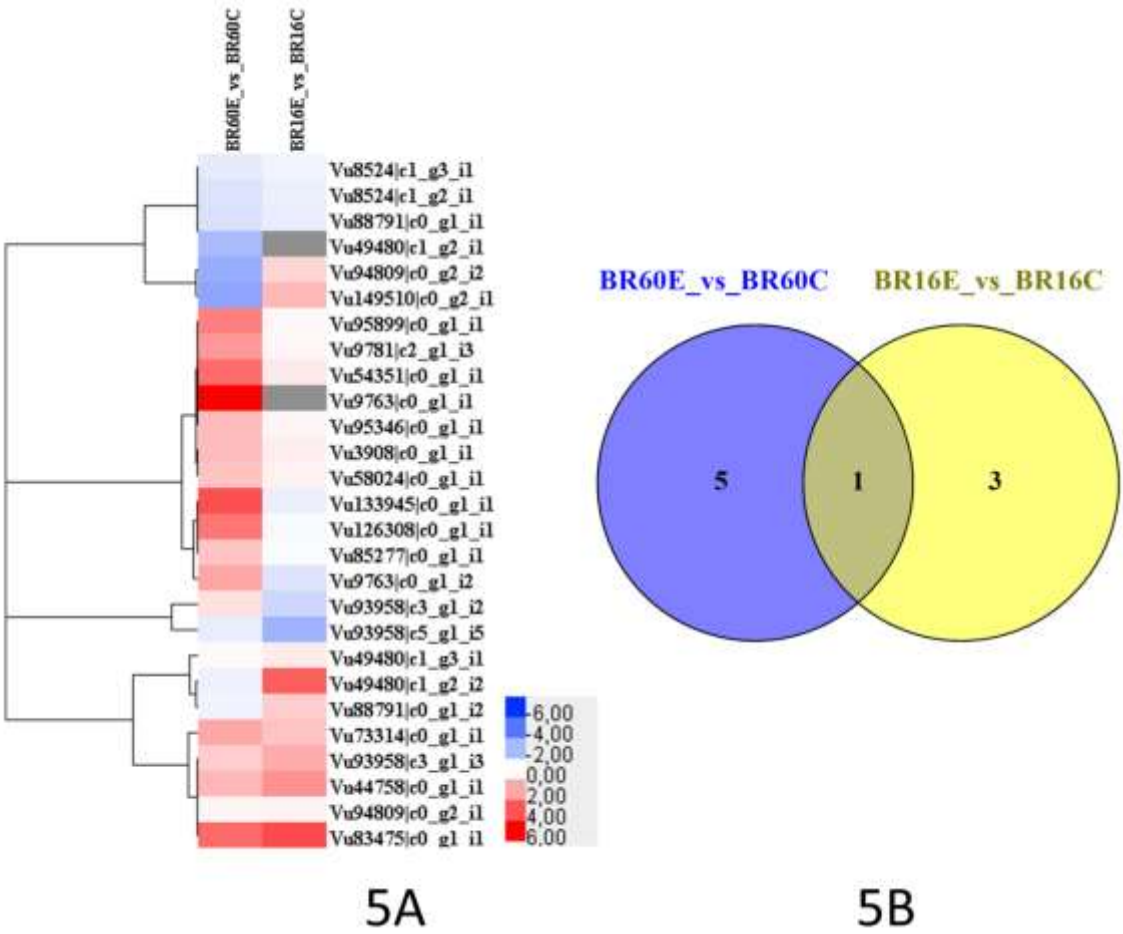
Suppl. Fig. S2. Neighbor-joining tree (distance matrix) generated from VuTLPs and thaumatin-like protein (TLPs) sequences from other plants. Values on the nodes regard bootstrap values (2000 replicates).



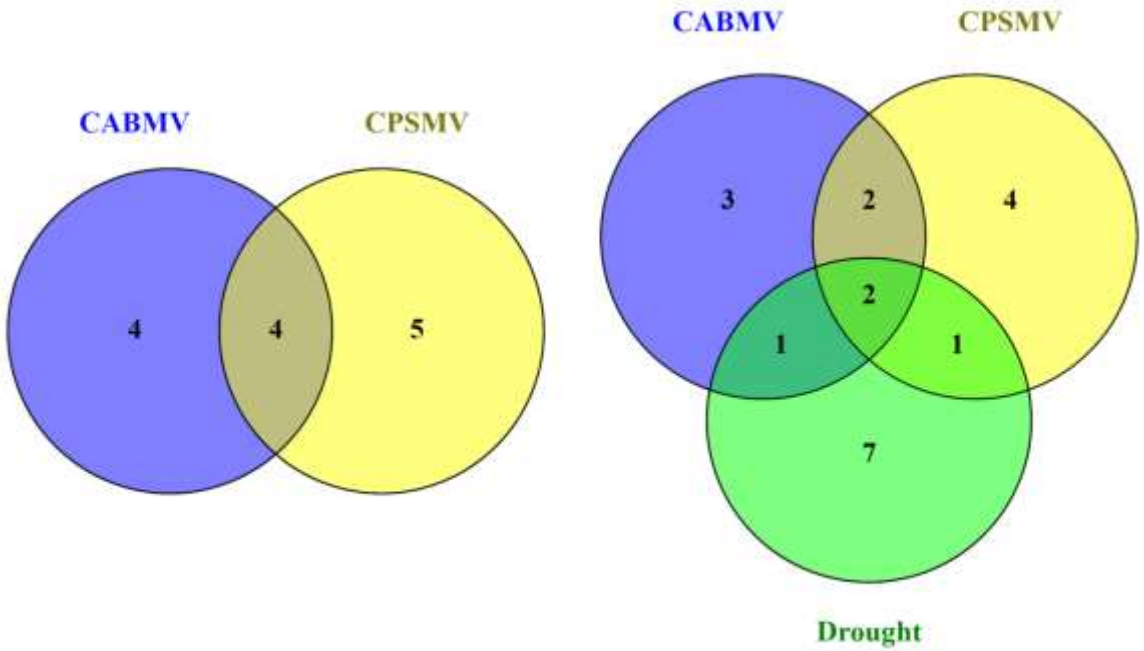
Suppl. Fig. S3. Candidate motifs of cis-regulatory candidates statistically validated, presenting their consensus sequence, e-value and number of anchoring sites (MEME software output underlined in blue) together with the annotation of the associated transcription factor and its Matrix JASPAR ID (in red) according to the Tomtom software output.



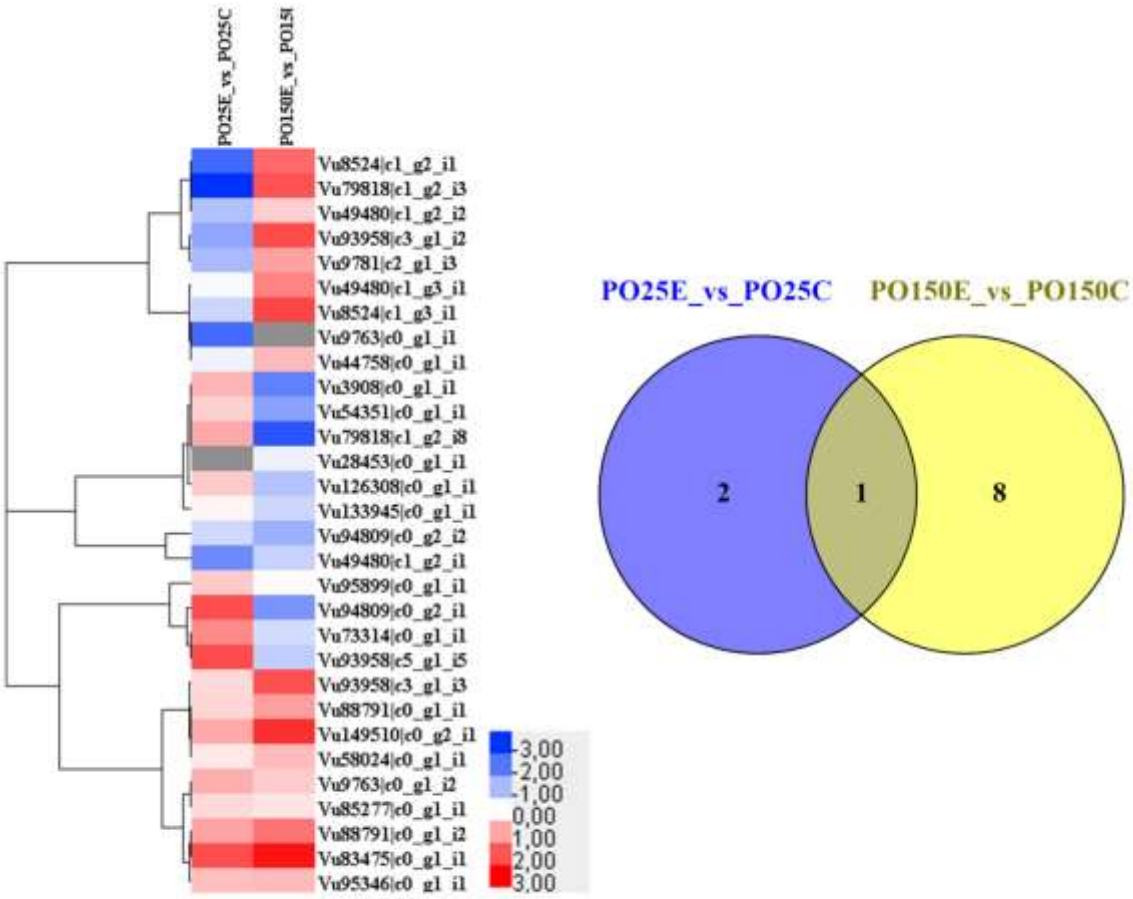
Suppl. Fig. S4. (A) Heat map illustrating the transcriptional regulation of 28 VuTLP-encoding transcripts after inoculation with *Cowpea Aphid-borne Mosaic Virus* (CABMV). **(B)** Venn diagram of VuTLP-candidates in each treatment / comparison. Libraries: IT60E: IT85F-2687, 60 minutes, stressed library; IT60C: IT85F-2687, 60 minutes, control library; IT16E: IT85F-2687, 16 hours, stressed library; IT16C: IT85F-2687, 16 hours, control library.



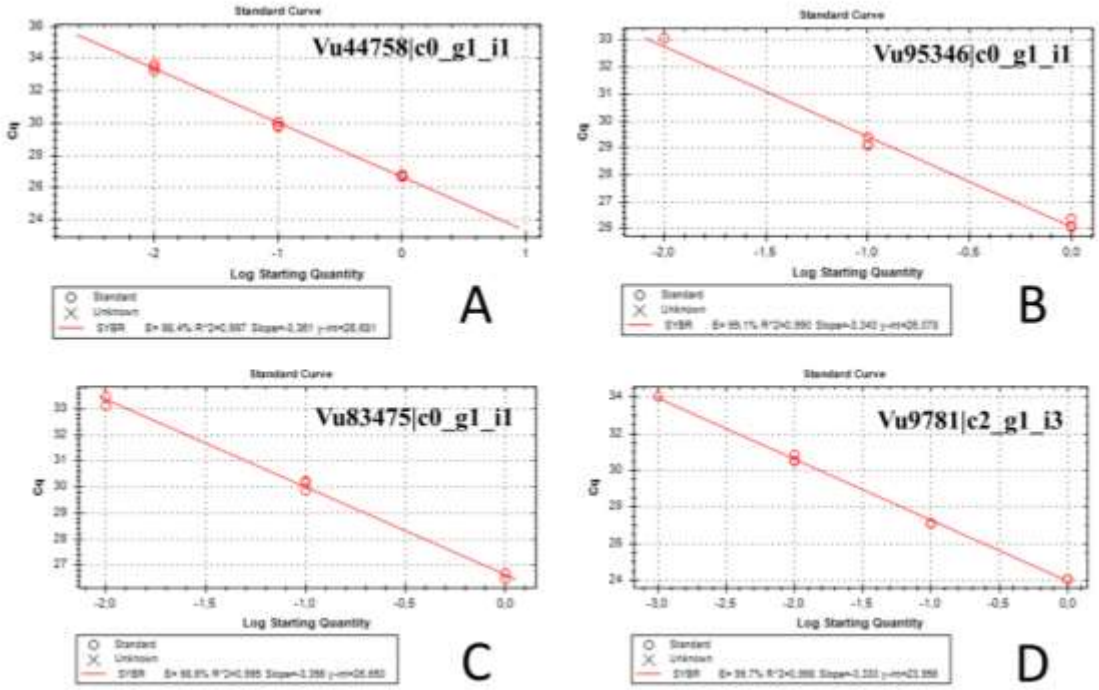
Suppl. Fig. S5. (A) Heat-map illustrating the transcriptional regulation of 27 VuTLP-encoding transcripts after inoculation with *Cowpea Severe Mosaic Virus* (CPSMV). **(B)** Venn diagram of induced VuTLP-candidates in each treatment / comparison. Libraries: BR60E: BR-14 Mulato, 60 minutes, stressed library; BR60C: BR-14 Mulato, 60 minutes, control library; BR16E: BR-14 Mulato, 16 hours, stressed library; BR16C: BR-14 Mulato, 16 hours, control library.



