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THIAGO FERNANDES MARTINS

IDENTIFICAÇÃO, CARACTERIZAÇÃO *IN SILICO* E ANÁLISE DA EXPRESSÃO DE MICRORNAS DE FEIJÃO-DE-CORDA (*Vigna unguiculata* [L.] WALP.) EM RESPOSTA AO VÍRUS DO MOSAICO SEVERO DO CAUPI

FORTALEZA
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Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Doutor em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. José Tadeu Abreu de Oliveira.
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Aprovada em: 28/02/2020.

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“A Deus, por me ceder tudo que há de bom em mim, sem Ele nada de bom posso fazer.
À minha mãe, Roselia Fernandes Martins, pelas orações e por não medir esforços em tornar essa caminhada mais fácil.
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"*Feliz é o homem que encontra sabedoria e o homem que adquire entendimento. Porque sua mercadoria é melhor do que mercadoria de prata, e seu lucro que o fino ouro. Ela é mais preciosa que os rubis, e todas as coisas que possas desejar não se comparam a ela*" (Pv. 3.13-15).

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“Para tudo há um tempo, e um tempo para todo o propósito debaixo do céu. Ec. 3.1”

RESUMO

Vários estresses bióticos afetam a produção e o rendimento do feijão-de-corda. O CPSMV se destaca por causar graves impactos negativos na cultura. As plantas têm dois principais sistemas imunológicos induzidos. No sistema basal (PTI, imunidade desencadeada por PAMP), as plantas reconhecem e respondem a padrões moleculares conservados associados a patógenos (PAMPs). O segundo tipo (ETI, imunidade desencadeada por Effector) é induzido após o reconhecimento da planta de fatores específicos de patógenos. O silenciamento de RNA é outro importante mecanismo de defesa nas plantas. Nosso grupo de pesquisa tem usado abordagens bioquímicas e proteômicas para aprender quais proteínas e vias estão envolvidas e poderia explicar por que alguns genótipos de feijão-de-corda são resistentes, enquanto outros são suscetíveis ao CPSMV. Este estudo atual foi conduzido para determinar o papel do miRNA do feijão caupi na interação entre um genótipo resistente do feijão caupi (BRS-Marataoã) e o CPSMV. Sequências de microRNAs vegetais previamente identificadas e depositadas foram usadas para descobrir todos os possíveis microRNAs no genoma do feijão-de-corda. Essa pesquisa detectou 617 microRNAs maduros, distribuídos em 89 famílias de microRNAs. Em seguida, 4 desses 617 miRNAs e seus possíveis genes-alvo que codificam as proteínas Kat-p80, DEAD-Box, GST e SPB9, todos envolvidos na resposta de defesa do feijão-de-corda ao CPSMV, tiveram sua expressão comparada entre as folhas de feijão-de-corda não inoculadas e inoculado com CPSMV. Além disso, a expressão diferencial de genes que codificam as proteínas Argonauta (AGO) 1, 2, 4, 6 e 10 é relatada. Em resumo, os miRNAs estudados e os genes associados à AGO2 e AGO4 apresentaram padrões de expressão diferenciais em resposta ao desafio do CPSMV, o que indica seu papel na defesa do feijão-de-corda.

Palavras-chave: Feijão-de-corda. CPSMV. MicroRNAs. Mecanismo de defesa.

ABSTRACT

Several biotic stresses affect cowpea production and yield. CPSMV stands out for causing severe negative impacts on cowpea. Plants have two main induced immune systems. In the basal system (PTI, PAMP-triggered immunity) plants recognize and respond to conserved molecular patterns associated with pathogens (PAMPs). The second type (ETI, Effector-triggered immunity) is induced after plant recognition of specific factors from pathogens. RNA silencing is another important defense mechanism in plants. Our research group has been using biochemical and proteomic approaches to learn which proteins and pathways are involved and could explain why some cowpea genotypes are resistant whereas others are susceptible to CPSMV. This current study was conducted to determine the role of cowpea miRNA in the interaction between a resistant cowpea genotype (BRS-Marataoã) and CPSMV. Previously identified and deposited plant microRNA sequences were used to find out all possible microRNAs in the cowpea genome. This search detected 617 mature microRNAs, which were distributed in 89 microRNA families. Next, 4 out of these 617 miRNAs and their possible target genes that encode the proteins Kat-p80, DEAD-Box, GST, and SPB9, all involved in the defense response of cowpea to CPSMV, had their expression compared between cowpea leaves uninoculated and inoculated with CPSMV. Additionally, the differential expression of genes that encode the Argonaute (AGO) proteins 1, 2, 4, 6, and 10 is reported. In summary, the studied miRNAs and AGO 2 and AGO4 associated genes showed differential expression patterns in response to CPSMV challenge, which indicate their role in cowpea defense.

Keywords: Cowpea. CPSMV. microRNAs. Defense mechanism.

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LISTA DE ABREVIATURAS E SIGLAS

BLAST	Ferramenta de busca de alinhamento local.
BLASTN	Ferramenta de busca de alinhamento local em bancos de dados de nucleotídeos usando uma consulta de nucleotídeos.
BLASTX	Ferramenta de busca de alinhamento local em bancos de dados de proteínas usando uma consulta de nucleotídeo traduzida.
cDNA	DNA complementar.
CDS	Sequência codificante.
CPSMV	Cowpea severe mosaic virus.
Ct	Cycle threshold.
dNTP	Desoxirribonucleotídeo trifosfato.
dsRNAs	RNA de fita dupla.
EST	Expressed Sequence Tag.
ETI	Effector-Triggered Immunity.
GSS	Genome Survey Sequences.
HAMP	Herbivore-Associated Molecular Pattern.
HR	Hypersensitive Response.
MAMP	Microbe-Associated Molecular Patterns.
MFE	Minimum Free Energy.
MFEI	Minimum Free Energy Index.
miRNA	Micro RNA.

miRNA*	Micro RNA complementar.
NBS-LRR	Nucleotide Binding Site-Leucine Rich Repeat.
NCBI	Centro nacional de informação biotecnológica.
ncRNA	RNA não codificante.
NGS	Next Generation Sequencing.
PAMP	Pathogens-Associated Molecular Patterns.
PCD	Programmed Cell Death.
PPFD	*Photosynthetic Photon Flux Density.*
PRR	Pattern Recognition Receptors.
PTGS	Post-Transcriptional Gene Silencing.
PTI	Pathogen-Triggered Immunity.
RISC	RNA-Induced Silencing Complex.
RT-PCR	Transcrição reversa seguida da reação em cadeia da polimerase.
RT-qPCR	Transcrição reversa seguida da reação em cadeia da polimerase quantitativa.
sRNA	pequeno RNA.
ssRNA	RNA de fita simples.
TGS	Transcriptional Gene Silencing.
UPE	Unpaired Energy.
UTR	Região não codificante.
VAMP	Viral-Associated Molecular Patterns
vsiRNAs	virus-derived siRNAs.

SUMÁRIO

1 INTRODUÇÃO

O feijão-de-corda (*Vigna unguiculata* [L.] Walp.) é uma das leguminosas mais importantes da África, Ásia e América do Sul (GONÇALVES et al., 2016), onde é cultivado para atender às necessidades alimentares e econômicas de grande parte da população. O continente africano é responsável por mais de 95% da produção mundial de feijão caupi (SILVA; ROCHA; JÚNIOR, 2016). Como alimento, os consumidores usam folhas de feijão-de-corda, vagens verdes e feijão verde e seco para preparar uma variedade de refeições.

Em todo o mundo, graves perdas na produção e no rendimento do feijão-de-corda são recorrentes devido a estresses abióticos, principalmente secas e hipersalinidade e doenças causadas por patógenos como vírus, bactérias, fungos, nematoides e várias pragas, como insetos herbívoros. O feijão-de-corda pode ser infectado por mais de 140 vírus, mas apenas cerca de 20 têm a distribuição mais difundida. Dentre estes vírus está o CPSMV (vírus do mosaico severo do caupi), que pertente à família Comoviridae, gênero Comovirus. Sua importância na redução da produção e do rendimento do feijão-de-corda reside na gravidade da doença que causa (BOOKER; UMAHARAN; MCDAVID, 2005).

Nas plantas, dois principais sistemas imunológicos inatos interconectados são induzidos após percepção de um ataque de patógenos ou pragas. No mecanismo de defesa basal, as plantas respondem aos seus inimigos ativando a PTI (do inglês, *pathogen-triggered immunity*) ou imunidade induzida por padrões moleculares associados a patógenos ou micróbios (PAMPs/MAMPs) ou ETI (do inglês, *effector-triggered immunity*) ou imunidade associada ao reconhecimento, pela planta, de moléculas específicas próprias de patogênicos ou pragas, denominadas efetores, resultantes da evolução, cuja função é de tentar suprimir a PTI. Receptores de reconhecimento (PRRs) detectam e interagem com PAMPs/MAMPs e HAMPs, que são moléculas conservadas em vários grupos de patógenos, micróbios e insetos (BASU; VARSANI; LOUIS, 2018; HAN, 2019; TANAKA et al., 2014; YU et al., 2017). Na ETI, genes de resistência ou genes R (do inglês, *Resistance*) da planta são expressos, gerando como produtos as proteínas intracelulares de resistência ou proteínas R, agrupadas na família NBS-LRR (do inglês, *Nucleotide Binding site-*

Leucine Rich Repeat), que podem interagir direta ou indiretamente com os efetores dos patógenos (proteínas Avr), anulando sua ação (JONES; DANGL, 2006).