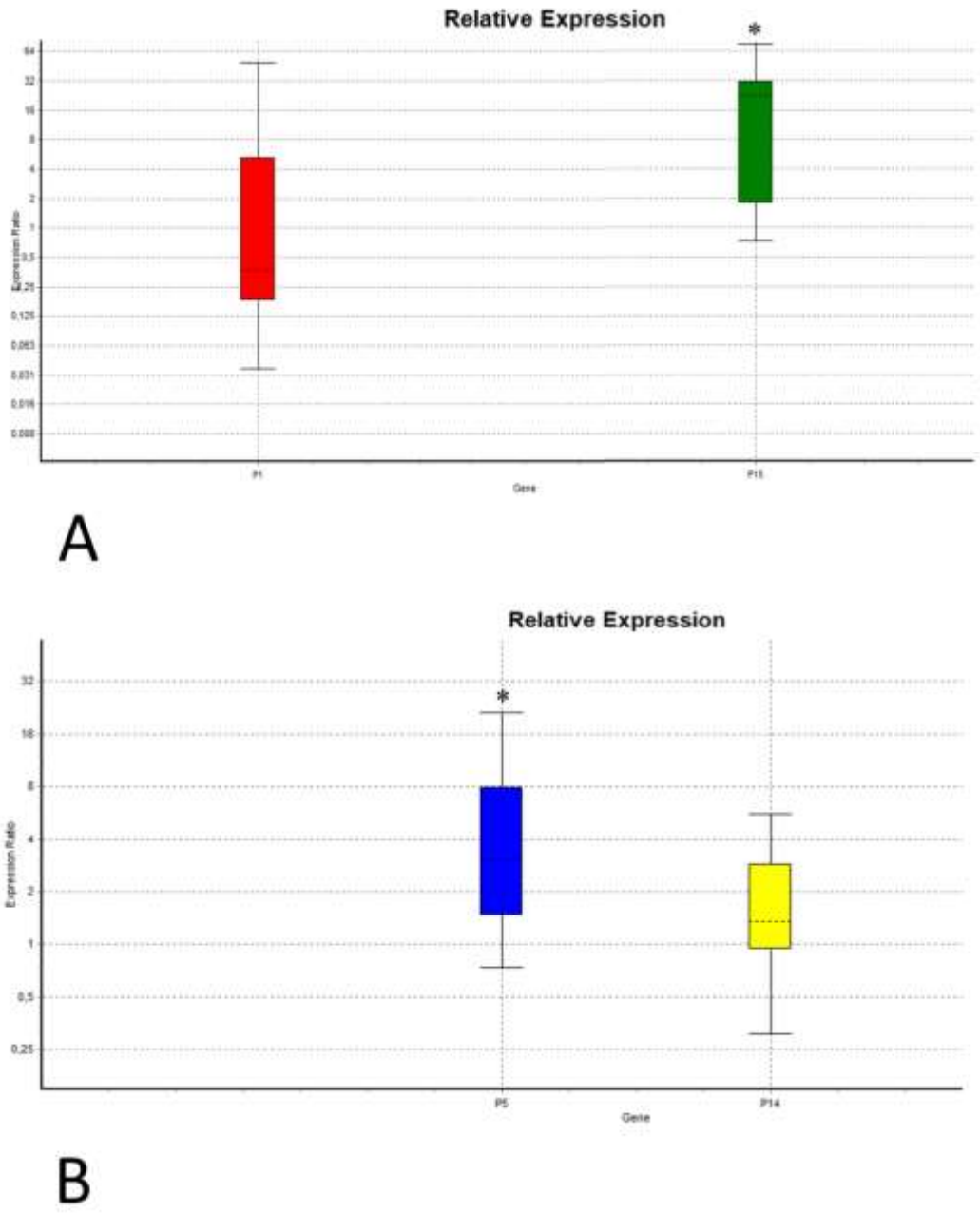
Suppl. Fig. S6. (A) Venn diagram with all VuTLP-candidates induced in each biotic assay (CABMV and CPSMV inoculation). **(B)** Venn diagram with all VuTLP-candidates induced in each assay (CABMV or CPSMV inoculation and radicular dehydration).



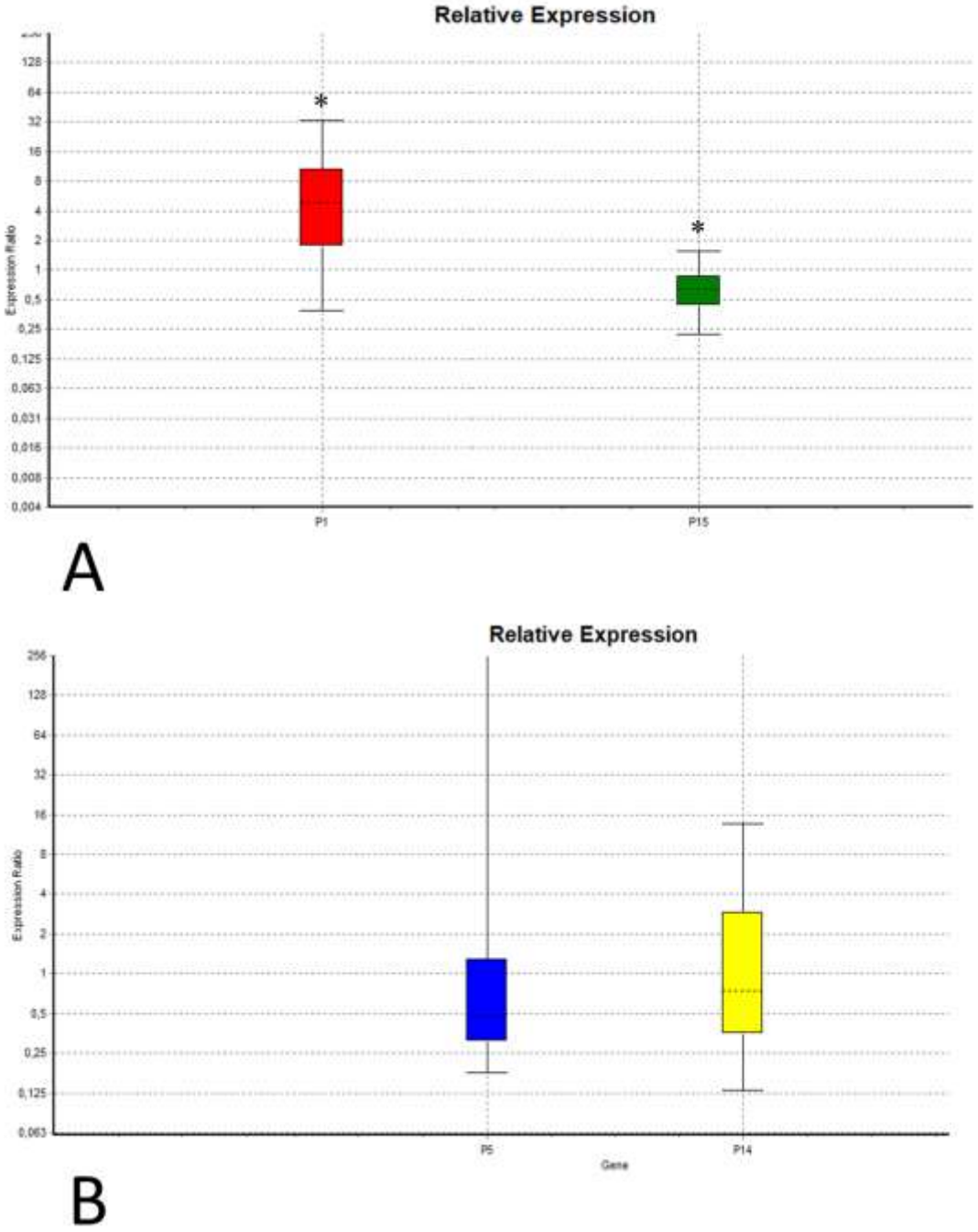
Suppl. Fig. S7. (A) Heat-map illustrating the transcriptional regulation of 30 VuTLP-encoding transcripts after radicular dehydration stress. **(B)** Venn diagram of induced VuTLP-candidates in each treatment / comparison. Libraries: PO25E: Pingo de Ouro, 25 minutes, stressed library; PO25C: Pingo de Ouro, 25 minutes, control library; PO150E: Pingo de Ouro, 150 minutes, stressed library; PO150C: Pingo de Ouro, 150 minutes, control library.



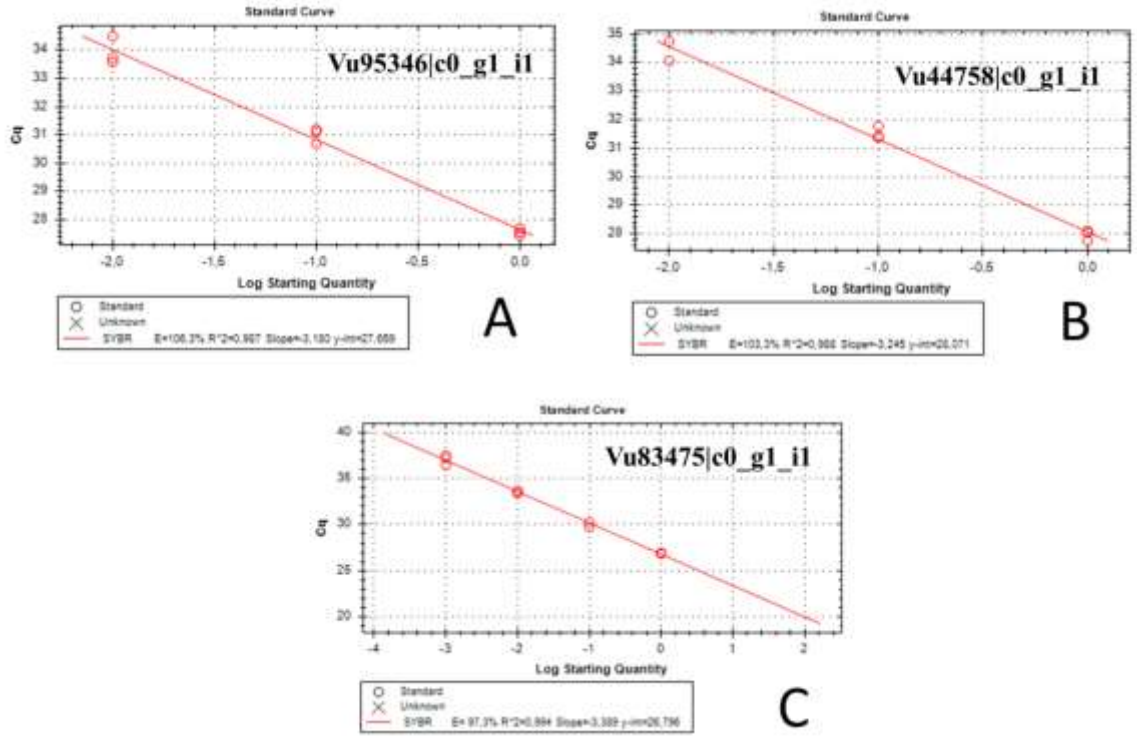
Suppl. Fig. S8. Dilution curve with the points used for the efficiency analysis of TLP-target transcripts in the CABMV experiment: **(A)** Vu44758|c0_g1_i1; **(B)** Vu95346|c0_g1_i1; **(C)** Vu83475|c0_g1_i1; **(D)** Vu9781|c2_g1_i3.



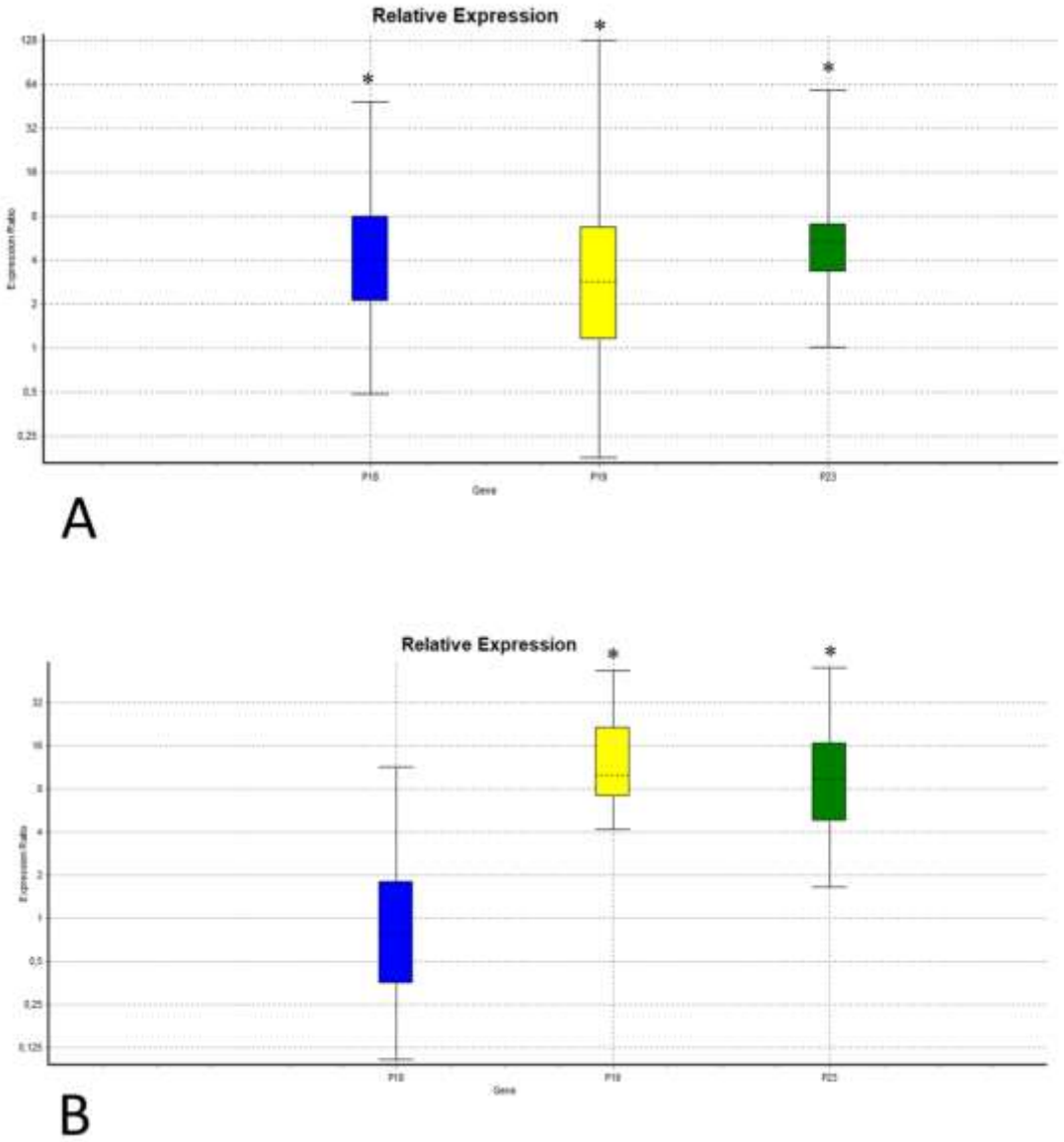
Suppl. Fig. S9. Relative expression of TLP-target transcripts at 60 minutes after CABMV inoculation, as compared with not stressed control. **(A)** Vu44758|c0_g1_i1 (red) and Vu9781|c2_g1_i3 (green); **(B)** Vu95346|c0_g1_i1 (blue) and Vu83475|c0_g1_i1 (yellow).