O silenciamento de RNA é outro processo de defesa importante que as plantas empregam para contra-atacar infecções por vírus de DNA e RNA (HUANG et al., 2019; ZHU et al., 2019). Na defesa baseada no silenciamento de RNA, a replicação do RNA viral induz a síntese de RNAs de fita dupla (dsRNAs). Os dsRNAs são, então, processados por proteínas do tipo dicer (Dicer RNase) para gerar pequenos RNAs (sRNAs) denominados de pequenos RNAs interferentes derivados de vírus (vsiRNAs), que interagem com as proteínas Argonauta (AGO) e formam complexos silenciadores induzidos (complexos AGO-RISC) que interagem com RNAs virais complementares, levando à inativação por fragmentação (PTGS, do inglês *Post-Transcriptional Gene Silencing*) ou inibição da tradução (TGS, do inglês *Transcriptional Gene Silencing*). Alternativamente, as AGOs podem associar-se a pequenos RNAs endógenos de plantas (sRNAs) para regular a expressão do gene hospedeiro que promove a imunidade das plantas contra vírus. Portanto, pode-se deduzir que as AGOs são componentes-chaves na defesa antiviral (HUANG et al., 2019; ZHU et al., 2019). A imunidade baseada no silenciamento de RNA também exibe respostas cruzadas com os genes R que codificam as proteínas NBS-LRR (proteínas R) associadas ao ETI (ZHU et al., 2019). Atualmente, vários processos celulares como desenvolvimento de plantas e mecanismos de defesa contra estresses bióticos são regulados por pequenos RNAs (sRNAs) (YANG; HUANG, 2014).

MicroRNAs (miRNAs) são pequenos RNAs (sRNAs) de 19 a 24 nucleotídeos, não codificantes, que promovem a inativação da expressão gênica por: (1) silenciamento pós-transcricional (PTGS), quando há clivagem de transcritos (mRNA) guiada por miRNA; (2) inibição da tradução mediada por miRNA (TGS); ou (3) inativação promovida por pequenos RNAs interferentes em fases [do Inglês, *Phased small interfering RNAs (pha-siRNAs ou phasiRNAs)*], que são um tipo de siRNAs secundários oriundos da clivagem de transcritos de RNAs, mediada por miRNAs, que aumentam a eficiência do silenciamento ou que, simultaneamente, inativam múltiplos genes alvos (DENG et al., 2018). Além de PTGS, os miRNAs podem induzir a inativação de genes codificantes por metilação do DNA dependente

de RNA (*RNA-dependent DNA methylation* – RdDM), tanto em plantas como em animais (ROGERS; CHEN, 2013).

Nosso grupo de pesquisa vem trabalhando no patossistema feijão-de-corda x CPSMV usando abordagens bioquímicas e proteômicas para descobrir quais proteínas e vias poderiam estar envolvidas e poderiam explicar a existência do fenótipo de resistência ou de suscetibilidade dos genótipos do feijão-de-corda ao CPSMV. Para dar continuidade a esta linha de pesquisa e alcançar seus objetivos, o presente estudo avaliou o papel de miRNAs do feijão-de-corda na interação incompatível entre um genótipo resistente do feijão-de-corda e o CPSMV.

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2 FUNDAMENTAÇÃO TEÓRICA

2.1 Feijão-de-corda

O feijão-de-corda, como é conhecido popularmente nas regiões Norte e Nordeste, possui outros sinônimos, tais como feijão-macassar e feijão-caupi nas regiões Sudoeste e Centro-Oeste do Brasil. É uma cultura pertencente ao grupo das dicotiledôneas, sendo classificada na ordem Fabales, família Fabaceae, subfamília Faboideae, tribo Phaseoleae, subtribo Phaseolineae, gênero *Vigna* e espécie *Vigna unguiculata* (L.) Walp. (SMARTT, 1990). Alguns estudos apontam o feijão-de-corda como uma cultura de origem africana, cuja introdução no Brasil se deu na metade do século XVI, pelos colonizadores portugueses no estado da Bahia, sendo, posteriormente, disseminado por todo o Brasil (FREIRE FILHO, 1988; PADULOSI; NG, 1997). O continente africano é a maior região produtora de feijão-de-corda do mundo. Nigéria, Niger e Burkina Faso são os três principais países produtores da cultura e juntos perfazem 83% da produção global (FAO, 2019). O Brasil, na safra de 2017/18, teve uma área de 1.020,4 mil hectares ocupada com a cultura de feijão-de-corda, produzindo 594,6 mil toneladas do grão (CONAB, 2018).

A cultura se destaca por apresentar grande importância socioeconômica, sendo uma das principais leguminosas produzidas no Brasil. Seu cultivo está concentrado nas regiões norte e nordeste, sendo esse fato atribuído à capacidade de adaptação a temperaturas elevadas (20-35 °C) e solos com pouca água (EHLERS; HALL, 1997; FILHO et al., 2011).

As folhas do feijão-de-corda possuem grande diversidade de micronutrientes (cálcio, magnésio, sódio, potássio, fósforo, ferro, zinco, manganês, cobre e selênio) e conteúdo proteico elevado, cerca de 15 a 17%, sendo de grande utilidade na dieta humana e animal no Brasil e continentes africano e asiático (SINGH; CHAMBLISS; SHARMA, 1997; WAWIRE et al., 2012). Afiukwa et al., (2013) analisaram 101 genótipos da espécie (*V. unguiculata*) e verificaram variação de 15 a 38% no conteúdo proteico das sementes. Além de alto teor proteico de boa qualidade, as sementes são fontes de grandes quantidades de fibras alimentares, carboidratos, vitaminas e minerais, podendo prevenir doenças nutricionais crônicas. Dois dos principais motivos que levam a maior utilização desta leguminosa, dentre outras espécies da família, são: alto valor nutritivo e baixo custo de produção (FROTA; SOARES; ARÊAS, 2008).

A produtividade do feijão-de-corda é prejudicada por diversos fatores bióticos (vírus, bactérias, fungos, nematoides e insetos pragas) e abióticos (escassez de chuvas, alta salinidade do solo, excesso de incidência solar, por exemplo (GOUFO et al., 2017; MELO et al., 2018; RIVAS et al., 2016). Dentre os fatores bióticos, os vírus compõem o principal grupo de patógenos que causam perdas agrícolas na produtividade do feijão-de-corda, possuindo 8 espécies distribuídas em 6 gêneros: vírus do mosaico do caupi (CPMV, *Comovirus*) (FAN et al., 2011); vírus do mosaico severo do caupi (CPSMV, *Comovirus*) (ABREU et al., 2012); vírus do mosaico do caupi transmitido pelo pulgão (CABMV, *Potyvirus*) (BARROS, 2010); vírus do mosaico da *blackeye* do caupi (BICMV, *Potyvirus*) (TAIWO et al., 2007); vírus do mosaico dourado do caupi (CGMV, *Germivirus*) (JOHN et al., 2008); vírus do mosaico do pepino (CMV, *Cucumovirus*) (HU et al., 2012); vírus do mosqueado clorótico do caupi (CCMV, *Bromovirus*) (ALI; ROOSSINCK, 2008) e vírus do mosqueado do caupi (CPMMV, *Carmovirus*) (BRITO et al., 2012), tendo o CPSMV a maior representatividade no Brasil.

2.2 *Vírus do mosaico severo do caupi* (CPSMV)

De acordo com o Comitê Internacional de Taxonomia de Vírus (ICTV, 2012), o CPSMV é um vírus pertencente à ordem Picornavirales, família Secoviridae, subfamília Comovirinae, gênero *Comovirus* e espécie (FAN et al., 2011); Vírus do mosaico severo do caupi. A espécie teve seu genoma sequenciado em 1992, por Chen e Bruening (1992a, 1992b). O CPSMV possui RNA (positivo) bipartido, denominados RNA 1 e RNA 2, como material genético, sendo desprovido de envelope viral. O capsídeo viral possui morfologia icosaédrica de 28 nm e encapsula ambas as moléculas de RNA pertencente à espécie (DARAI, 2002). Duas características inerentes aos vírus da subfamília Comovirinae é a poliadenilação da extremidade 3' em ambas as fitas de RNA, além da presença de uma proteína denominada VPg (do inglês, *Viral genome-linked protein*) ligada à extremidade 5'.

O RNA 1 (5'→3') do CPSMV é formado por 5957 pares de bases que codificam uma poliproteína de 1858 resíduos de aminoácidos. Após sofrer processamentos pós-traducionais, a poliproteína dará origem a cinco proteínas importantes para a replicação viral, sendo estas, um cofator da protease, uma helicase, uma VPg, uma protease e uma RNA polimerase RNA-dependente (CHEN; BRUENING, 1992b; FAN et al., 2011). O RNA 2 (5'→3') é constituído por 3732 pares de bases que codificam uma poliproteína de 1002 resíduos de aminoácidos composta por uma proteína de movimento célula a célula e duas subunidades proteicas, uma maior (~41 kDa) e uma menor (~21 kDa) formadoras da capa viral (CHEN; BRUENING, 1992b).

O processo de replicação do CPSMV, como todos os vírus de RNA positivo que infectam plantas, se dá após a penetração do vírus na célula do hospedeiro onde, no ambiente intracelular, o material genético é colocado para fora do capsídeo icosaédrico. Em seguida, o RNA (+) será transcrito pela RNA polimerase RNA-dependente viral em uma fita complementar (-) que servirá de molde para a síntese de inúmeras cópias da fita senso (LALIBERTÉ; SANFAÇON, 2010). Esse processo ocorre devido à interação dos ribossomos do hospedeiro com a VPg ligada na

extremidade 5’ do RNA viral, dando início à tradução e, consequentemente síntese das poliproteínas virais.