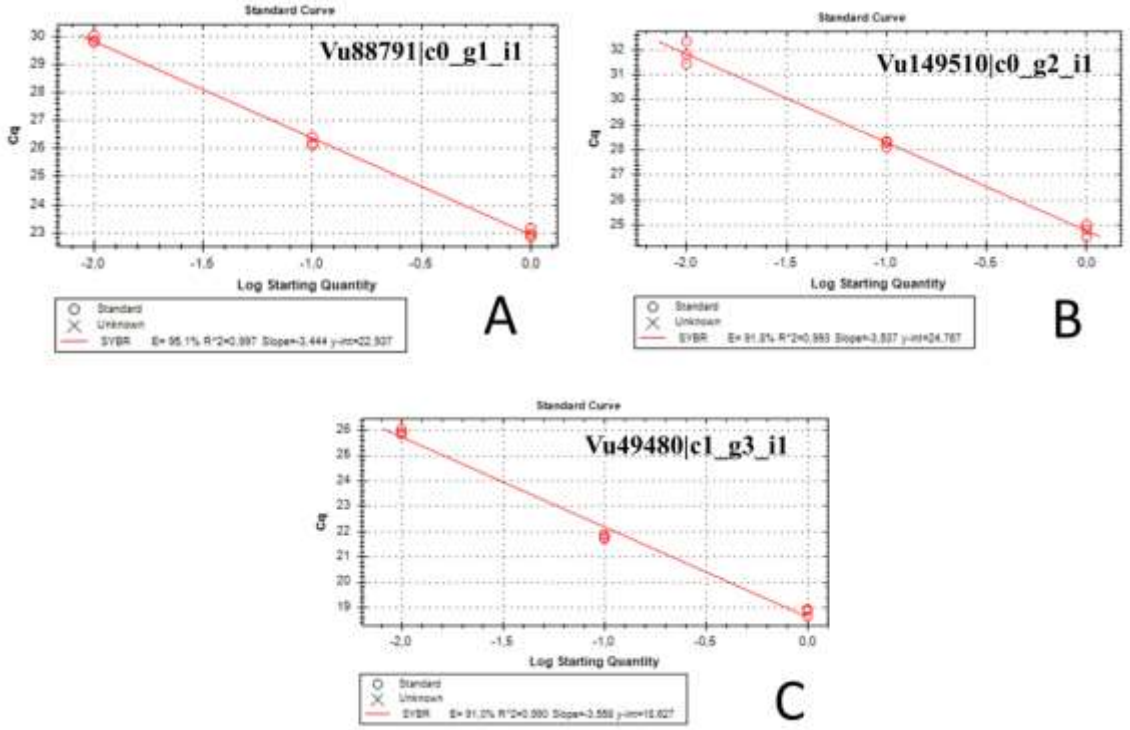
Suppl. Fig. S10. Relative expression of TLP-target transcripts at 16 h after CABMV inoculation, as compared with not stressed control. **(A)** Vu44758|c0_g1_i1 (red) and Vu9781|c2_g1_i3 (green); **(B)** Vu95346|c0_g1_i1 (blue) and Vu83475|c0_g1_i1 (yellow).



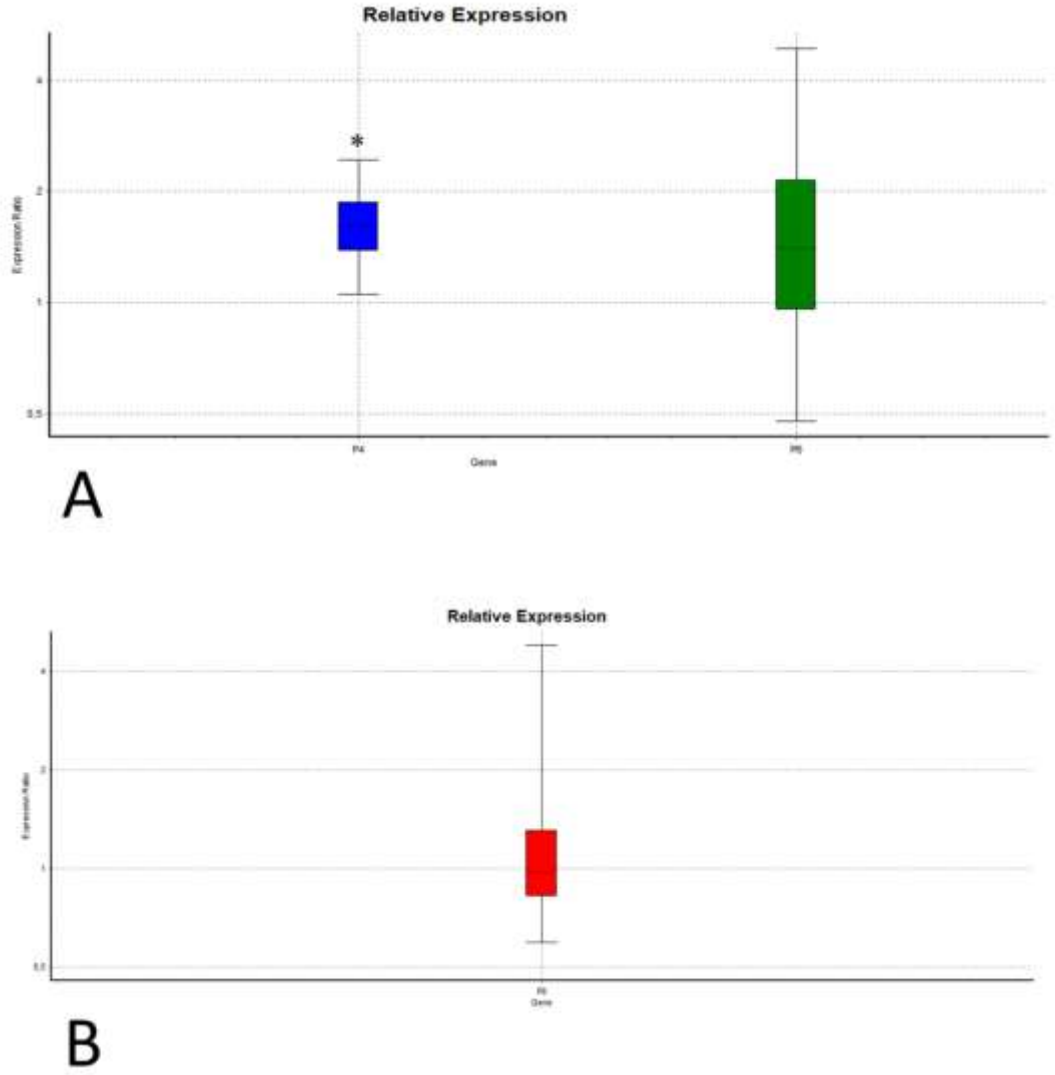
Suppl. Fig. S11. Dilution curve with the points used for the efficiency analysis of TLP-target transcripts in the CPSMV experiment: **(A)** Vu95346|c0_g1_i1; **(B)** Vu44758|c0_g1_i1; **(C)** Vu83475|c0_g1_i1.



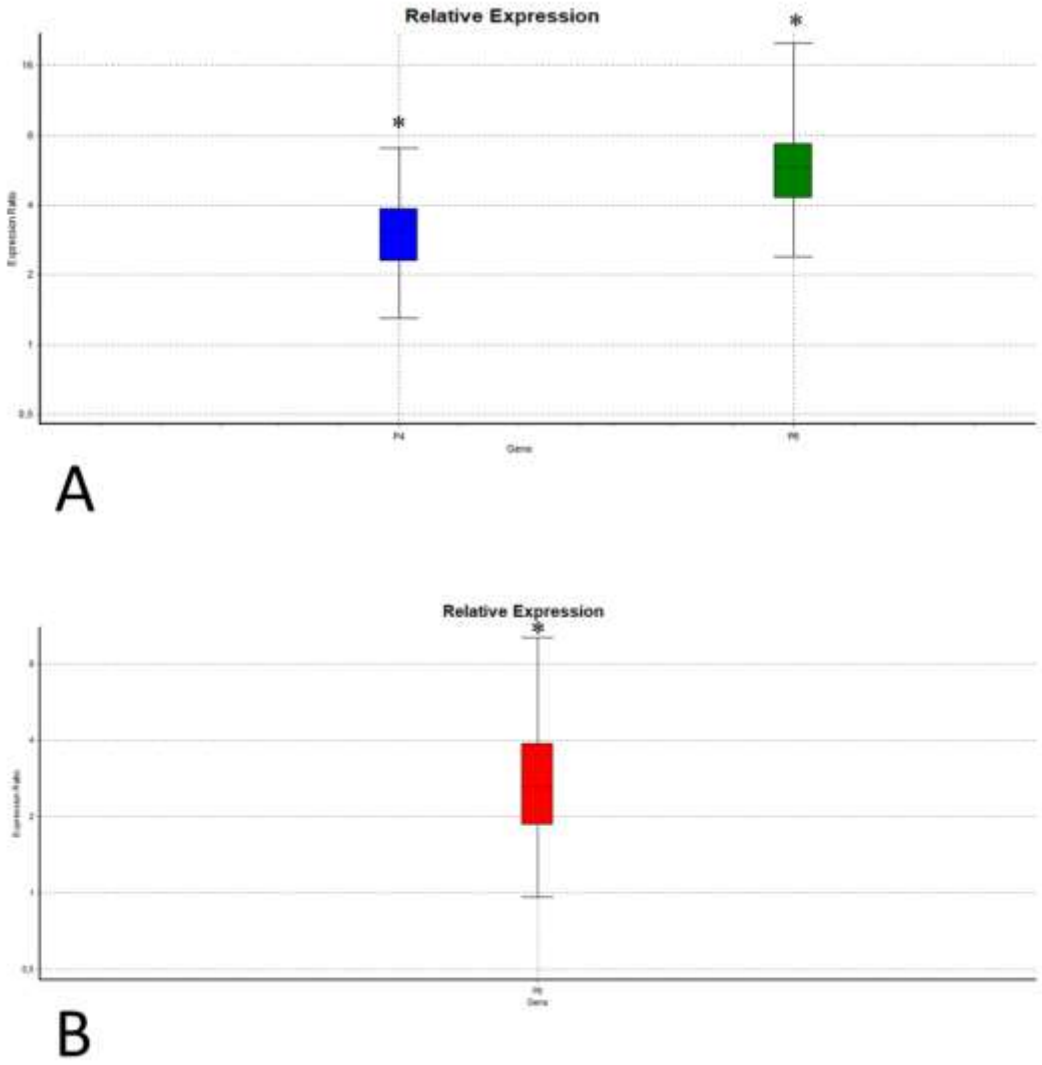
Suppl. Fig. S12. (A) Relative expression of TLP-target transcripts at 60 minutes after CPSMV inoculation, as compared with not stressed control. Vu95346|c0_g1_i1 (blue); Vu44758|c0_g1_i1 (yellow); Vu83475|c0_g1_i1 (green). **(B)** 16 h after CPSMV inoculation (compared with negative control): Vu95346|c0_g1_i1 (blue); Vu44758|c0_g1_i1 (yellow); Vu83475|c0_g1_i1 (green).



Suppl. Fig. S13. Dilution curve with the points used for the efficiency analysis of TLP-target transcripts in the root dehydration experiment: **(A)** Vu88791|c0_g1_i1; **(B)** Vu149510|c0_g2_i1; **(C)** Vu49480|c1_g3_i1.



Suppl. Fig. S14. Relative expression of TLP-target transcripts at 25 minutes after root dehydration, as compared with not stressed control. **(A)** Vu88791|c0_g1_i1 (blue) and Vu149510|c0_g2_i1 (green); **(B)** Vu49480|c1_g3_i1 (red).



Suppl. Fig. S15. Relative expression of TLP-target transcripts at 150 minutes after root dehydration imposition, as compared with not stressed control. **(A)** Vu88791|c0_g1_i1 (blue) and Vu149510|c0_g2_i1 (green); **(B)** Vu49480|c1_g3_i1 (red).