Em 1947, o primeiro relato da ocorrência do CPSMV no Brasil, na região Sul do país, foi publicado. Posteriormente, no ano de 1977, sua incidência no nordeste brasileiro foi descrita, e, desde então, sua presença tem sido relatada em todas as regiões produtoras do feijão-de-corda no país, ocasionando prejuízos aos produtores da cultura (LIMA; NELSON, 1977; LIMA; SITTOLIN; LIMA, 2005; OLIVEIRA, 1947). Os sintomas severos da doença causada pelo CPSMV são observados no limbo foliar, apresentando bolhosidade associadas a manchas cloróticas, mosaico severo, lesões necróticas pontuais, mal desenvolvimento das nervuras principais, alteração no formato e tamanho do limbo. Suas sementes apresentam deformidades e baixa taxa de germinação, e, em alguns casos, quando as plantas infectadas são jovens, podem apresentar nanismo (PIO-RIBEIRO; ASSIS-FILHO, 2005; ZERBINI-JR; CARVALHO; ZAMBOLIM, 2002).

Algumas estratégias são utilizadas na tentativa de controle do patógeno. A eliminação de plantas de feijão-de-corda infectadas de cultivos anteriores, bem como o combate a insetos vetores por meio do uso de agrotóxicos embora, esta prática, tenha limitação tanto econômica quanto ecológica (PEREIRA, 2006). Atualmente, a opção mais econômica e ambientalmente mais favorável do controle do CPSMV se dá pelo cultivo de genótipos resistentes ou imunes ao vírus, a exemplo do genótipo denominado Macaibo, que foi identificado como sendo imune ao CPSMV, estando essa imunidade associada a uma característica monogênica recessiva (CAMARÇO et al., 2009; LIMA et al., 2012).

2.3 Mecanismos de defesa vegetal contra patógenos

De maneira geral, as interações entre plantas e patógenos podem ser classificadas em dois tipos: compatível, onde o organismo tem sucesso em estabelecer a infecção; e incompatível, na qual o organismo não consegue se desenvolver na planta alvo. Alguns fatores contribuem para a incompatibilidade dessa relação, sendo estes as barreiras constitutivas, que são subdivididas em bioquímicas e/ou físicas (fenóis, alcaloides, glicosídeos, fototoxinas, inibidores de proteases, cutícula, estômatos, tricomas e vasos condutores), que limitam o acesso dos

organismos patogênicos às células vegetais e as barreiras pós-formadas ou induzidas, que, da mesma forma, são subdivididas em bioquímicas e/ou físicas, tais como fitoalexinas, espécies reativas de oxigênio (EROs), PR-proteínas (do inglês, *Pathogenesis-Related Proteins*), lignificação, camada de abscisão, tiloses, sendo necessário, inicialmente, o reconhecimento do patógeno pela planta e, consequentemente, indução de diversos sinais que resultam na resposta de defesa pós-formada (YEATS; ROSE, 2013).

Os mecanismos de defesa induzidos nas plantas podem ser divididos em dois tipos principais. O primeiro se constitui na linha de defesa imune inata, basal, sendo denominada de “imunidade induzida pelo patógeno” ou PTI (do inglês, *Pathogen-Triggered Immunity*), que é desencadeada após o reconhecimento extracelular de padrões moleculares conservados, associados aos patógenos (PAMP, do inglês, *Pathogens-Associated Molecular Patterns*) ou a micróbios (MAMPs, *Microbe-Associated Molecular Patterns*) ou a vírus (VAMPs, *Viral-Associated Molecular Patterns*). Estes padrões são estruturas essenciais à sobrevivência destes organismos e são conservados entre os patógenos. São exemplos de MAMPs bacterianos a Flagelina (Flg; flg22), o Fator de elongação TU (EF-Tu; elf18/26), Peptidoglicanano (PGN), Lipopolissacarideo (LPS), Proteínas bacterianas de choque frio (motivo RNP1), Superóxido dismutase bacteriana (SOD) e Ativador de XA21 (Ax21). Em relação às MAMPs de fungos e oomicetos, há a quitina e β-Glucanos. Estes MAMPs são detectados por receptores correspondentes de reconhecimento de padrões moleculares associados a patógenos (PAMPs) ou, abreviadamente, PRRs (do inglês, *Pattern Recognition Receptors*) presentes nos hospedeiros (NEWMAN et al., 2013). Após o reconhecimento desses padrões por proteínas PRRs, cascatas sinalizadoras são ativadas na tentativa de conter a infecção. Eventos a jusante incluem sinalização mediada por íons Ca^{2+}, explosão oxidativa mediada por EROs e espécies reativas de nitrogênio (ERN), bem como ativação de rotas metabólicas que culminam na expressão de genes codificadores de proteínas-PR (NICAISE; ROUX; ZIPFEL, 2009; STOTZ et al., 2014).

Analisando essas respostas, é possível observar que a interação planta-patógeno é bastante dinâmica. Resultou da co-evolução de plantas e patógenos num modelo tipo ping-pong. Também denominada de “corrida armamentista”, essa co-

evolução permitiu que alguns patógenos, no decorrer de sua história evolutiva, passassem a sintetizar moléculas efetoras, chamadas de proteínas de avirulência ou Avr (do inglês, *Avirulence proteins*) capazes de suprimir a PTI e, dessa forma, obter sucesso na infecção (ANDERSON et al., 2010). As plantas, por sua vez, em resposta à evolução desses patógenos co-evoluíram e passaram a exibir um tipo de mecanismo de defesa denominado imunidade induzida por efetores ou ETI (do inglês, *Effector-Triggered Immunity*). Neste mecanismo, genes de resistência ou genes R (do inglês, *Resistance*) da planta são expressos, gerando como produtos as proteínas de resistência ou proteínas R, agrupadas na família NBS-LRR (do inglês, *Nucleotide Binding site-Leucine Rich Repeat*). As NBS-LRR podem interagir direta ou indiretamente com os efetores dos patógenos (proteínas Avr), neutralizando sua ação (JONES; DANGL, 2006). A ETI, responsiva ao reconhecimento dos efetores pelas proteínas Avr, é caracterizada pelo rápido reestabelecimento e amplificação das vias transcricionais de defesa basal da PTI, levando à resposta hipersensitiva ou HR (do inglês, *Hypersensitive Response*), seguida, eventualmente, com a morte celular programada ou PCD (do Inglês *Programmed Cell Death*) no local da tentativa da infecção (CUI; TSUDA; PARKER, 2015).

Entretanto, recentemente, estudos têm demonstrado que a regulação de diversos processos celulares são realizadas por pequenos RNAs (sRNA ou do Inglês, small RNAs) e, dentre esses processos, estão mecanismos de defesa a patógenos e desenvolvimento normal das plantas, que envolvem a intervenção destes sRNA (YANG; HUANG, 2014).

2.4 Aspectos gerais dos microRNAs

Os microRNAs (miRNA) compõem uma grande classe de sRNA. São ubíquos na natureza, sendo moléculas de fita simples não-codificadoras, formadas por, aproximadamente, 19-22 nucleotídeos (nt) (CHEN et al., 2014; HAAR et al., 2015; LUDWIG et al., 2016; MEGRAW et al., 2016). São moléculas evolutivamente conservadas, possuindo papel de grande relevância na regulação gênica em plantas e animais (LUDWIG et al., 2016; LUO; GUO; LI, 2013). Os miRNAs podem modular a expressão gênica por meio dos seguintes mecanismos: indução de modificações na

cromatina; repressão da tradução de seus alvos; ou indução da degradação do mRNA. Neste último caso, é necessário que o pareamento completo do miRNA com o mRNA alvo ocorra. Esse elevado padrão de complementariedade é mais observado em plantas, enquanto que ,em animais, esta alta complementariedade é menos observada (AXTELL; WESTHOLM; LAI, 2011).

Os primeiros miRNAs relatados na literatura foram lin-4 e let-7, ambos descritos como reguladores do desenvolvimento larval do nematoide *Caenorhabditis elegans* (LEE; FEINBAUM; AMBROS, 1993; REINHART et al., 2000). Em plantas, os miRNAs tiveram sua primeira descrição em *Arabidopsis thaliana* (PARK et al., 2002). Eles concluíram que essas moléculas desempenham papel fundamental no desenvolvimento vegetativo da planta. Até o presente momento, no banco de dados especializado miRBase (http://www.mirbase.org/) estão depositadas 6.965 sequências de precursores de miRNAs e 8.495 sequências de miRNAs maduros distribuídos em 73 espécies de plantas.