4 DISCUSSÃO GERAL

As TLPs (*Thaumatin-Like Proteins*) de vegetais são abundantes e altamente diversificadas. Porém, um número maior de isoformas em plantas superiores foi identificado, comparativamente a grupos basais (como briófitas e algas), possivelmente devido a eventos de duplicação, incluindo duplicações segmentais e WGD (*Whole Genome Duplication* - duplicação do genoma inteiro). Entretanto, as TLPs de plantas têm sido pouco exploradas, considerando a crescente disponibilidade de genomas e transcriptomas disponíveis em bancos de dados públicos. Além disso, a identificação de TLPs no genoma ou transcriptoma de plantas nativas (não cultivadas) ainda é limitada, com ênfase para espécies tropicais. Considerando a perda de diversidade genética (erosão genética) prevalente em espécies cultivadas, isoformas únicas são esperadas em táxons tropicais não cultivados.

Quanto ao seu potencial, estudos biotecnológicos de TLPs impactaram em diversas áreas e aplicações. No melhoramento genético, o gene codificante dessa proteína tem sido expresso com sucesso em plantas, especialmente para aumentar a doçura e qualidade dos frutos. Além disso, a maioria das plantas transformadas visou resistência contra fungos, mas em muitos casos, foi obtida uma tolerância a estresse abiótico (por exemplo, tolerância à seca, salinidade, ferimentos e estresse oxidativo), isoladamente ou em conjunto com a resistência a um determinado patógeno.

Embora o papel mais reportado para as TLPs seja a ação antifúngica, estudos tem apontado para um papel na resposta à invasão por outros tipos de patógenos (por exemplo, bactérias, vírus ou protozoários), abrindo novas possibilidades que demandam análises funcionais. Desta forma, está claro que os genes que codificam proteínas com o domínio taumatina representam candidatos valiosos para o melhoramento de plantas com seleção assistida por marcadores e também para a geração de plantas transformadas. Apesar do potencial sucesso no uso de TLPs - especialmente considerando alguns resultados com benefícios multifuncionais sob ambos os estresses bióticos e abióticos - até onde sabemos, nenhuma planta transgênica expressando TLPs está comercialmente disponível até o momento.

Assim, a descoberta de novas TLPs está abaixo de seu potencial, quando consideramos a quantidade de dados ômicos disponíveis. Dessa forma, um exemplo de procedimento de anotação de bioinformática é apresentado e aplicado ao transcriptoma de feijão caupi (*Vigna unguiculata*), incluindo bibliotecas de dois tecidos (raiz e folha) e dois tipos de estresse (biótico / abiótico). O pipeline utilizado no presente estudo permitiu identificar TLPs no genoma de feijão-caupi (VuTLPs), sendo o quantitativo observado semelhante aos de outras espécies diploides, como arroz e *Arabidopsis thaliana*.

A maioria das VuTLPs apresentou a configuração canônica encontrada em outras plantas superiores. Por sua vez, um grupo reduzido de TLPs não apresentou todos os aminoácidos constituintes do motivo REDDD, nem todas as 16 cisteínas conservadas, sugerindo possíveis diferenças na estrutura e na funcionalidade desses peptídeos, sem descartar uma possível especificidade estrutural desse grupo proteico em feijão-caupi.

A análise fenética das sequências de aminoácidos dividiu as VuTLPs escrutinadas em grupos funcionais, possivelmente devido a eventos de neofuncionalização ocorridos após duplicações em tandem de algumas regiões do genoma. Além disso, sugere-se que a estrutura primária, ou seja, a sequência linear de aminoácidos das VuTLPs deve ser considerada como critério de classificação, em detrimento de sua indução a um determinado tipo de estresse ou a espécie na qual foi inicialmente identificada. Desta forma, o uso de termos como “osmotina” e “zeamatina” é desaconselhável e ineficiente.

A análise de motivos de candidatos a elementos cis-regulatórios (CCREs), ancorados em regiões promotoras de genes codificadores de VuTLPs, permitiu a identificação de quatro CCREs *bona fide*, associados aos seguintes fatores de transcrição: bHLH (dois motivos), MYB e Dof. Os CCREs *bona fide* detectados podem ser usados para identificar potenciais processos fisiológicos ou adaptativos nos quais as TLPs participam, associando-as a fatores de transcrição e seus processos biológicos intrínsecos. Nesse caso, os CCREs *bona fide* associados aos TLPs apresentam reconhecido envolvimento na resposta vegetal a estresses (ex: frio, seca e estresse salino) e outros processos fisiológicos (ex: processos metabólicos e de desenvolvimento da planta), sugerindo uma possível participação de TLPs de feijão-caupi nesses mecanismos.

Uma vez que TLPs têm sua participação bem documentada na resposta aos estresses bióticos, um maior quantitativo de VuTLPs induzidas seria esperado na reposta a viroses. Os dados RNA-Seq apontam, entretanto, para um maior número de VuTLPs induzidas sob as condições abióticas aqui analisadas (desidratação radicular), comparativamente à sua contraparte biótica (inoculação com dois vírus CPSMV e CABMV). Apesar dessa maior indução de TLPs, destaca-se que o experimento de inoculação viral está associado a uma etapa crucial que envolve a injúria da folha (imprescindível para a inoculação de cada vírus). Apesar disso, comparando-se ambos os experimentos de inoculação viral (CPSMV e CABMV) realizados com a mesma metodologia, fica clara a existência de TLPs exclusivas para cada tratamento, indicando uma possível especificidade a ser validada experimentalmente no futuro.

No conjunto, os resultados agregam valor ao entendimento da dinâmica das TLPs na resposta de vegetais a estresse, evidenciando sua pluralidade estrutural e funcional.

5 CONCLUSÃO

- O feijão-caupi apresenta 34 membros das *Thaumatin-Like Proteins* (VuTLPs), tratando-se de um número comparável ao observado em outras espécies diploides, como arroz e *Arabidopsis thaliana*.
- A maioria das VuTLPs apresenta a configuração canônica semelhante a TLPs encontradas em outras plantas superiores. Um grupo reduzido, porém, não contém todos os aminoácidos esperados no motivo REDDD. A conservação das 16 cisteínas, adicionalmente, foi uma característica prevalente nas VuTLPs, indicando possíveis diferenças na funcionalidade desses peptídeos.
- A análise fenética de VuTLPs, por sua vez, reflete provavelmente grupos funcionais em relação a TLPs neofuncionalizadas após duplicações em tandem de algumas regiões do genoma, conforme confirmado pelo agrupamento de TLPs de *V. unguiculata* em genoma de *P. vulgaris*.
- A análise de promotores associados a TLPs em bibliotecas de RNA-Seq sob diferentes estresses (bióticos e abióticos) indicam uma pluralidade do papel desse grupo proteico.
- As análises de expressão gênica dos dados RNA-Seq apontam para um maior número de VuTLPs induzidas sob condições abióticas (desidratação radicular) comparativamente à sua contraparte biótica (inoculação com dois vírus CPSMV e CABMV), considerando os dados do presente estudo. Tal fato agrega valor à dinâmica das TLPs na fisiologia de vegetais, nos evidenciando sua pluralidade de ações. Além disso, o fato da inoculação com os vírus em estudo (CABMV e CPSMV) depender de injúria do tecido foliar, deve ser considerado. Portanto a associação de TLPs com resposta a cada vírus estudado depende de validação experimental.

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APÊNDICE A – ARTIGO PUBLICADO NA REVISTA *Current Protein & Peptide Science*

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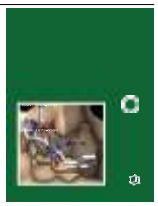
Plant Thaumatin-like Proteins: Function, Evolution and Biotechnological Applications

Current Protein and Peptide Science, 2020, *21*, 36-51

REVIEW ARTICLE

Plant Thaumatin-like Proteins: Function, Evolution and Biotechnological Applications



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Abstract: Thaumatin-like proteins (TLPs) are a highly complex protein family associated with host defense and developmental processes in plants, animals, and fungi. They are highly diverse in angiosperms, for which they are classified as the PR-5 (Pathogenesis-Related-5) protein family. In plants, TLPs have a variety of properties associated with their structural diversity. They are mostly associated with responses to biotic stresses, in addition to some predicted activities under drought and osmotic stresses. The present review covers aspects related to the structure, evolution, gene expression, and biotechnological potential of TLPs. The efficiency of the discovery of new TLPs is below its potential, considering the availability of omics data. Furthermore, we present an exemplary bioinformatics annotation procedure that was applied to cowpea (*Vigna unguiculata*) transcriptome, including libraries of two tissues (root and leaf), and two stress types (biotic/abiotic) generated using different sequencing approaches. Even without using genomic sequences, the pipeline uncovered 56 TLP candidates in both tissues and stresses. Interestingly, abiotic stress (root dehydration) was associated with a high number of modulated TLP isoforms. The nomenclature used so far for TLPs was also evaluated, considering TLP structure and possible functions identified to date. It is clear that plant TLPs are promising candidates for breeding purposes and for plant transformation aiming a better performance under biotic and abiotic stresses. The development of new therapeutic drugs against human fungal pathogens also deserves attention. Despite that, applications derived from TLP molecules are still below their potential, as it is evident in our review.

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1. INTRODUCTION

In the course of their evolution, plants have developed a series of components to fight pathogens, including the expression of PR (Pathogenesis-Related) proteins. PR proteins are encoded by genes rapidly induced after pathogen attacks, and by the accumulation of hormones associated with plant defense [1]. They are classified into 17 protein families based on sequence features, putative function, and serological, immunological or enzymatic properties [2]. The PR-5 family comprises the "Thaumatin-like Proteins" (TLPs), a name related to their structural similarity with thaumatin, which is a protein purified from the fruit of *Thaumatococcus daniellii* Benth. (Marantaceae), a plant from West Africa. Thaumatin gained attention because studies have reported it to be up to 100,000 times sweeter than sucrose on a molar basis [3]. Despite this property, many studies have reported that some TLPs identified later have no sweet taste [4]. The comparison of the conserved domain organization of TLPs shows that the thaumatin domain (Pfam: PF00314) covers almost 95% of the entire mature peptide. For example, this was verified in 49 of 59 TLPs analyzed in poplar (*Populus trichocarpa*) [5].

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Velazhahan *et al*. [4] highlighted that many seed proteins associated with thaumatin domains of cereal crops are considered homologs of TLPs and have antifungal activity. However, the authors highlighted that their presence was not always associated with the presence of pathogens, which contradicts with the classification of TLP within the PR-5 family. Sensitive detection methods (such as RT-qPCR; western and northern blots) have allowed the identification of TLPs that are developmentally regulated. Some are also responsive to abiotic stresses [4], thus indicating other roles for TLPs besides those related to pathogenesis.

Moreover, there are nomenclature problems regarding the designation of a TLP protein. As highlighted by Petre *et al.* [5], depending on the type of stress to which this protein group is responsive, a different nomenclature was attributed. If a particular protein presented a sequence similarity to a TLP but was isolated under osmotic stress (or other associated stress), this protein was termed as osmotin or osmotin-like protein [5]. There is also a report on a TLP named according to the species in which it was discovered, as is the case of zeamatin, a TLP identified in maize (*Zea mays*) [6]. According to Shatters *et al*. [7] and Petre *et al*. [5], this controversial nomenclature is not applicable any longer since similarity analyses do not support a separate nomenclature that can generate semantic confusion in the literature, as will be discussed hereinafter.

The present review encompasses aspects of stress transcriptional regulation with some proteins containing thaumatin (G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)-[GQ]-x(2,3)-C; PROSITE: PS00316; Pfam: PF00314), besides reporting data on annotation, evolution, function, applications, and biotechnological aspects of TLPs.

Considering potential biological functions, most TLPs evaluated have an antifungal activity, which probably occurs through fungal membrane permeabilization [8]. There is also evidence that some TLP representatives act on invading fungi through bond activities, by hydrolysis of β-1,3-glucans [9, 10] or by inhibiting fungal enzymes such as xylanases [11]. Lopes *et al.* [12] reported that TLPs have a selective anticandidal activity by inducing apoptosis via a membrane receptor. TLP members have been intimately associated with resistance features in genetic maps, as was the case of chickpea (*Cicer arietinum*), in which a TLP gene was located near the *Fusarium oxysporum* fsp. *ciceri* resistance locus [13, 14]. Another aspect is the allergenic properties of some fruit TLPs, such as Mal d2, an allergenic TLP from apple that was first described as having allergenic properties [15]. TLP expression is also regulated in response to certain stress factors such as drought [16], wounding [17], freezing [18], as well as under infection by virus or bacteria [19]. However, its mechanism of action in such situations remains undetermined.

In relation to the occurrence of TLPs in eukaryotes, besides plants, TLPs were observed in various organisms, including fungi [20], insects [21] and nematodes [22], but are absent in vertebrates. Fungal TLPs were initially reported in the basidiomycetes *Irpex lacteus* (Fr.), *Lentinula edodes* (Berk.), and in *Rhizoctonia solani* (J.G. Kühn) [23]. In turn, animal TLPs have been reported in the nematode *Caenorhabditis elegans* (Maupas) [22] and in insects of the orders Coleoptera, Hemiptera, Hymenoptera, and Orthoptera [7]. A three-dimensional model of locust (*Schistocerca gregaria*) TLP suggests a glucanase function, indicating a defensive role, as proposed for plant TLPs.

In plants, the distribution of TLP is observed at several taxonomic levels. In a survey conducted on the UniProt database - which includes experimentally validated TLPs -, 1,816 TLPs from 187 different species were identified (Supplementary Table **S1**; Fig. **1**), although it is considered that all plant groups have representatives. 1,720 TLPs were reported for angiosperms (1,332 in dicots and 388 in monocots), 82 for gymnosperms, seven for bryophytes and seven for algae species (five for Klebsormidiophyceae and two for Chlorophyceae). A higher number of gene/protein isoforms in higher plants are evident compared to basal groups (bryophytes and algae). This is possibly due to duplication events, including WGD (whole genome duplication) in angiosperms (Fig. **1A**), as discussed later in this review.

Considering the ten plant families with the highest number of TLP representatives and the species of greater prominence in each family (Fig. **1B**), there is a prevalence of Solanaceae (285 TLPs), being 66 in tobacco (*Nicotiana tabacum*); 284 of Poaceae (106 in maize, *Z. mays*), 279 of Fabaceae (56 in barrel medic, *Medicago truncatula*), 159 of Brassicaceae (51 in *Arabidopsis thaliana*), 137 of Malvaceae (97 in cotton, *Gossypium hirsutum*), 71 of Rosaceae (30 in Yoshino cherry, *Prunus yedoensis*), 63 of Juglandaceae (all in walnut, *Juglans regia*), 47 of Fagaceae (40 in cork oak, *Quercus suber*), 44 TLPs of Cucurbitaceae (31 in melon, *Cucumis melo*) and 36 TLPs of Actinidiaceae (28 in kiwi-fruit, *Actinidia chinensis*). Taking into account plant species that have more TLPs deposited in the UniProt databank (Fig. **1C**), cultivated and model species stand out, such as maize (106 TLPs), cotton (97), rice (*Oryza sativa*, 78), tobacco (66), walnut (66), pepper (*Capsicum annuum*, 62), barrel medic (56), *A. thaliana* (51), rape (*Brassica napus* 50) and alpine penny-cress (*Noccaea caerulescens*, Brassicaceae) (46 TLPs). Considering the availability of omics data (especially genomics and transcriptomics), it is expected that the number of studied species increase in the next years, covering a much higher number of taxonomic groups, including native (not cultivated) tropical species.

2. PREVIOUS WET LAB APPROACHES: ISOLATION AND ANALYSIS OF PLANT TLPs

For every living organism, the instructions of composition, development, and survival are contained in the DNA. Previously unreleased possibilities are now available for the characterization of these structures and manipulation of crops through genetic engineering techniques. This set of methods aims, *a priori*, to isolate a gene, changing specific amino acid sequences followed by its introduction in the same species or another. Such action has become essential to uncover fundamental processes performed in living organisms. All methods available to carry out the isolation of genes exploit one or more out of four characteristics that define these structures [24]: (1) to have a primary structure (sequence) set; (2) to occupy a particular location within the genome; (3) to encode RNA with a particular expression pattern; and (4) to encode proteins or RNAs with a given function.

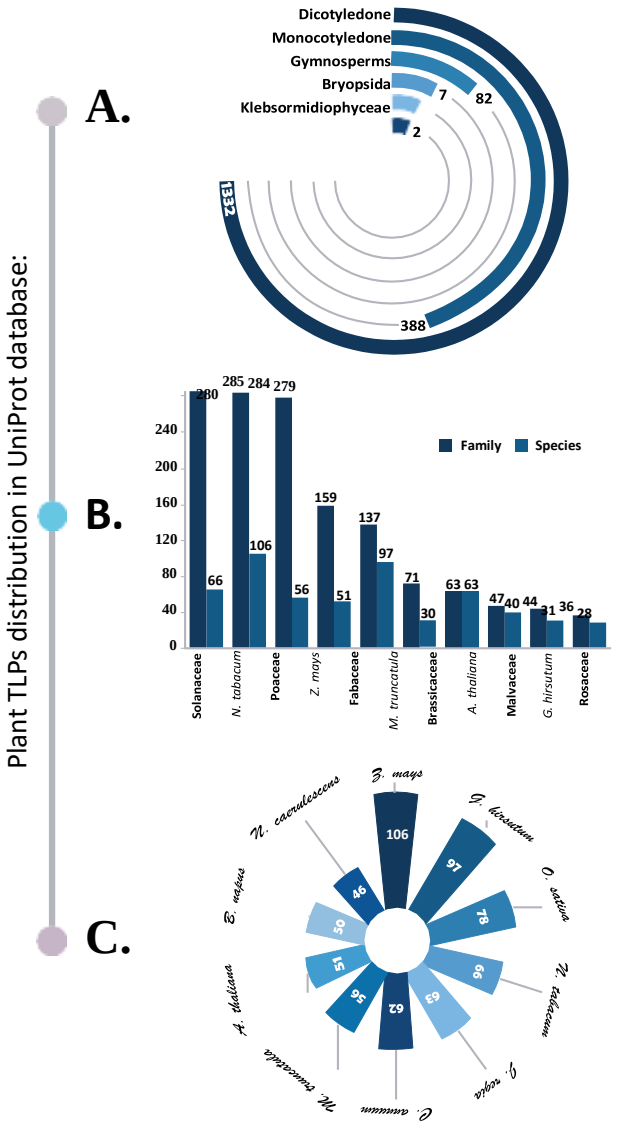

Fig. (1). Taxonomic distribution of 1,816 plant TLPs deposited in the UniProt database. **A.** Higher taxonomic groups. **B.** Top ten plant families with the most represented species in each family. **C.** Top ten plant species with TLPs available at UniProt.

TLP-coding genes have been extensively isolated from various plant species, allowing the analysis of their genetic and biochemical properties. To this end, researchers have taken advantage of both, *i.e.*, the existence of conserved motifs (alternative 1, above) present in their DNA/RNA sequences for the anchoring of specific primer pairs (Table **1**), or the prior knowledge of their coding loci position in species with genomic or transcriptomic sequences available in public databases (alternative 2, above; Table **1**). Isolated sequences may serve as a template for a series of additional actions, such as genetic transformation, to obtain transgenic individuals. Additionally, primer pairs can be used for the quantification of transcripts encoding these proteins. Through various quantification techniques, it has been possible to analyze the activity of such proteins in different situations. A review of such information is available (Table **1**), including primer pairs, strategies and conditions (stress type) used for the isolation and quantification of genes/transcripts encoding such proteins. Such information is imperative for research groups interested in isolating and analyzing TLPs.

Considering existing studies (Table **1**), it is evident that TLPs are expressed in a range of species and tissues in response to various stressor agents (biotic and abiotic [25]). Such information is relevant and has a biotechnological potential. It provides tools for the analysis of this protein family in related species that have limited genomic information available, or in contrasting genotypes of the same species. The use of primers anchored at conserved sites enables the identification of relevant molecular targets. These primers can allow the identification of polymorphisms that are useful for breeding purposes or selection of tolerant/resistant crop plants.

3. IN SILICO IDENTIFICATION AND CHARACTERIZATION OF PLANT TLP TRANSCRIPTS AND PROTEINS

Besides the analysis of single TLP candidates (compiled in Table **1**) and the isolation of TLP proteins using biochemical inferences, bioinformatic approaches have the potential to provide a large-scale identification of TLP candidates under different experimental conditions. Despite their importance, TLPs have not been appropriately studied considering the currently available databases and omics resources, with an emphasis on genomics and transcriptomics. To exemplify the potential of data mining on omics data to identify TLPs, the present work presents an exemplary *in silico* evaluation of TLPs in cowpea (*V. unguiculata*) transcriptome using bioinformatics (for details on the recommended annotation steps, see Supplementary Material **SM1**).

The outlined mining approach (Fig. **2**) was based on the use of TLPs as seed sequences. For this purpose, all protein sequences containing the thaumatin domain (PROSITE: PS00316; Pfam: PF00314) deposited in the UniProt database were downloaded (Supplementary Table **S1**), including 1,816 proteins from 187 different plant taxa of 58 plant families. Using these seed sequences, a search for cowpea TLP candidates was carried out in the cowpea RNA-Seq libraries generated (Supplementary Table **S2**), which were chosen to exemplify the participation of TLPs in response to different tissues under stress types. The choice was justified due to the diversity of sequences available in the Cowpea Genomics Consortium (CpGC), including transcriptomics data of different types and ESTs (Expressed Sequence Tags), SuperSAGE (Super Serial Analysis of Gene Expression; Supplementary Fig. **S1**) and RNA-Seq (these last two with Next Generation Sequencing methods) (see Supplementary Table **S2**). The data are suitable for a demonstration because it covers the response of this species to abiotic (root dehydration, high salinity and leave injury) and biotic stresses [inoculation with two viruses: *Cowpea Severe Mosaic Virus* - CPSMV, OR *Cowpea Aphid-borne Mosaic Virus* - CABMV]. Transcripts from leaves and roots under different biotic/abiotic conditions that were clustered together with 264,945 ESTs deposited in the Cowpea HarvEST database V.1.33 (http://harvest.ucr.edu/) and GenBank (NCBI) were used for anchoring and annotating TLP transcripts. Thus,

Table 1. Main molecular approaches and primer pairs used for TLP isolation or quantification in plants, including gene name, source species, associated stress type where their coding transcripts, associated tissues, and literature source are presented.

Gene Name	Species	Induced By	Tissue	Name of Primer	Left Primer	Right Primer	Aim	Strategy	Refs.
ATLP-3	*Arabidopsis thaliana*	Pst DC3000 inoculation and salicylic acid	Floral bud	ATLP-3S	TGATGCTAG-CACCGTATT-CACTTTA	TAATACGACTCACT ATAGGG**	Isolation	-	[26]
Cry j 3.4	*Cryptomeria japonica*	UV-B, salt stress (NaCl 200 mM), ethephon AS, ABA, Arachidonic acid	Pollen	Cry j 3.4	TCTAGAAC-TAGTGGATCCCCCG	TCCCTATTCGACTG CATAAAGAC	Isolation	-	[27]
Cry j 3.5				Cry j 3.5	TGGGGGCGGACG-GACTG	CGGCA-GAACAAGTGTAAA-GACTGG			
Cry j 3.6				Cry j 3.6	GATATATGGAGAC-TACTGCCATTC	GCCTTGGCAGTT-GAGCGTG			
Osmotin-like protein, b isoform	*Glycine max*	High salt (NaCl, 100mM)	Seedlings	GmOLPb	CCCAACA-CACTTGCGGAATT	TTCTGTCCATTGGG AGAACC	Isolation	RACE-PCR	[28]
PmTLP*	*Pinus monticola*	Seasonal regulation, response to abiotic/biotic stresses	Shoots, stems, needles, and roots	PmTLP	TGG GGN CGN CAN GGN TGT TCC TT	oligo dT	Isolation	RACE-PCR	[25]
TaPR5	*Triticum aestivum*	*Puccinia striiformis* f. sp. Tritici infection, SA, JA and ABA, wounding, cold temperature, and high salinity	Leaves	S11_CY23_ Contig112	GAGATGTCGTA-GAAGTCTGGGTGCC GC	GCGGCACCCAG-GACTTCTACGACAT	Isolation	RACE-PCR	[29]
				TaPR5	CAAGCAGTGGTAT-CAACGCAGAG	GTGAAGCCA-CAGTTGTTCTTGAT GTT	Quantification	RT-qPCR	
PopTLP1	*P. trichocarpa* X *Populus deltoides*	*M. laricipopulina* infection	Leaves	*PopTLP1*	CCA-GACTTGGTATCTTA ATG	GTTAC-CAAACTGATTTAAC G	Quantification	RT-qPCR	[5]
PpTLP	*Pyrus pyrifolia* cv. Huobali	Self-defense during fruit development	Pericarp and leaves	PpTLP	GCAAACAGGCAAT-TAAGACATATTCA	CTGCATATAT AATCCCATTTCGTG C	Isolation	RACE-PCR	[30]
Osmotin-like protein	*Beta vulgaris*	Water deficit	Leaves	OLP	GCAAGTGCCCGCAA CAC	CGTAAGCGGAGT-GATCCCTATT	Quantification	RT-qPCR	[31]
AdTLP	*Arachis diogoi*	*Phaeoisariopsis personata* infection, JA and ABA treatments	Leaves	AdDR11-64R	-	CATTAGGGCACTGG TTGCTA	Isolation	RACE-PCR	[32]
				AdDR11-117R	-	GCCTCCTGAACAAG TGAAAG			
				PCR anchor primer	GAC-CACGCGTATCGAT-GTCGAC	-			

(Table 1) contd….