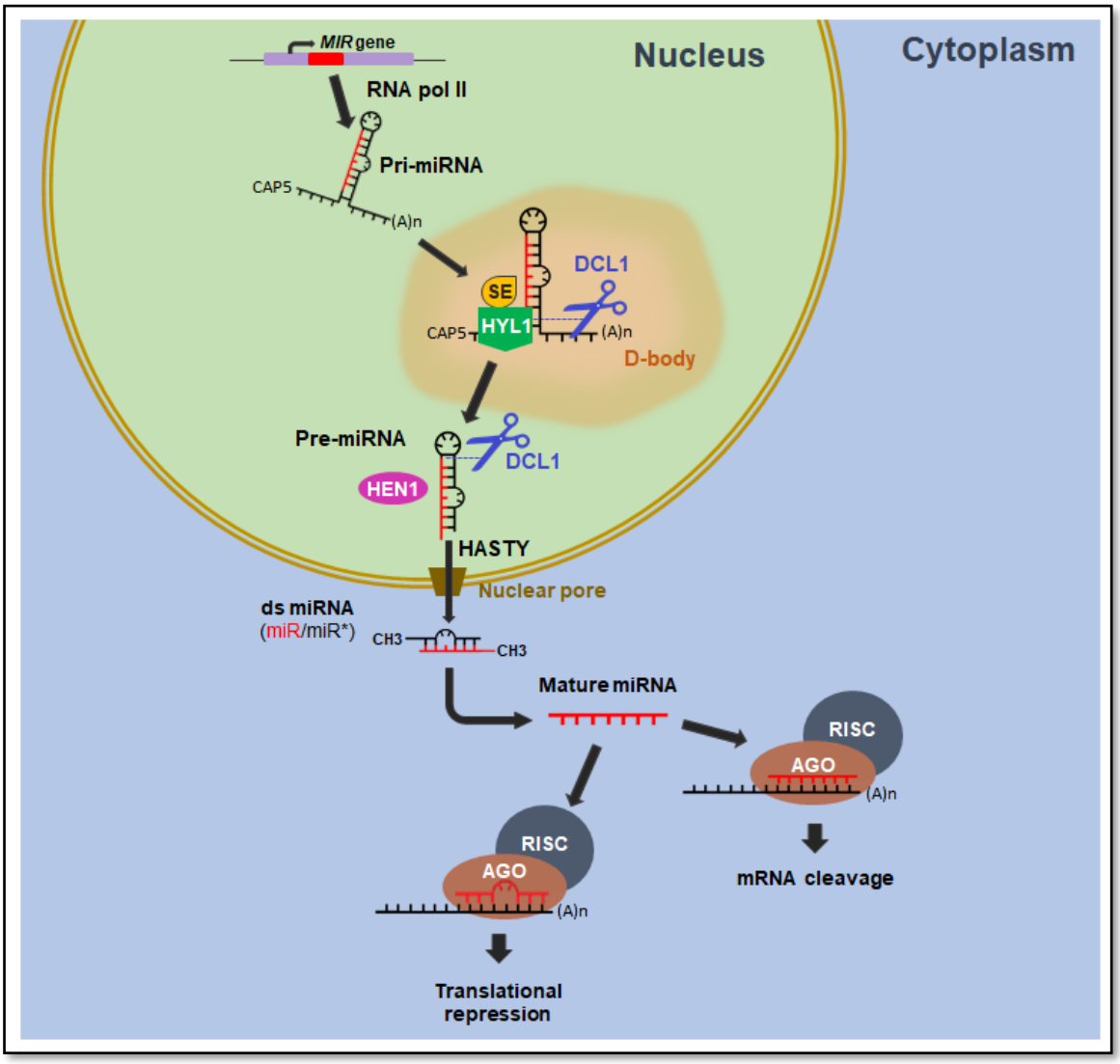
Na maioria das plantas, segundo Nozawa e colaboradores (2012), existem, aproximadamente, mais de 100 genes codificadores de miRNA (*MIR*) distribuídos em regiões intergênicas. Em plantas, a maioria dos genes de miRNA são monocistrônicos, mas, raramente, em algumas famílias de miRNAs, há transcrição em unidades policistrônicas (MERCHAN et al., 2009). A transcrição de genes codificadores de miRNAs em plantas é um processo controlado por diversos fatores de transcrição e, também, modificações da cromatina (metilação de DNA e modificação de histonas), semelhante à transcrição de genes codificadores de proteínas (MENG et al., 2011). Esta dinâmica de expressão pode ser modulada na planta mediante estresses bióticos e abióticos (KHAKSEFIDI et al., 2015).

2.5 Biogênese dos microRNAs

A síntese dos miRNAs pode ser dividida, didaticamente, em: transcrição, que ocorre no núcleo; transporte da molécula precursora para o citoplasma; e, subsequentemente, processamento e maturação (Figura 1) (AXTELL, 2013). Uma RNA polimerase do tipo II (RNA pol-II) da planta é requerida para realizar a transcrição e originar moléculas de miRNAs primário (pri-miRNA). Após sua síntese, ainda no

núcleo, os pri-miRNA são estabilizados por proteínas Dawdle (DDL), e apresentam estruturas secundárias em forma de grampo (*hairpin*), imperfeitas, contendo uma cauda poli-A na extremidade 3', bem como o *cap* 5'. Posteriormente, no centro de processamento nuclear denominado corpo-D (do inglês, *D-body*) são convertidos em pré-miRNAs, que contêm duas fitas fortemente ligadas, formando apenas um grampo, pela ação de uma RNA polimerase tipo III denominada de *Dicer-Like 1* (DCL 1) (VOINNET, 2009). Ainda no núcleo, os pré-miRNAs são processados pela interação físico-química das proteínas DCL 1, HYL 1, da proteína SE que contém o domínio "*C_2H_2 zinc finger*", e do CBC (do inglês, *cap-binding complex*). O pré-miRNA, assim processado, forma um duplex de miRNA que consiste de uma fita guia (miRNA) e sua fita complementar denominada de fita passageira complementar (miRNA*). Após formado, o duplex miRNA: miRNA* contém dois nucleotídeos não pareados na extremidade 3' em ambas as fitas. Esse duplex pode sofrer degradação por exonucleases do tipo SDN (do inglês, *Small RNA Degradading Nuclease*). Assim, um passo adicional ao processamento do pré-miRNA em plantas é necessário para que o duplex não seja degradado. Uma metil-transferase (HEN 1) é responsável pela estabilização da molécula, ao adicionar um grupamento metil na extremidade 3' de ambas as fitas (BARANAUSKĖ et al., 2015), prevenindo a uridilação das fitas e posterior degradação. O transporte em plantas dos pré-miRNAs (miRNA: miRNA*) para o citoplasma não está totalmente elucidado, mas é descrito o envolvimento de uma proteína de membrana homóloga à exportina-5 em animais, a proteína HASTY (PARK et al., 2005).

Figura 1 – Biogênese de microRNAs vegetal e modo de ação



Fonte: Elaborada pelo autor.

No citoplasma, o pré-miRNAs (miRNA: miRNA*) é processado por uma RNA helicase (SDE3), separando as duas fitas. Embora haja consenso de que após essa separação os miRNA* sejam degradadas e não possuam atividades regulatórias, trabalhos recentes demonstram que essa atividade tem sido subestimada. Posteriormente, os miRNA são, preferencialmente, incorporados ao complexo proteico RISC (do inglês, *RNA-Induced Silencing Complex*) formando o miR-RISC,

que estabelecendo pareamento de bases nucleotídicas com o mRNA alvo mediam o silenciamento do mRNA por meio de proteínas argonauta 1 (AGO 1) presentes no complexo RISC (MAH et al., 2010).

2.6 Defesa vegetal mediada por miRNA

Várias doenças de plantas são causadas por patógenos vegetais específicos, causando grandes perdas de produtividade em culturas de importância agrícola e consequentemente, danos à economia (CERDA et al., 2017). Esses patógenos são vírus, bactérias, fungos e nematoides, que estimulam as plantas hospedeiras a exibirem uma miríade de respostas de defesa (Tabela 1). Entre as moléculas, siRNAs e miRNAs podem ser mobilizados para regular a imunidade da planta após o reconhecimento de PAMPs e outros efetores de patógenos (DALIO et al., 2017).

Tabela 1 – O papel defensivo de vários miRNA contra infecções por patógenos

miRNA	Target	Target general function	Pathogen	Plant	Referece
miR168	AGO1	Involved in RNA-mediated post-transcriptional gene silencing	viruses	*A. thaliana, N. benthamiana*	(BORTOLAMIOL et al., 2007; VÁRALLYAY et al., 2010a)
miR162	DCL1		viruses	*A. thaliana*	(ZHANG et al., 2006)
miR159	P69 and HC-Pro	gene silencing suppressors	viruses	*A. thaliana*	(NIU et al., 2006)
miR167b	P1/HC-Pro	gene silencing suppressor	viruses	*N. benthamiana*	(SIMÓN-MATEO; GARCÍA, 2006a)
miR171a	TGBp1/p25	gene silencing suppressor	viruses	*N. tabacum*	(AI et al., 2011a)
miR166	NPK1	ROS	viruses	*C. papaya*	(ABREU et al., 2014)
miR396	HIRP and CAP	ROS	viruses	*C. papaya*	(ABREU et al., 2014)
miR171b	SCL6-IIa, SCL6-IIb and SCL6-IIc	DNA-binding transcription factor activity	viruses	*O. sativa*	(TONG et al., 2017)
miR393	TIR1	Is involved in the pathway protein ubiquitination	bacteria	*A. thaliana*	(NAVARRO et al., 2006a)
miR393	AFB2 and AFB3	Is involved in the pathway protein ubiquitination	bacteria	*A. thaliana*	(FAHLGREN et al., 2007)
miR399	PHO2	Regulator in phosphate metabolism	bacteria	*Citrus sinensis*	(ZHAO et al., 2013)
miR408	ARPN	electron transfer activity	bacteria	*A. thaliana*	(ZHANG et al., 2011)
miR472	CC-NBS-LRR	involved in the recognition of specialized pathogen effectors	bacteria	*A. thaliana*	(BOCCARA et al., 2014)
miR169o	NF-YA4	Stimulates the transcription of various genes	bacteria	*O. sativa*	(YU et al., 2018)
miR5300	tm-2 protein	ADP binding	fungus	*S. lycopersicum*	(OUYANG et al., 2014)
miR408	CLP1	electron transfer activity	fungus	*Triticum aestivum*	(FENG et al., 2013)
miR2118	TIR-NBS-LRR	involved in the recognition of specialized pathogen effectors	fungus	*Gossypium hirsutum*	(YIN et al., 2012)

miR1447	Disease resistance protein	Nucleotide binding	fungus	*Populus beijingensis*	(CHEN et al., 2012)
miR1448	Glutathione	Glutathione transferase activity	fungus	*Populus beijingensis*	(ZHAO et al., 2012)
miR482	Disease resistance protein	Nucleotide binding	fungus	*Gossypium hirsutum*	(LU; SUN; CHIANG, 2008; YIN et al., 2012)
miR396	GRF		fungus	*A. thaliana*	(SOTO-SUÁREZ et al., 2017)
miR169	NF-YA	Stimulates the transcription of various genes	fungus	*O. sativa*	(LI et al., 2017)
miR161	PPR	binding with specific RNA sequences	nematode	*A. thaliana*	(HEWEZI et al., 2008)
miR164	NAC1	Nucleotide binding	nematode	*A. thaliana*	(HEWEZI et al., 2008)
miR9750	Peroxidase	oxidative stress control	nematode	*G. max*	(TIAN et al., 2017)
miR2119	Zn-binding dehydrogenase	electron transfer activity	nematode	*G. max*	(TIAN et al., 2017)
miR1512	G6PD	electron transfer activity	nematode	*G. max*	(TIAN et al., 2017)

Fonte: Elaborada pelo autor.