Gene name	Species	Induced by	Tissue	Name of Primer	Left Primer	Right Primer	Aim	Strategy	Refs.
				TLP ORF	GGGATCCATGGCGATTACTCGTGTTGT	CCTCGAGT-CATGGACA-GAAGTTGATAGC	Quantification	Semi-quantitative RT-PCR	
				TLP ORF (1)	GGGGCCCATGGCGATTACTCGTGTTGT	GGGATCCTCATGGACAGAAGTTGATAGC			
				TLP ORF (2)	GGGGCCCATGGCGATTACTCGTGTTGT	CCCCGGGTCATGGACAGAAGTTGATAGC			
CaOSM1	*Capsicum annuum* cv. Nockwang	*Xanthomonas campestris* pv. vesicatoria (Xcv) infection	Leaves	CaOSM1	ATGGGTCACTTGACA	AG-CAACTTCATGTGTAC	Quantification	RT-qPCR	[33]
				CaOSM1ORF	TCTA-GATGGGTCACTTGACAACTTGTTTA	GGATCCTTAAG-CAACTTCATGTGTACTTG	Isolation	-	
TLP 1	*Manilkara zapota*	†	Leaves	SAP5 / SAP9	CGACATATCTCTGGTTGACGG	GGTTGTCTTCTGCCCTTGA AATTG	Isolation	-	[34]
PeTLP	*Populus deltoides* × *P. euramericana* cv. Nanlin895	200 mM NaCl, 10 % PEG6000, 100 µM gibberellin, 1 mM salicylic acid and 100 µM abscisic acid.	Leaves	PeTLP	GTCA CATTAGCCTCTTAAACCAG	CAAA-GAAGCGGAAGA-TAGACATA	Isolation	RACE-PCR	[35]
Rj4	*Glycine max*	‡	Root	Rj4	ATGGCATC-CATCGTGACTATGG	GGAACAGGGACGATCTTGAAGTC	Isolation	Positional Cloning	[36]
ObOLP	*Ocimum basilicum*	Methyl jasmonate (MeJA) and mechanical wounding	Leaves	OSMful	TTTCCATGGAAAACTCAAAAATTTCACCCAAAC	TTTAAGCTTATAC-CATC-TAAAAATGGCAA-GAC	Isolation	Cloning of full length ObOLP cDNA	[37]
				RT osm	CAAACCTGGACCCTATCCTTC	TGGGCAGAAGAC-TACCCGGTA	Quantification	RT-qPCR	
SindOLP	*Solanum nigrum* var. indica	Methyl jasmonate, salicylic acid, H2O2, ABA, and NaCl	Leaves	OLP1	CGCGGATC-CATGGGCTACTTGA-GATCT	CCCAAGCTTTTACTTGGCCACTTCATC	Isolation	-	[38]
TaLr35PR5	*Triticum aestivum*	*Puccinia triticina* infection, ABA and salicilic acid	Leaves	TaLr35PR5	CCCAAGCTTTCA-GAAGCTGATGGGGTAGAC	CGGGATCCTGGGTCAGGAGCAACATC-CAG	Isolation	-	[39]
PR-5a	*Cicer arietinum*	*Ascochyta rabiei* infection	Leaves	tlp l-1/ tlp-con-1	TTY-GAYATHGTNAAY-CARTG	ACGGGNCTYCGNAT	Isolation	PCR for isolation of full-length cDNAs encoding PR-5a	[40]

(Table 1) contd….

Gene name	Species	Induced by	Tissue	Name of Primer	Left Primer	Right Primer	Aim	Strategy	Refs.
CpOsm	*Calotropis Procera*	*Fusarium solani* spores	Leaves	CpOsm	GCCACNTTYACNATHCGNAACAAYT-GYCC	GGGRCARAANAYAACYCTRTARTTDGT	Isolation	PCR to amplify a cDNA segment encoding CpOsm mature sequence	[41]
CkTLP	*Cynanchum komarovii*	ABA, SA, MeJA, NaCl (300 mM) and drought	Roots, stems, leaves, cotyledons and mature seeds	TLP-F/ TLP-R	CGACATTTCGCTGGTGGATG	CTGTAAGCAT-CAGGGCACCT	Quantification	RT-qPCR	[42]
TaLr19TLP1	*Triticum aestivum*	SA, MeJA, ETH, ABA and infection with *Puccinia triticina*	Leaves, stems, and roots	qTcLr19-F/qTcLr19-R	CAACGAGAACCA-GAAGGACAGC	TACGGACG-GACATACGGACACT	Quantification	RT-qPCR	[43]

Legend for abbreviations: *10 different TLPs were isolated; † Isolated due to its food allergenic role; ‡ Rj4 (rj4) was constitutively transcribed in roots (including nodules), regardless of the presence of nodule symbiosis; - Strategy was not reported; **T7 universal primers; OLP (osmotin-like proteins).

gene families still not addressed in global transcriptome analyses, as is the case of TLPs, can be studied under different experimental conditions. The inclusion of libraries under root dehydration is an interesting approach since it regards an abiotic stress of global importance, especially considering that few works have reported TLPs under abiotic stress. Most of the RNA-Seq reads available are between 30-400 bp in length, depending on the sequencing technology used [44]. This provides a direct structural characterization, as is the case of the present demonstrative approach.

The tBLASTn search allowed identifying 56 putative transcripts coding TLPs in *V. unguiculata* (VuTLPs) transcriptome (Supplementary Table **S3**). The recent improvement of available genome sequences for model and non-model plant species provides an opportunity to study the abundance of the TLP gene family using computational tools. The identification based on computational methods is efficient and faster than the traditional molecular methods outlined in Table **1**. Considering the significant roles associated with TLPs, it is logical to expect that the number of coding-genes varies widely among plant taxa. Thus, computational approaches are an efficient way to evaluate the abundance of these proteins in plant genomes, evidencing their evolutionary relationships in different species or in regard to their intrinsic characteristics such as structural features and allergenicity [45].

The pipeline presented to identify and characterize TLP transcripts using bioinformatics (Fig. **2**; Supplementary Material **SM1**) can be applied to any plant with omics data available. This analysis is simple and can be applied to all repositories available, including identification based on seed-sequence, functional annotation, and TLP characterization. The only disadvantage resides in the fact that this is not a comprehensive analysis, requiring some time to increase the number of seed sequences (considering that new sequences are increasingly available in databanks). To be sure that the selected candidates belong to the TLP group, all proteins for which no thaumatin domains (PROSITE: PS00316; Pfam: PF00314) were identified and excluded from the sampling (Supplementary Table **S3**). 39 completely expressed TLP-transcripts were identified in cowpea under root dehydration. As previously reported, TLPs are rich in Cys (cysteine) residues [46] that promote protein stability [47]. TLPs also present five additional conserved amino acids - an arginine (R), a glutamic acid (E), and three aspartic acid residues (D) (Supplementary Fig. **S2**) -, known as REDDD motif. This configuration is responsible for the reported antifungal activity of plant TLPs [48, 49]. In addition, VuTLP candidates had a molecular weight between 10.61 and 36.27 kDa. Among all candidates, 18 cowpea TLP predicted sequences presented the most typical molecular weights (from 20 to 26 kDa) and isoelectric points (from 3.9 to 8.76), while 20 members presented a more basic isoelectric point (Supplementary Table **S3**).

The TargetP prediction revealed hydrophobic signal peptide sequences, indicating that these proteins are predominantly secreted. All 39 predicted VuTLPs presented a thaumatin domain in at least one database searched (NCBI CDD: cd09218 and cl02511, Pfam: pfam00314, SMART ID: smart00205; Supplementary Table **S3**). Five highly conserved amino acids of the REDDD motif in an acidic cleft were detected in most VuTLPs predicted. The alignment of cowpea candidates with other TLPs from *Arabidopsis*, rice and soybean (Supplementary Material **SM1**; **SM2**, multifasta

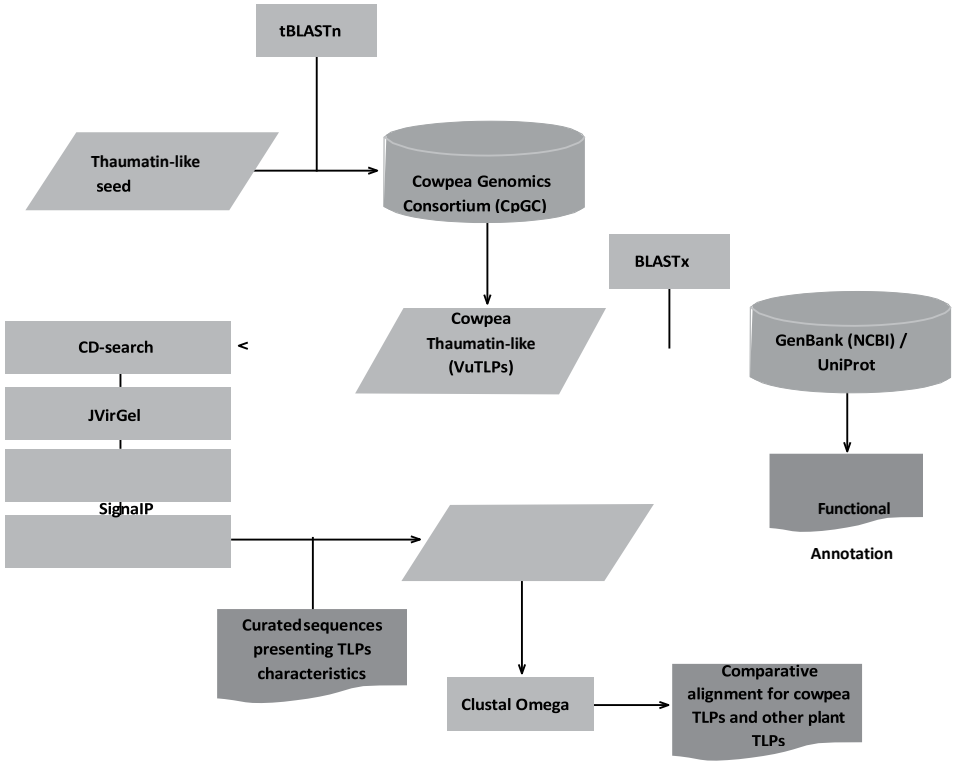

Fig. (2). Workflow for *in silico* identification and prediction of TLPs, here exemplarily applied to cowpea (*Vigna unguiculata*) transcriptome as an illustrative case study. The *in silico* approach presented can be used for any available plant database to improve the knowledge of TLP-related gene representatives.

format) showed that some VuTLPs do not contain all expected amino acids. There is a replacement of the second glutamic acid (E) in REDDD motif for glutamine (Q), and the third aspartic acid (D) for glycine (G), similar to that observed in the soybean sequence used in our alignment (Supplementary Fig. **S2**). Regarding the 16 conserved cysteines, only one cowpea sequence (TR101189|c0_g1_i1; Supplementary Fig. **S2**) lacked the last cysteine, while all other TLPs presented the 16 residues that form eight disulfide bonds necessary for correct folding and for ensuring a high level of thermostability and pH constancy [30].

The exemplary pipeline presented here can be successfully applied to any plant species (including genomic and transcriptomic data) with the recommendation of an increase in the selected seed sequences with newly deposited sequences in GenBank (NCBI) and plant databases listed in the Supplementary Table **S1**.

4. TLP EVOLUTION

Previous reports have indicated that evolutionary relationships within the TLP family are not fully understood. Among them, we can highlight the phylogenetic and structural analysis of PR-5 gene family members performed by Shatters *et al*. [7]. According to the authors, such a family was formed by thaumatins, osmotins and TLP-related proteins (considered here as synonyms), many of which have antimicrobial activity. They identified some conservation regarding sequences of this multigene family in plants and suggested that plant PR5 is paraphyletic in angiosperms and related to insect and nematode TLPs. However, a later study on TLP phylogeny associated with protein structure and function in eukaryotes indicated three monophyletic groups [5].

In the present work, we generated a neighbor-joining tree (Fig. **3**) including 12 cowpea TLPs and 24 TLPs from other plant species (Supplementary Material **SM1**; **SM3**, multifasta format). Based on the dendrogram (node I), two cowpea sequences (Vu_C6 and Vu_C1587) emerged in a basal position with a bootstrap value of 78. Subsequently, a second basal group emerged with two cowpea sequences (Vu_C1 and Vu_C5651, bootstrap 41). From these basal groups, a second node (II) emerged, including proteins of the Osmotin (from Solanaceae and Brassicaceae families) and the Zeamatin group (Poaceae family). Both the groups (Osmotin and Zeamatin) were taken together with a high bootstrap value (100), indicating a close structural similarity between both the categories. Osmotins are reported as responsive to osmotic stress, while zeamatins (which are more frequent in grasses) have an antifungal activity due to membrane permeabilization. They also present a thaumatin-like conserved domain, which justifies the position of some cowpea candidates in a more basal position in the generated tree.

The neighbor-joining analysis presented here confirms that the classification of some TLPs in different protein families (osmotin and zeamatin) is inappropriate since both the categories are structurally similar and were positioned together with other TLPs, with no evident particularity or specificity. A similar observation was reported by Petre *et al.* [5], who analyzed eukaryotic TLPs and highlighted that phenetic analysis associated with the structure and function

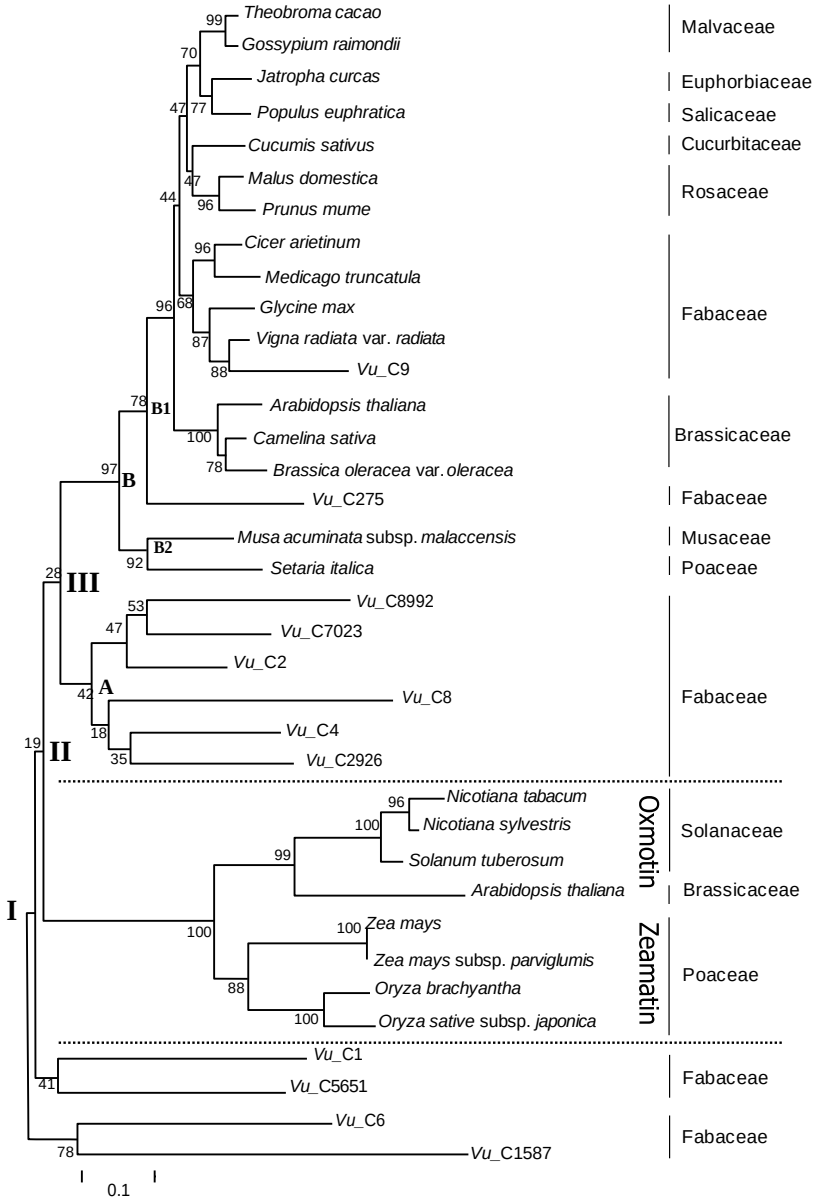

Fig. (3). Neighbor-joining tree (distance matrix) generated from plant thaumatin-like protein (TLP) sequences. Values on the nodes are the percentage of bootstrap values (1,000 replicates). Scale bar (based on the dendrogram) indicate 0.1 amino acid substitutions per site.

of these proteins was not enough to justify the adoption of different nomenclatures associated with TLPs responsive to a given biotic or abiotic stress.