Alguns miRNAs vegetais atuam de forma semelhante aos miRNAs de animais, interferindo na tradução do mRNA alvo. Esse silenciamento gênico se dá através da associação do mRNA alvo em uma região do miRNA contendo 6-8 nt, denominada região "*seed"*, localizada na extremidade 5' (LEE; FEINBAUM; AMBROS, 1993). A grande maioria dos alvos da região "*seed*" está localizada na porção não codificante (UTR, do inglês, *Untranslated Region*) na extremidade 3' do mRNA alvo (KUME et al., 2014). Outro modo de regulação da expressão gênica em plantas por miRNA, e o mais comum no reino vegetal, é através de sua elevada complementariedade com o mRNA alvo, levando à inativação deste último por clivagem (GERMAN et al., 2008).

O primeiro estudo demonstrando o envolvimento de miRNAs na defesa vegetal foi realizado por Navarro e colaboradores (2006). Eles relataram que plantas de *Arabidopsis thaliana* respondem com aumento da expressão do miR393 após o reconhecimento da presença do elicitor flagelina (proteína bacteriana). Como consequência, as plantas diminuíram a expressão de mRNAs codificadores de receptores de auxina (TIR1, AFB1, AFB2 e AFB3). As plantas com menor sensibilidade à auxina apresentaram fenótipo de resistência mais elevada à bactéria *Pseudomonas syringae* (NAVARRO et al., 2006b). Em alguns casos, a regulação positiva de miRNAs não é favorável para as plantas. Recentemente, Salvador-Guirao e colaboradores (2018) relataram que a redução no miR773, responsável por controlar a expressão de uma metiltransferase 2 (MET2), é crucial para a resistência das plantas a vários patógenos fúngicos. Da mesma forma, dois miRNAs (nta-miR6019 e nta-miR6020) foram encontrados em *Nicotiana benthamiana*, que regulam negativamente a expressão dos genes que codificam TIR-NBS-LRR, estando envolvidos na suscetibilidade das plantas ao vírus do mosaico do tabaco (TMV) (LI et al., 2012).

2.7 miRNA vegetal e defesa contra vírus

A interação planta-vírus é estudada desde 1898 (BEIJERINCK, 1898). Por mais de 120 anos, esforços foram direcionados à pesquisa envolvendo o patossistema planta-vírus. Nos últimos 50 anos, o trabalho não tem mais foco em ecologia, mas em abordagens moleculares para essa interação (VAN DER WANT; DIJKSTRA, 2006). Vários estudos relatam o impacto da infecção viral na biogênese e nos miRNAs para

funcionar nas plantas, sugerindo que essas moléculas desempenham papel fundamental na defesa das plantas contra vírus (MAGHULY; RAMKAT; LAIMER, 2014; PERTERMANN et al., 2018).

Os vírus têm uma variedade de configurações de material genético, que podem ser compostas por ssRNA (do inglês, *single-stranded RNA*), dsRNA (do inglês, *double - stranded RNA*), ssDNA ou dsDNA (JOHNSON; ARGOS, 1996). Um dos mecanismos de defesa antiviral das plantas ocorre durante a infecção do hospedeiro por vírus ssRNA. Durante a replicação, o vírus produz moléculas de dsRNA pela RNA polimerase RNA-dependente viral (ISLAM et al., 2017). A fita dupla é um padrão molecular associado ao patógeno viral (VAMP), que é reconhecido pelo sistema de silenciamento de RNA vegetal, iniciando a degradação do RNA viral e produzindo siRNAs pela atividade das proteínas DCL da planta. Os siRNAs derivados de sequências virais (vsiRNA), contendo 21-22 (nt), exibem resposta antiviral específica, sendo suas sequências complementares ao RNA viral alvo, levando à degradação por RISC (GARCIA-RUIZ et al., 2010, 2017). Esta é a via natural da resposta antiviral pela produção de siRNA a partir de dsRNA exógeno, como dsRNA viral. No entanto, em relação à defesa mediada por miRNA, o processo é bem diferente, considerando que os miRNAs são produzidos a partir de genes endógenos presentes no DNA da planta. Nesse caso, a imunidade antiviral mediada por miRNA pode ser direcionada a vírus de DNA e RNA, que são capazes de inserir seu genoma no genoma da planta (BECHER et al., 2014; GHOSHAL; SANFAÇON, 2015). Por exemplo, Becher e colaboradores (2014), realizando o sequenciamento de nova geração (NGS, do inglês, *Next Generation Sequencing*), identificaram que o DNA inserido de um *Caulimovirus* no genoma de *Fritillaria imperiailis* é usado para produzir miRNA empregado na imunidade antiviral de plantas. Isso não é uma surpresa, porque os vírus de DNA normalmente se replicam no núcleo.

Uma informação interessante foi relatada por Gao et al. (2012), relacionada ao acúmulo do vírus do *Hibiscus chlorotic ringspot virus* (HCRSV), um não-retrovírus de RNA no núcleo. O acúmulo de HCRSV no núcleo, por si, foi um achado muito interessante, mas, além disso, os autores relataram a detecção de miRNA viral direcionado à proteína p23 do HCRSV, responsável pela localização nuclear do HCRSV. Os mecanismos por trás da capacidade da planta de produzir miRNA a partir do RNA do HCRSV no núcleo não são entendidos, mas trouxeram mais curiosidade e atenção ao papel exibido pelo miRNA na defesa das plantas à infecção viral.

O sucesso da defesa da planta durante a infecção viral depende da capacidade dela iniciar muitos mecanismos contra o vírus ao mesmo tempo. Ao usar o silenciamento de RNA como a defesa mais importante para o vírus, as plantas podem obter duas vantagens: (1) capacidade de espalhar o siRNA para o tecido sistêmico; e (2) transferência da resistência a células e tecidos não infectados (LU et al., 2008).

Simón-Mateo e García (2006a) relataram a produção de miRNA (miR171, miR167 e miR159) em plantas de *Arabidopsis* em resposta ao *Plum pox virus* (PPV) HC-Pro. Vários estudos mostraram o HC-Pro como alvo geral de muitos miRNA, como pré-miRNA159 e miRNA159a (SIMÓN-MATEO; GARCÍA, 2006a; AI et al., 2011b; GAO et al., 2015), O componente auxiliar da proteinase (HC-Pro) de muitos vírus é uma proteína importante para a infecção viral, devido à sua atividade supressora de silenciamento (GARCIA-RUIZ et al., 2017). Esse fato pode explicar por que muitos miRNA têm como alvo essa proteína. Muitos estudos, ao longo dos anos, mostraram que o supressor de silenciamento viral é um alvo geral do miRNA produzido pelas plantas (JELLY et al., 2012; MITTER et al., 2016; SONG et al., 2014). Esse é um ponto muito interessante, porque o supressor de silenciamento é a molécula viral mais importante envolvida no processo de infecção, com base na capacidade de interromper a defesa da planta.

A proteína P19 de *Tombusvirus* é o supressor de silenciamento mais bem estudado e sabe-se que desempenha papéis críticos nas interações planta-vírus (VÁRALLYAY et al., 2010a). Até o momento, nenhum miRNA envolvido na supressão do P19 foi descoberto. No entanto, tem sido amplamente discutida a capacidade do P19 de se ligar e sequestrar miRNAs, impedindo o carregamento da AGO no complexo RISC e, assim, inibindo sua atividade (VÁRALLYAY et al., 2010b). Um segundo alvo viral comum do miRNA é a proteína de revestimento (CP), uma proteína estrutural envolvida na montagem das partículas virais (SUN et al., 2016; WAGABA et al., 2016). Nesse caso, o vírus pode concluir todo o processo de replicação, mas, no momento da montagem do vírus, a ausência da proteína CP vai causar sua morte.

2.8 Os microRNAs em feijão-de-corda

Utilizando abordagem computacional (análise *in silico*), dados de miRNAs conhecidos de plantas foram analisados utilizando Blastn contra dados de ESTs e

GSS de *V. unguiculata.* Esta busca revelou a presença de 47 miRNAs distribuídos em 13 famílias (LU; YANG, 2010a). Adicionalmente, 30 possíveis genes alvos destas moléculas foram preditos, sendo a maioria codificadora de fatores de transcrição ou de enzimas de grande importância no metabolismo primário da planta (LU; YANG, 2010a).

Atualmente, existem apenas 18 sequências miRNAs identificadas em *V. unguiculata*, depositados no miRBase (http://www.mirbase.org/). Todas as sequências depositadas foram obtidas no estudo de Paul et al. (2011). O estudo foi baseado em uma abordagem genômica comparativa, possibilitando a identificação de 18 miRNAs conservados em *V. unguiculata* e classificados em 16 famílias. Análises de bioinformática revelaram os alvos desses miRNAs, tendo sido possível identificar 15 deles como fatores de transcrição. Também, RT-qPCR foi realizada para validação de sete dos 18 miRNAs identificado. Os miRNAs que foram validados como *up-regulated* em raízes de plantas submetidas ao estresse salino foram: vun-miR156a, vun-miR159b, vun-miR160a, vun-miR162a, vun-miR168a, vun-miR169b e vun-miR408.

Existem alguns poucos genótipos de feijão-de-corda que são resistentes, mas vários que são susceptíveis ao CPSMV. Contudo, poucos relatos, na literatura, explicam os fatores que definem a resistência ou susceptibilidade de genótipos de feijão-de-corda ao CPSMV. Vários destes trabalhos atribuem a herança recessiva como principal fator que tornam alguns genótipos resistentes ao CPSMV. Mas, como já relatado, nenhum destes trabalhos explica os mecanismos de defesa envolvendo miRNAs como mediador de defesa.

Assim, devido à inexistência de trabalhos relacionados ao patossistema feijão-de-corda x CPSMV, com enfoque nos mecanismos de defesa e rotas metabólicas que sofram intervenção de miRNAs, este trabalho tem como proposta investigar o papel dos miRNAs na interação incompatível entre feijão-de-corda (genótipo BRS-Marataoã) e CPSMV.

3 HIPÓTESE

Com base no que há na literatura, sabe-se que os miRNAs estão envolvidos na modulação de diversos processos biológicos, incluindo o envolvimento de alguns deles nos mecanismos de defesa da planta contra estresses bióticos em outras espécies vegetais que não *Vigna unguiculata*.

Diante dessa ausência de qualquer dado referente à participação de miRNAs na defesa do feijão-de-corda ao CPSMV, a seguinte hipótese foi levantada:

Plantas de feijão-de-corda resistentes respondem ativamente à infecção pelo CPSMV expressando, diferencialmente, miRNAs específicos que intervêm, direta ou indiretamente, nos mecanismos de defesa ao vírus.

4 OBJETIVOS

4.1 Objetivo geral

Avaliar se há intervenção de miRNAs de feijão-de-corda nos seus mecanismos de defesa contra o CPSMV.

4.2 Objetivos específicos

- Identificar, *in silico,* candidatos a miRNAs presentes no genoma de *V. unguiculata*;
- Selecionar genes estáveis em feijão-de-corda, adequados para a normalização da RT-qPCR em folhas de *V. unguiculata* inoculadas com CPSMV;
- Analisar o perfil transcricional de 4 miRNAs e seus respectivos alvos, possivelmente envolvidos na defesa do feijão-de-corda (genótipo BRS-Marataoã) contra o CPSMV;
- Avaliar a expressão dos genes de ARGONAUTAS de *V. unguiculata* em plantas não-inoculadas e inoculadas com CPSMV.

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5 ARTIGO REFERENTE À TESE

- **Revista científica**

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Título

Identification, characterization and expression analysis of cowpea (*Vigna unguiculata* [L.] Walp.) miRNA in response to cowpea severe mosaic virus (CPSMV) challenge

Autoria

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Identification, characterization, and expression analysis of cowpea (*Vigna unguiculata* [L.] Walp.) miRNAs in response to cowpea severe mosaic virus (CPSMV) challenge

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Abstract

Key message

Cowpea miRNAs and Argonaute genes showed differential expression patterns in response to CPSMV challenge

Abstract

Several biotic stresses affect cowpea production and yield. CPSMV stands out for causing severe negative impacts on cowpea. Plants have two main induced immune systems. In the basal system (PTI, PAMP-triggered immunity) plants recognize and respond to conserved molecular patterns associated with pathogens (PAMPs). The second type (ETI, Effector-triggered immunity) is induced after plant recognition of specific factors from pathogens. RNA silencing is another important defense mechanism in plants. Our research group has been using biochemical and proteomic approaches to learn which proteins and pathways are involved and could explain why some cowpea genotypes are resistant whereas others are susceptible to CPSMV. This current study was conducted to determine the role of cowpea miRNA in the interaction between a resistant cowpea genotype (BRS-Marataoã) and CPSMV. Previously identified and deposited plant microRNA sequences were used to find out all possible microRNAs in the cowpea genome. This search detected 617 mature microRNAs, which were distributed in 89 microRNA families. Next, 4 out of these 617 miRNAs and their possible target genes that encode the proteins Kat-p80, DEAD-Box, GST, and SPB9, all involved in the defense response of cowpea to CPSMV, had their expression compared between cowpea leaves uninoculated and inoculated with CPSMV. Additionally, the differential expression of genes that encode the Argonaute (AGO)

proteins 1, 2, 4, 6, and 10 is reported. In summary, the studied miRNAs and AGO 2 and AGO4 associated genes showed differential expression patterns in response to CPSMV challenge, which indicate their role in cowpea defense.

Keywords: Cowpea, CPSMV, microRNAs, defense mechanism

5.1 Introduction

Cowpea (*Vigna unguiculata* [L.] Walp.) is one of the most important legumes in Africa, Asia, and South America (Gonçalves et al. 2016), where it has been cultivated to satisfy food requirements and cash needs. The world area harvested and production of dry cowpea in 2017 were 12,577,845 ha and 7,407,924 tons, respectively (FAO 2017). The African continent accounts for more than 95% of the world's cowpea production (Silva et al. 2016). The most important producers are Nigeria (3,409,992 tons), Niger (1,959,082 tons), Burkina Faso (603,966 tons), Tanzania (200,940 tons), Cameroon (198,201 tons), Myanmar (178,582 tons), Kenya (146,342 tons), and Mali (145,018 tons) (FAO 2017). As food, consumers use cowpea leaves, green pods, and green and dry beans.

Worldwide, severe losses in cowpea production and yield is recurrent due to abiotic stresses especially drought and hypersalinity, and diseases caused by pathogens like viruses, bacteria, fungi, nematodes, and treats from various pests like herbivorous insects (Carvalho et al. 2019). Plant pathogens and pests are considered the principal constraints to crop productivity globally (Savary et al. 2019). For instance, cowpea can be infected by over 140 viruses, but only around 20 have the most widespread distribution (Hampton and Thottappilly, 2003). They include BCMV-blackeye cowpea mosaic strain (BCMV-BlCM), Cowpea aphid-borne mosaic virus

(CABMV), Cowpea chlorotic mottle virus (CCMV), Cowpea golden mosaic virus (CGMV), Cowpea mottle virus (CPMoV), Cowpea mosaic virus (CPMV), Cowpea severe mosaic virus (CPSMV), and Southern bean mosaic virus (SBMV) (Hema et al. 2014). CPSMV belongs to the family Comoviridae, genus *Comovirus* and its importance in reducing cowpea production and yield resides in the disease severity it causes (Booker et al. 2005).

In plants, two main interconnected innate immune systems are induced after perception of a pathogen or pest attack. In the basal defense mechanism, plants respond to their enemies by activating PTI (pathogen- or microbe-associated molecular patterns [PAMPs/MAMPs]-triggered immunity) or HTI (Herbivore-Associated Molecular Patterns [HAMPs]-triggered immunity). Plants also detect and interact with DAMPs (Damage-Associated Molecular Patterns) that consist of plant endogenous molecules recognized as danger signals. The other defense mechanism is ETI (effector-triggered immunity), which rely on the recognition of specific pathogen/pest effectors. The pattern-recognition receptors (PRRs) detect and interact with PAMPs/MAMPs and HAMPs, which are molecules conserved in various groups of pathogens, microbes, and insects (Tanaka et al. 2014; Yu et al. 2017; Basu et al. 2018; Han 2019). Effectors are particular molecules produced by pathogens and pests to subvert PTI and to facilitate pathogen colonization and herbivorous feeding (Kachroo et al. 2017; Basu et al. 2018). In retaliation, resistant plants developed specialized resistance proteins (R-proteins) that recognize the presence of specific pathogen effector proteins (Avr) and trigger ETI (Kachroo et al. 2017; Saijo and Loo 2019). R-proteins are ubiquitous in plants and belong to a class of immune receptors designated NLRs because they possess a central nucleotide-binding (NB) domain and

a C-terminal leucine-rich repeat (LRR) domain, as well as a variable N-terminal domain (Kachroo et al. 2017; Feng and Tang 2019).

RNA silencing is other important defense process that plants employ to counterattack DNA and RNA virus infections (Huang et al. 2019; Zhu et al. 2019). In RNA silencing-based defense, replication of viral RNA induces the synthesis of double-stranded RNAs (dsRNAs). dsRNAs are then processed by dicer-like proteins (Dicer RNase) to generate 21- to 24-nucleotide small RNAs (sRNAs), named virus-derived small interfering RNAs (vsiRNAs), which interact with Argonaute proteins (AGO) and form RNA-induced silencing complexes (AGO-RISC complexes). AGO-RISC complexes interact with sequence-specific complementary viral RNAs and leads to inactivation by fragmentation (post-transcriptional gene silencing, PTGS) or translational inhibition (TGS). Alternatively, AGOs can associate with plant endogenous small RNAs (sRNAs) to regulate host gene expression that promotes plant immunity against viruses. Therefore, it can be deduced that AGOs are key components in antiviral defense (Huang et al. 2019; Zhu et al. 2019). RNA silencing-based immunity also exhibits cross-talk with R genes encoding NBS-LRR proteins (R-proteins) associated with ETI (Zhu et al. 2019). Indeed, it has been reported that microRNAs (miRNAs) are involved in R-gene regulation in wheat, tobacco, and cotton, for example (Brant and Budak 2018). Several cellular processes, such as plant development and defense mechanisms against biotic stresses, are regulated by small RNAs (sRNAs) (Yang and Huang 2014). sRNA are 20–30 nucleotide long small non-coding RNAs (ncRNAs), which include the small interfering RNAs (siRNAs), microRNAs (miRNAs), and Piwi-interacting RNAs (piRNAs) (Brant and Budak 2018).