Node III included two subclades (A and B), where clade A comprised of six distinct cowpea TLPs and clade B (with bootstrap 97) included monocot and dicot TLPs. This branch included no putative osmotin or zeamatin members. Within this branch, terminal clades comprised of related species (often from the same family or class) with the most significant bootstrap values, indicating conservation. By analyzing eukaryote TLPs, Liu *et al.* [50] observed the formation of nine distinct groups designated as subtypes of the highly diversified TLP superfamily. A possible explanation for the formation of these groups can be attributed to the TLP structural diversity. According to Petre *et al.* [5], such diversity may influence biological and biochemical functions, and differences in the topology around the cleft could determine TLP specificity to a given target ligand [51]. This may be true also when considering cowpea TLPs, since their diversity and position in different clades is possibly due to the presence of protein sequences with different structures and functions. Thus, the grouping of cowpea candidates at various levels in the dendrogram possibly reflects the inclusion of gene products that represent paralogs, putatively also representing TLP candidates with different functional roles and transcript isoforms, as reported for *P. trichocarpa* with 55 putative TLP gene candidates [5].

Recently, Cao *et al.* [49] studied the evolution of PR-5 gene family by identifying and comparing genic sequences containing the thaumatin domain. As highlighted by the authors, the green algae *Chlamydomonas reinhardtii* and the moss *P. patens* present few TLP genes (one and six, respectively), a condition also highlighted in Fig. (**1**). In turn, higher plants (*e.g.*, *A. thaliana*, *O. sativa*, *P. trichocarpa* and

Z. mays) have a higher number of representatives. This suggests that the expansion of the TLP family took place after the divergence of vascular plants (embryophyte group). Additionally, Cao *et al.* [49] suggested that tandem and segmental duplications are possibly the main drivers for the existing diversity in plants. Wanderley *et al.* [52] observed a similar feature for R (resistance) and other PR protein-coding genes in soybean and *M. truncatula* genomes.

To evaluate the distribution of TLPs in a plant species of interest, it is recommended to anchor TLP candidates in the genome of the target species, or in a near-related species. In the exemplary annotation presented here, we anchored cowpea VuTLPs candidates against the genome of *Phaseolus vulgaris* (common bean). Such an anchoring allows inference on the distribution, relative position and abundance of TLPs (for recommended procedures, see Supplementary Material **SM1**). This approach revealed a distribution of 26 TLPs in the most common bean chromosomes, except for the chromosomes 5, 7 and 10. A high clustering in some chromosome regions was evident, mainly on chromosomes 1, 2 and 11 (Fig. **4**; Supplementary Table **S4**). For example, in the subterminal region of the short arm of chromosome 1, four clustered TLP coding genes were detected, indicating the occurrence of tandem duplications in the generation of new TLP isoforms.

Cowpea (as common bean) is a diploid species with 2n = 22 [53, 54]. It presents a number of TLPs similar to *A. thaliana* (51) and lower than *P. trichocarpa*, and is considered a tetraploid species [55]. However, such a correlation may be not true, since other species (such as rice and maize, with 78 and 106 TLPs, respectively) are diploid and present a higher number of representatives described (Fig. **1**). Additionally, a higher number of cowpea representatives is expected if the search includes genomic sequences.

Another aspect associated with TLP evolution and worth mentioning is the plant-pathogen co-evolution. Over the time, plants have been acquired new mechanisms to promote resistance [56, 57]. Pathogens also continue to evolve, causing pressure on the structure and the diversity of some associated gene families, increasing the size of some protein families [58], including PR-genes. This increase represents a major significance of the functional diversity via sub- or neofunctionalization of paralogs [59]. In this scenario, the natural selection of genes with new functions under environmental pressure plays an important role in TLP evolution [50]. Some studies have investigated the function and evolution of these proteins as a result of natural selection [5, 49, 60]. For example, Petre *et al.* [5] found that amino acids of various TLPs were under diversifying selection selection, while amino acids forming the acidic cleft were under purifying selection, thus being highly conserved. According to Misas-Villamil and van der Hoorn [61], the preservation of acidic cleft is required to maintain antifungal activity, while diversification of the exposed amino acids can prevent pathogen recognition. Thus, the antifungal activity has been suggested as a universal property presented by most TLPs, as indicated by Petre *et al.* [5]. In turn, Cao *et al.* [49] observed that the positive selection of some TLP nucleotides in poplar might have accelerated functional divergence, resulting in the formation of functional subgroups.

Some studies focused on three-dimensional (3D) modeling using various proteins containing the thaumatin domain, similar to what carried out by Batalia *et al.* [8] and Ogata *et al.* [62]. They suggest that the 3D model of TLPs consists of a small protein with three characteristic domains sustained in a compact fold by disulfide bridges. Due to the high conservation of PR-5 homologs and their common structural features, slight differences in their 3D features may reflect the proposed functional specificity under different development and stress conditions. In addition, other differences have been reported, including isoelectric value, subcellular localization, and regulation of gene expression [63]. For instance, studies have reported that tobacco osmotin has an antifungal

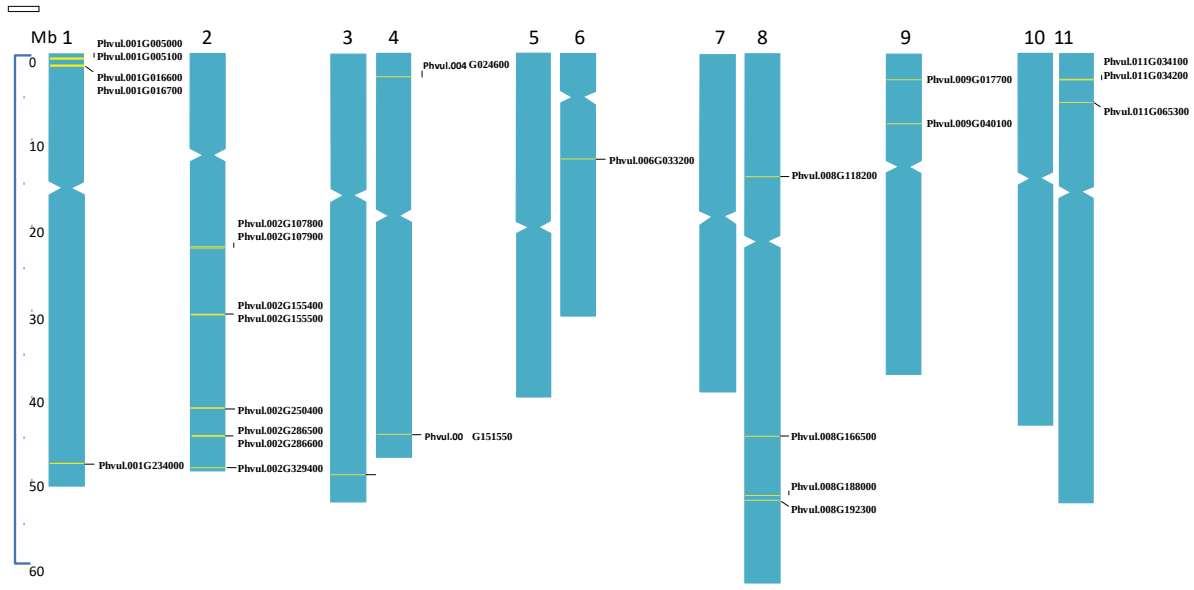

Fig. (4). Distribution of cowpea thaumatin-like genes in *Phaseolus vulgaris* virtual chromosomes (Chr1 to Chr11). Positions of TLP anchored genes or clusters are indicated in yellow.

activity [64] and protects against abiotic stress [65], among other functions. In turn, zeamatin from maize exhibited membrane permeabilization activity [66] and α - amylase/trypsin inhibitory activity [67].

5. DATA MINING IN THE US PATENT AND TRADEMARK OFFICE

A search for patents was initially conducted for thaumatin and plant stress. It resulted in 1,444 hits (for pipeline, see Supplementary Material **SM1**). 260 patents were selected according to their descriptions, summaries and "claims." Moreover, the patents selected were arbitrarily categorized into four groups based on their agronomic applications: (1) characterization, (2) stress tolerance mechanism, (3) plant breeding and biotechnology, and (4) plant varieties and cultivars (Fig. **5A**).

The most representative group of patents is "varieties and cultivars," with 173 patents on five plant species (157 patents for wheat, seven for barley, five for tomato, three for cantaloupe, and one for pumpkin). Patents describe (i) new genotypes produced by conventional breeding (Fig. **5A**); (ii) application of alternative breeding technologies such as transgenesis (*e.g*., mutation, genetic transformation, and gene conversion) and haploidization for breeding purposes; (iii) potential agronomic advantages such as resistance to herbicides, insects, diseases and abiotic stresses. The group "breeding and plant biotechnology" included 50 patents (Fig. **5A**), with an emphasis on methods for transgenic plant production and their applications. 27 patents (Fig. **5A**) have been described for different approaches or applications, such as response to biotic and abiotic stresses (classified as "mechanisms of tolerance to stress"). The "characterization" group had ten patents (Fig. **5A**) for plant resources, describing data on molecular evaluation and correlation between the gene expression and production traits.

Despite several patents covering different aspects of stress and plant performance, no available patent described the direct application of thaumatins for stress amelioration. Thus, a second search was performed using "thaumatin OR thaumatin-like OR TLP" as queries in the title or description sections in order to search for patent applications for a direct application of thaumatins. This search resulted in 157 patents; 45 were hits, with a direct relationship with thaumatins (Supplementary Table **S5**). They were classified accordingly to their major applications (Fig. **5B**).

Thaumatins have been used in diverse industrial applications mainly due to their flavoring potential. Despite the availability of alternative flavoring molecules such as sucrose, thaumatins hold advantages such as increased flavoring potential and low caloric content. Initial patents referred to thaumatin purification methods from plant extracts, as well as methods for thaumatin identification and quantification. The technological progress has led to the development of thaumatin variants with a higher flavoring potential and large-scale production using heterologous systems. The simultaneous development of solutions for thaumatin application and strategies to prolong its effects was the focus of research, mainly by the food industry.

The second group of patents (Supplementary Table **S5**) focused on the development of *in vitro* systems for thaumatin production based on recombinant DNA technology. Such patents describe variations in cloned sequences (propeptide, mature peptide and modified peptides) and alternative applications such as the production of thaumatins in an extracellular matrix targeting human cancer cells.

The third patent category referred to thaumatins used as model proteins intended for improvement of plant traits associated with specific transgenesis protocols. Although they did not exclusively cover exogenous production in plants, thaumatins were also used in proof-of-principle experiments.

A single patent applies a genetic transformation to thaumatin exogenous expression aiming to increase fruit flavor in transgenic plants. Although the role of thaumatins in toler-

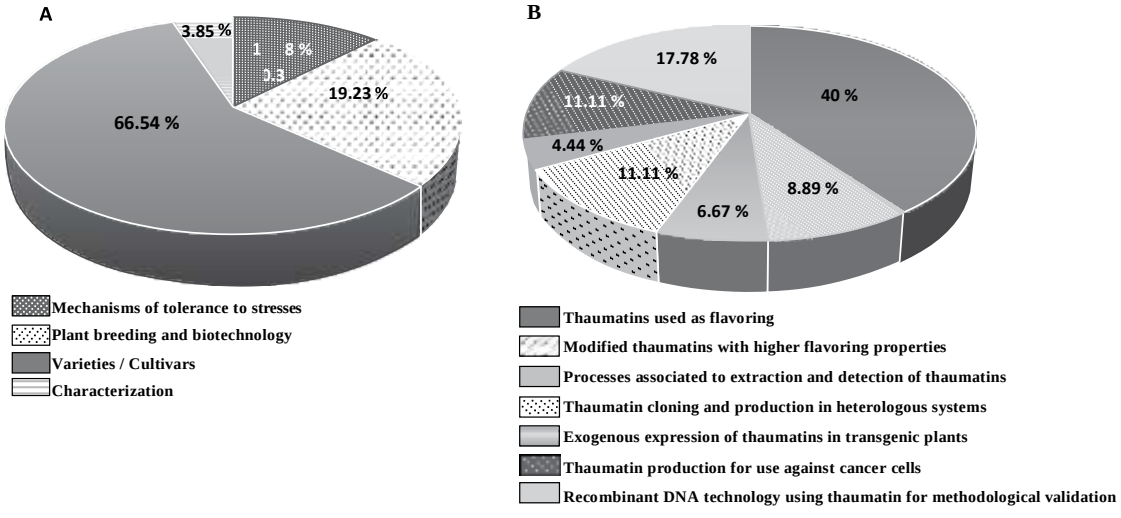

Fig. (5). Patents retrieved after search in the US Patent and Trademark Office (USPTO). **(A)** Patents found using "thaumatin" OR "thaumatin-like" OR "TLP" AND "stress" as keywords. Patents were classified into four categories: (1) characterization, (2) stress tolerance mechanism, (3) plant breeding and biotechnology, and (4) varieties/cultivars; **(B)** Main applications for patents identified by searching "thaumatin" OR "thaumatin-like" OR "TLP" as keywords in titles or abstracts.

ance to biotic stress has been under intense research, there is no report on patents for this agronomic application. The simultaneous development of plants with increased flavoring features and improved resistance to biotic stress would represent a significant step forward, with implications on scientific, agronomic and industrial scenarios.

6. BIOTECHNOLOGICAL APPLICATIONS

Because of the intense sweet taste (on a molar basis, some thaumatin domains contain sequences are 100,000 times sweeter than sucrose from 300 to 3,000 the weight basis) [68], some proteins containing a thaumatin domain have been explored since the 1990s by the food and pharmaceutical industry as a "natural flavor modifier or enhancer," replacing synthetic sweeteners [69, 70]. However, limitations related to obtaining this protein from its natural source (fruits of *T. daniellii*) have hindered its use [69, 71]. In fact, few potential sweeteners can meet all market requirements, such as large-scale production, taste quality, safety, solubility, cost *etc*. [72]. Thus, efforts have been directed to produce TLPs with a sweet taste using efficient methods that meet business requirements.

The production of a recombinant TLP has been tested in different organisms (including bacteria, yeasts and fungi), as well as in transgenic plants. Although the results are still not encouraging (*e.g.*, low scale production and, in some cases, production of an inactive protein), biotechnological approaches are promising regarding the efficiency of their production and use as a sweetener or as a flavor enhancer [71, 73]. Based on previous reports, different strategies have been adopted to produce TLPs by recombinant microorganisms and transgenic plants using the genes encoding these proteins or synthetic genes with codon use adjusted to each host organism [69, 73].

6.1. Strategies for Thaumatin Production Using Microorganisms and Plants

After cloning the first thaumatin, several attempts to produce this type of protein using bacterial expression systems (particularly *Escherichia coli*) have been adopted over the past decades [74, 75]. The use of *E. coli* as a recombinant production system has increased due to its simplicity, fast growth, relatively low cost and compatibility with almost all commercially available vectors [76]. According to Daniell *et al.* [77], the synthetic gene thaumatin II expressed in *E. coli* resulted in the production of a similar native protein considering biochemical, spectroscopic and organoleptic properties.

Filamentous fungi are also attractive hosts for the expression of recombinant proteins given their capacity to grow in a large scale and to produce high densities of biomass using low-cost substrates and given the relatively simple and well-established fermentation processes [78]. Some works in this sense have been performed especially on fungi from *Aspergillus* and *Penicillium* genera. For example, Hahm and Batt [79] reported the expression and secretion of 50 ng/ml of thaumatin in *Aspergillus oryzae* transformed with the cDNA of thaumatin II (from *T. daniellii*) using a glyceraldehyde 3-phosphate dehydrogenase-promoter from *Saccharomyces cerevisiae*). Using *A. niger* var. awamori, Faus *et al.* [80] obtained the secretion of thaumatin at higher concentrations (5-7 mg/L). Thaumatin II was also expressed in *Penicillium roquefortii* [81]. According to this study, different expression cassettes resulted in the production of thaumatin in the intracellular environment and in the extracellular medium at the concentrations of 1-2 mg/L. Higher thaumatin levels were obtained in *Aspergillus* (150 mg/L) due to the introduction of the *pdi*A gene (that codifies a protein disulfide isomerase, PDA) with chaperone and foldase activity in the thaumatin expression cassette [82].

Considering the necessity of high protein concentrations in the active form, the expression system in yeast (in particular *Pichia pastoris*) deserves attention. Although some restrictions concerning the processing and secretion of thaumatins are known, some vectors, promoters and transformation protocols were combined with efficient fermentation techniques and ensured a significant production of recombinant proteins [83, 84]. For example, using *P. pastoris*, Masuda *et al.* [85] achieved a considerable level of thaumatin II (approximately 25 mg/L of culture), which had the same sweet taste as that obtained from the plant *T. daniellii.* Ide *et al.* [86] identified a thaumatin I candidate (except for the residues at position 113, which regarded an Asp instead of an Asn) that was transformed by *P. pastoris*, producing approximately 30 mg/L in the culture medium. The authors also demonstrated that the insertion of a sequence (NH2-A- T-F-E-I-V-N) at the N-terminal region of the protein pro- vided a secretion signal and increased the efficiency of TLP secretion in *P. pastoris* compared with the native sequence alone. Furthermore, Ohta *et al.* [87] found that basic amino acid residues (especially R82 and K67) in the cleft region of thaumatin sequences appear to be critical for enhancing the sweet taste.

The adoption of plants as thaumatin expression systems has advantages compared with the production in microorganisms since they produce larger quantities of inexpensive raw materials for processing without the need for a thorough purification of the end product from foreign proteins and medium components [88].

Using the thaumatin II gene from *T. daniellii*, Firsov *et al.* [88] developed two transgenic tomato plants with accumulation of thaumatin II. The authors obtained 4.6 and 4.1% of total soluble proteins, respectively. Such values were considered promising since comparable studies on the heterologous expression of target proteins in plants resulted in up to 1% of total soluble proteins.

Another strategy of the genetic transformation with *Agrobacterium rhizogenes*, is referred to as an attractive option for mass production of plant metabolites and target proteins [89]. Using this methodology, Pham *et al.* [90] reported a successful production and secretion of a recombinant thaumatin I in tobacco hairy root cultures. The presence of an ER (Endoplasmic Reticulum) signal peptide was crucial for protein secretion, increasing the production by about 12 times. Such results show that tobacco hairy roots may be a suitable system for thaumatin production.

These and many other examples demonstrate the range of thaumatin production systems currently available using plant biotechnology, which has the potential to favor the commer-

cial use, particularly in the production of food and food additives [91].

6.2. TLP & Tolerance to Biotic and Abiotic Stresses

The interest in TLPs has also increased in plant breeding programs due to their potential use in the development of transgenic plants with an increased performance under biotic and abiotic stresses [32, 92]. Considering that proteins containing the thaumatin domain are present in all plant groups and that most species have not yet been studied, the possibilities for the exploration of these proteins are immense [93, 94]. Such roles under stress have been exploited using different methodological approaches, including analysis of expression of transgenic plants (*in vitro* and *in vivo*) regarding antifungal activity, and the investigation of protein activity [50, 92]. Sripriya *et al.* [95] found that transgenic rice lines expressing a combination of rice chitinase (*chi11*) and tobacco osmotin (ap24) in the same T-DNA presented higher resistance levels to sheath blight than those expressing *chi11* or *ap24* individually. Three transgenic plants (CO1, CO2, and CO3) had complete copies of T-DNAs with both *chi11* and *ap24*, but only CO1 and CO2 had higher expression levels of both the genes.

Misra *et al.* [96], in turn, identified and characterized a basil TLP (ObTLP1; *Ocimum basilicum*) in EST libraries treated with Methyl Jasmonate (MeJA). The isolated ObTLP1 was later expressed ectopically in *A. thaliana*, indicating resistance to fungal pathogens (*Scleretonia sclerotiorum* and *B. cinerea*) and tolerance to abiotic stresses (drought and high salinity).

Transgenic lines of oilseed rape (*Brassica napus*) transformed via *Agrobacterium* with the insertion of a cDNA coding, a TLP isolated from barley (named Hv-TLP8), had increased resistance to the soil pathogen *Plasmodiophora brassicae (protozoa*) [97]. Using another approach, the gene PpTLP isolated from *Pyrus pyrifolia* was overexpressed in transgenic tobacco lines leading to an increased *in vitro* activity against the fungi *S. sclerotiorum*, *Phomopsis* sp., *P. parasitica* var. *nicotianae*, and *Alternaria* sp. [30]. The TLP-3 gene from *M. truncatula*, without the signal peptide, was efficiently transferred (via *A. tumefaciens*) and expressed in tobacco plants [98]. Extracts of transformed and untransformed plants were used to test the inhibition of fungal growth in culture medium, preventing *Alternaria alternate* spore germination and conferring resistance to this critical pathogenic fungus.

Transgenic tobacco plants overexpressing the gene GbTLP1 (a cotton TLP) showed resistance to different stress agents, in particular to the fungus *Verticillium dahliae*. The plants acquired a considerable resistance to *Fusarium oxysporum*, besides tolerance to salinity and drought [99]. In another study, the heterologous expression of TLP isolated from *Camellia sinensis* (CsTLP) conferred resistance to the fungi *Macrophomina phaseolina* and *Phytophthora infestans* in potato plants [100].

Rajam *et al.* [92] reported that tobacco plants transformed with the thaumatin gene from *T. daniellii* presented higher resistance to a fungal disease caused by *Pythium aphanidermatum* and *Rhizoctonia solani*, but also showed tolerance to stress caused by salinity and PEG treatment. This study revealed this gene can confer tolerance to both types tested stresses, also confirming its possible gene multifunction as pointed in our *in silico* analysis.

Similarly, Singh *et al.* [32] tested transgenic tobacco plants containing a cDNA sequence encoding a TLP isolated from *Arachis diogoi* (wild peanut). Apart from the expected resistance to *Rhizoctonia solani*, transgenic plants showed tolerance to salinity (200-300 mM NaCl) and oxidative stress. Using a relatively straightforward chromatographic procedure, Ye *et al.* [101] isolated a TLP from French bean legume (*P. vulgaris* cv. Kentucky wonder) that had an inhibitory effect on *Fusarium oxysporum*, *Pleurotus ostreatus* and *Coprinus comatus* growth. Recently, using a combination of affinity chromatography, ion-exchange chromatography and gel filtration, Yasmin *et al.* [102] purified a TLP from Basdai banana fruits, which had activity against *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus fumigatus* and *Trichoderma viride*. Interestingly, this protein was also effective against the growth of *Aspergillus phoenicis* and *Aspergillus flavus* in white bread, indicating potential use as a food preservative.

Such results indicated potential applications of TLP in crop improvement through a genetic transformation with applications in both biotic and abiotic stress protection, with strong evidence of its role in the crosstalk between both the stress types, as shown in the present review and suggested by Velázquez *et al*. [103] for different proteins.

CONCLUDING REMARKS AND PERSPECTIVES

Plant TLPs are abundant and highly diversified, but still scarcely explored considering existing omics approaches and available database resources. This is evident for cowpea after application of an *in silico* annotation routine.

Considering the existing evidence, higher plants have many different TLP isoforms that may be activated by biotic or abiotic stresses (or both), as shown for cowpea and other plant species. It indicates that their structure (and not the type of stress or the source species) should be considered as a classification criteria for TLPs. Therefore, the use of terms such as “osmotin” and “zeamatin” is inadvisable.

The phenetic analysis of amino acid sequences reflects functional groups regarding neo-functionalized TLPs after tandem duplications of some genome regions, as confirmed by the clustering of TLPs observed in the diploid Fabaceae species *P. vulgaris*. Considering that most plant species are polyploids (or diploidized polyploids), the redundancy of TLPs within a single genome provides possibilities for the generation of new gene and protein isoforms, by alternative splicing, different expression control systems, and post-transcriptional processing. Therefore, an in-depth analysis of differential expressions in plants (*e.g.*, in model plant species) may lead to a better understanding of the processes involving TLPs in tissues, developmental stages and under different stress types.

Most of the previous biotechnological approaches regarded TLP production aiming to develop flavors for food, and pharmaceutical industry. Regarding plant breeding, most transformed plants targeted resistance to fungi. However, in many cases, other abiotic stress tolerances have been

achieved (*e.g.*, tolerance to drought, salinity, wounding and oxidative stress). In addition, the role of TLPs considering the response to the invasion of other pathogen types (*e.g.*, bacteria, virus or protozoa, as discussed here) needs to be scrutinized.

A targeted analysis of the role of TLPs in molecular networks is needed, especially to understand the role of TLPs crosstalk (as an inducer or induced by other proteins) by associating responses to biotic and abiotic stresses. In addition, the identification of associated transcription factors and promoters may help not only in the understanding of this dual role, but also in the design of genetic transformation procedures.

The identification of TLPs in the genome or transcriptome of native, non-crop, non-model plants is still limited. Considering the loss of genetic diversity (genetic erosion) prevalent in crop species (including the loss of individual genes or particular combinations of genes due to intensive breeding and selection), unique isoforms are expected to uncultivated tropical taxa, whereas differences may exist when comparing woody plants with long generation times to herbs and annual herbs.

It is clear that genes coding proteins containing the thaumatin domain are valuable candidates for plant breeding with marker-assisted selection, and for the generation of transformed plants. Despite the potential successful use of TLPs - especially considering some results of multifunctional benefits under both biotic and abiotic stresses -, to our knowledge, no transgenic plant expressing TLPs is currently commercially available.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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