MicroRNAs (miRNAs) are 20–24 nucleotide long single-stranded, non-coding sRNA that promote post-transcriptional gene expression silence (PTGS)

through miRNA-guided transcript cleavage, miRNA-mediated translation inhibition (TGS) or miRNA-triggered biogenesis of phased secondary small interfering RNAs (siRNAs) from their target transcripts (Yu et al. 2017).This latter mechanism being a widespread and conserved mode of action in plants (Rogers and Chen 2013). Additionally to PTGS, miRNAs can induce transcriptional gene silencing by RNA-directed DNA methylation in plants and animals (Rogers and Chen 2013).

In plants, miRNAs were first described in *Arabidopsis thaliana* (Park et al., 2002). To date, 6,965 miRNA precursor sequences (pre-miRNA) and 8,495 mature sequences distributed in 73 plant species are deposited in the miRBase specialized database (http://www.mirbase.org/, access in Jan. 2019).

The first study demonstrating the involvement of miRNAs in plant defense was carried out by Navarro et al. (2006). These researchers showed that after perception and recognition of the *Pseudomonas syringae* flagellin-derived peptide elicitor, *Arabidopsis thaliana* responded (PTI) with increased expression of miR393. This miRNA specifically cleaved and thus negatively regulated the messenger RNAs that are decoded to produce the F-box auxin receptors TIR1, AFB2, and AFB3. Therefore, the absence of these receptors contributes to the antibacterial resistance to *P. syringae* by repressing auxin signaling (Navarro et al., 2006).

Our research group has been working on the pathosystem cowpea x CPSMV using biochemical and proteomics approaches to find out which proteins and pathways are involved and could explain the resistance and susceptibility trait of cowpea genotypes to CPSMV. To give continuity to this research and achieve these objectives, the present study focuses on the role of cowpea miRNA in an incompatible interaction between a resistant cowpea genotype and CPSMV.

5.2 Material and methods

5.2.1 Annotation of genes that encode cowpea proteins responsive to CPSMV

Selected cowpea proteins that changed in abundance upon CPSMV challenge, as previously shown by using proteomics studies (Paiva et al., 2016; Varela et al., 2017), were used to annotate the nucleotide sequences of their corresponding encoding genes in the genome of cowpea.

5.2.2 In silico identification of conserved miRNA precursors

The cowpea genome was downloaded from: https://www.ncbi.nlm.nih.gov/genome/?term=vigna%20unguiculata. The C-mii software (http://www.biotec.or.th/isl/c-mii) was used to identify miRNAs (Numnark et al. 2012). Putative miRNA candidates identified in the cowpea genome were searched against mature miRNAs of Viridiplantae deposited in the miRBase bank (http://www.mirbase.org/) using BLASTN (*e*-value = 10; number of allowed mismatches ≤ 4). A BLASTX search against UniProt and Swiss-Prot protein databases was performed to exclude any mRNA nucleotide sequence that decode into known cowpea protein (E-value ≤ 1e-20). The Rfam database 10 was used to search RNA sequences to predict and annotate for homologues to non-coding RNA (ncRNAs) families. The flanking sequences of the miRNA candidates were retrieved from the genome of cowpea using a window of 200 nucleotides in length. Prediction of the secondary structure of miRNA precursors was done using Mfold (Zuker 2003), with the

following parameters: maximum base pair distance, 3000; maximum interior/bulge loop size, 30; and fixed temperature at 37 °C.

5.2.3 Prediction of possible cowpea genes regulated by miRNAs

Possible target genes for cowpea miRNAs were identified using two predictive schemas. Firstly, putative cowpea miRNA gene targets were identified based on homology by submitting mature miRNA sequences as a query against the *V. unguiculata g*ene sequences, in the DFCI Gene Index (VUGI) project deposited in the server psRNATarget (http://plantgrn.noble.org/psRNATarget/). In the second schema, genes annotated [3'-untranslated region + coding sequence + 5'-untranslated region (3' UTR+CDS+5' UTR)] based on the primary sequences of cowpea proteins that were responsive to CPSMV (Paiva et al., 2016; Varela et al., 2017) were subject to psRNATarget program (http://plantgrn.noble.org/psRNATarget/analysis?function=2) and searched for homologous mature miRNA sequences available in miRBase (http://www.mirbase.org/). The parameters used to predict putative target genes of cowpea miRNAs and the regulatory miRNAs of the specifically selected cowpea target genes that encode the proteins Kat -p80, DEAD-Box, GST, and SPB9, all involved in the defense response of cowpea, were as follows (Dai and Zhao 2018): (1) maximum expectation equal or less than 4.0; (2) length for complementarity scoring (hspsize) shorter than 20 nt; (3) target accessibility-allowed maximum energy to unpair the target site (UPE) shorter than 20; (4) flanking length around target site for target accessibility analysis of 17 bp in upstream and 13 bp in downstream; (5) range of central mismatch that leads to translational inhibition of 9–11 nt; (5) Poor incompatibility at the complementary site equal or less than 4 without gaps.

5.2.4 Prediction of the secondary structures of the studied cowpea miRNAs

First, the predicted miRNA sequences complementary to cowpea genes were aligned with the genome of *V. unguiculata*. The cowpea miRNA sequences that achieved exact or near-exact matches with the genome were retrieved together with 200 nt flanking regions. MFOLD v3.6 (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) was used to predict the secondary structures of cowpea pre-miRNA candidates (Zuker 2003). The following MFOLD web server parameters were adjusted for identification of miRNAs in various plant species as previously published by several researchers: (1) the RNA sequence should fold into hairpin secondary structure with a high-defined stem and terminal loop; (2) the stem of hairpin structure should harbor all potential miRNA sequence; (3) in the predicted miRNA sequence strand (guide strand) and its complementary sequence (miRNA*, passenger strand), in the miRNA duplex, no more than 4 mismatches should be allowed; (4) the predicted secondary structure should be stable with highly-negative (≤ -18.0 kcal mol^{-1}) minimum free energy (MFE) and minimum free energy index (MFEI) ≤ -0.8 kcal mol^{-1} (Biswas et al., 2016; Hussain et al., 2018; Singh et al., 2016). The miRNA precursor sequences passing these criteria were considered potential miRNAs of *V. unguiculata*. The MFE value was obtained after folding the pre-miRNA sequence in the MFOLD web server using a method based on the dynamic programming algorithm of the applied mathematics (Zuker and Stiegler 1981). The following equation was used to calculate MFEI: MFEI = [(MFE/length of the RNA sequence) x 100] / (G+C)%.

5.2.5 Plant material and growth conditions

Cowpea (*Vigna unguiculata* [L.] Walp.) mature seeds, BRS-Marataoã genotype, which is resistant to CPSMV (Filho et al. 2005), were obtained from EMBRAPA Meio Norte (Terezina, Piaui, Brazil). Seeds were surface sanitized by incubation with 1% (v/v) sodium hypochlorite solution (0.05% active chlorine) for 3 min, followed by soaking with distilled water for 1 minute. To break dormancy, seeds were soaked in distilled water for 20 minutes. Seeds without visible damage were placed on two overlapping sheets of Germitest® paper (GERMILAB -A3034-8, 28 cm x 38 cm, neutral pH, Brazil) previously wetted with autoclaved (121 °C, 1.5 x 10^5 Pa, 30 min), sterilized distilled water (2x paper dry weight). The set formed by the two overlapping paper sheets containing the cowpea seeds was rolled over itself and the seeds allowed to germinate in a humid environment close to 100% relative humidity, in the dark, for three days. Next, the seedlings were transferred to 0.8 L pots (3 seedlings per pot) containing river sand previously washed (7x) with tap water followed by autoclaved (121 °C, 1.5 x 10^5 Pa, 30 min) distilled water (1x). The plants were maintained in a greenhouse under a 12 h photoperiod, photosynthetic photon flux density (PPFD) varying from 300 to 650 mmol m^2 s^{-1} (190SA quantum sensor, LI-COR, USA), temperature of 32 ± 2 °C (day) and 27 ± 2 °C (night), and 79.8 ± 10.9% relative humidity. Plants were watered daily with distilled water until the appearance of the primary leaves and next with a nutritive solution (Hoagland and Arnon 1950) modified by Silveira et al. (2001) and diluted (1:10, v/v) with distilled water, until the end of the experimental period.

5.2.6 Inoculum preparation and inoculation of cowpea leaves with CPSMV

The virus inoculums were prepared according to Silva et al., (2016). In brief, leaves were collected from the highly susceptible cowpea genotype CE-31 (syn. Pitiuba) (Lima et al., 2011) infected with $CPSMV_{CE}$, an isolate obtained at Ceara State, Brazil (Lima and Nelson, 1973), thereafter referred as CPSMV. The CPSMV-infected leaves were macerated (1:10, m/v) with 0.010 M sodium phosphate buffer, pH 7.0, containing 0.01% (m/v) sodium bisulfate, and the suspension obtained was mixed (1:10, m/v) with 500-600 mesh carborundum powder, which was used as abrasive. Inoculation of experimental plants (V group) with CPSMV was done manually at 14 days after planting by rubbing the inoculum on the adaxial and abaxial sides of the cowpea (BRS-Marataoã genotype) secondary leaves using the tips of the index finger and thumb with hands protected with surgical gloves (Silva et al. 2016). Mock-inoculated (MI group) control cowpea (BRS-Marataoã genotype) plants received similar treatment, but with inoculums prepared from the CE-31 cowpea leaves not infected with CPSMV. The secondary leaves from 9 cowpea plants from each experimental group (V and MI) and at every studied time point were collected after 0, 1, 2- and 6-days after inoculation (DAI) with CPSMV, immediately frozen in liquid nitrogen, and stored in a freezer at -80 °C until use.

5.2.7 Extraction and purification of total RNA from cowpea leaves

Total RNA was extracted from 100 mg frozen cowpea secondary leaves from V and MI plant groups and purified using the RNeasy Plant mini Kit (Qiagen),

according to the manufacturer's instructions. Total extracted RNA was stored at -20 °C until analysis.

5.2.8 Quantification and integrity analysis of the purified cowpea RNA

RNA quantification was done using Epoch™ Microplate Spectrophotometer (BioTek Instruments, Winooski, USA) by taken absorbance readings at 260 and 280 nm and calculating the A_{260}/A_{280} ratio. RNA integrity was analyzed by electrophoresis in 1.2% (m/v) agarose gel containing ethidium bromide (0.05 µL/mL). Visualization of rRNAs (18S and 28S) was performed using an UV light transilluminator. Photodocumentation was done with the MiniBIS Pro system (Bio-Imaging Systems) and the GelCapture™ software.

5.2.9 Reference gene identification in cowpea for quantitative real-time polymerase chain reaction (qRT-PCR) analyses

5.2.9.1 Analysis of candidate reference cowpea genes and selection for expression validation

A set of nine candidate reference genes was selected: *Act5* (Actin 5); *F-box* (F-box protein); *Insu* (Insulinase); *EF1α2* (Elongation factor 1-alfa-2); *EF1α3* (Elongation factor 1-alfa-3); *EF1 β* (Elongation factor 1 beta); PP2A (Phosphatase 2A); *L23α1*; and *L23α3* (60S ribosomal proteins). These genes are conventionally used as reference in qPCR data normalization, as they have expression stability during *A. thaliana*, *O. sativa*, *G. max*, and *Brassica napus* development and also when these plants are grown under various stress conditions (Lilly et al., 2011; Fang et al., 2015;

Wan et al., 2017; Ling et al., 2018). All these candidate genes mentioned above had their expression stability assessed in cowpea plants inoculated with CPSMV (V group) at 0, 6, 12, 24, 48, 96, and 144 hours after inoculation in comparison with their respective mock-inoculated (MI group) controls. Expression levels of these genes were determined by the number of amplification cycles required for detection of the fluorescence emitted by BRYT® Green that exceed the threshold during the exponential phase of the PCR reaction, which is determined by the values of the detection threshold, denominated Cycle threshold (*Ct*). The Ct data obtained were exported from the Realplex program and imported into the GeNorm program (Version 2.4). From these data, the expression stability of the candidate genes was calculated using the means of the *Ct* values, means of expression stability (M value), and the number of genes necessary for normalization under the tested conditions established herein (Vandesompele et al. 2002).

5.2.9.2 Expression stability analysis of the candidate reference genes in cowpea plants inoculated with CPSMV

Analysis of the relative gene expression was carried out by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Normalization was performed using two reference genes (phaFbox and L23a3) chosen from the 5 tested genes and validated using the genormPLUS module in qbasePLUS (Vandesompele et al. 2002). Calculation of normalized relative gene expression levels was done using the qbasePLUS software version 1.5 (Biogazelle) (Hellemans et al. 2007). RT-qPCR reactions were performed in triplicate and Ct values were averaged. Primer efficiency was determined by the dilution method from which values of 80-100% efficiency was

obtained. Reaction products were analyzed by melting curves to verify the absence of unspecific products and/or primer/dimer formation. Table 1 shows the primer sequences of the cowpea reference (normalizers), targets, and Argonaute genes.

Table 2 – Primer sequences of cowpea reference (normalizers), target, and argonaute genes, fragment size to be amplified (amplicon), accession number, and optimal melting temperature of the evaluated genes

Gene	Gene product	Primer sequence (5' - 3')	Amplicon (bp)	Tm (°C)	Accesion Number
CpCpSMV	Viral Coat Proteins	F: ATGGCAGTTGAGAACATTGG R: GAAACAAACCTTCTTTGGCTTC	154	57.5	KF793280
PP2a2	phospholipase A2	F: GTCGCTGTGTAGGATTGGAG R: AAGAAGAAACTCGTGCTGTG	130	57.5	XM_014658159
L23a1	60S ribosomal protein L23a	F: GCTGTTTCAACCCTAAACCCT R: AGAAGAAGATCAAGGACGCA	103	57.5	XM_014641092.1
L23a3	60S ribosomal protein L23a	F: CAGGGCATCATAGTCAGGT R: AGGCTTAACATCGAGTAGG	125	57.5	XM_014641511.1
Insu	Insulinase	F: GCAACCAACCTTTCATCAG R: TCCTTTGCTCAATGTTCCC	143	57.5	FG9062104.1
F-Box	F-Box protein	F: GCTTATTCAATCCGCTTGTC R: GTCCTATAACAGCTTCTCCA	148	57.5	FG812521.1
DEAD-Box	DEAD-box ATP-dependent RNA helicase 3, chloroplastic-like	F: GTTGTACACCAGTAGCCAG R: GACTCCAAGACCTCTTCCA	105	49.7	NM_147920.4
SPB9	Squamosa promoter-binding-like protein 9	F: ATCCATCAACTTCCAAATGCC R: ATGTAATGCCTCCTACCCTG	155	50.5	XM_017558424.1
Kat-p80	Katanin p80 WD40 repeat-containing subunit B1 homolog	F: CAAAGTTACAGGTTGTGCGG R: ACAAGGACACTGATAACATCTG	115	50.5	XM_017581340.1
GST	Glutathione S-transferase U18-like	F: AGTTGGATCAGAGTGTTGGA R: TTTGTCAGTGTCTGGTATAAGG	135	49.0	XM_017582271.1
AGO1	Argonaute 1	F: ATCTGTTTCAATTGTGCCTCCT R: AATACCGCCACCCATTCCA	139	53.0	XM_017584420.1
AGO2	Argonaute 2	F: GGCTACAGATTCCGTTGTTCCC R: TCGTGCCACCATTATCAGGTCTC	128	53.0	XM_014661518.1
AGO4	Argonaute 4	F: GAAGGGTAAAGGTGGAGGG R: CAATTCACAAAGCTCAATGGG	169	53.0	XM_017584598.1
AGO6	Argonaute 6	F: GACACAGCAATCACGCATCC R: CTTTGGTTCACATAGGACAGCGA	167	53.0	XM_017575963.1
AGO10	Argonaute 10	F: ATCATCTAAGAGGTTCTGTCCA R: GCCAACATATTCCACAACTG	199	53.0	XM_017559797.1

Source: the author.

5.2.10 Detection of CPSMV infection by RT-PCR

Total RNA obtained from the cowpea secondary leaves from V and MI plant groups were transcribed into cDNA using ImProm-II™ reverse transcriptase (Promega). To a volume containing 1.0 µg RNA, 1.0 µL of oligo(dT)^{15} was added and the volume was adjusted to 1.16 µL with RNase free water. The mixture was incubated in a thermocycler (PTC 200-MJ Research, USA) at 70 °C for 5 min and immediately cooled at 4 °C for 5 minutes. Next, 4.0 µL the RT buffer (Reverse Transcriptase), 1.0 µL dNTPs (1.0 mM), 2.4 µL $MgCl_2$ (25 mM), 1.0 µL reverse transcriptase (1,0 U/µL, Promega) and RNA-free water were added to complete 20 µL total reaction volume. Thermocycling was conducted according to the following schedule: 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 minutes. The reaction products were stored at -20 °C for further analysis.

To confirm the presence or absence of CPSMV in the leaves of control (MI) and virus-inoculated (V) cowpea plants, degenerate primers that correspond to the specific and conserved sequences of the gene that encodes the virus-like protein within the genus Comovirus was used as template: 5'-YTCRAAWCCVYTRTTKGGMCCACA-3' (reverse) and 5'-GCATGGTCCACWCAGGT-3' (forward) (Brioso et al., 1996). Different amounts of cDNA (1.5 x 10^{-3}, 3.0 x 10^{-3}, and 6.0 x 10^{-3} µg) were added to 2 µL [5x] reaction buffer containing the primers (1.0 µL each primer at 20 mM), 0.1 µL dNTPs (10 mM), 0.1 µL (1.0 U µL^{-1}) GoTaq DNA Polymerase (Promega), and 4.8 µL RNase-free water. PCR was conducted according to the following schedule: 94 °C for 3 min, 27 cycles of 1 min at 94 °C, 41 °C for 30 s, 72 °C for 45 seconds and an additional step of 10 min at 72 °C to finish elongation.

The PCR products were subjected to agarose gel (1.2%, m/v) electrophoresis run under 100 mA constant current for 30 min at 26 °C. After electrophoresis, the gel was immersed in ethidium bromide (0.5 µg/µL) for 10 minutes. Visualization of the PCR products was done using a transilluminator of UV light and photo-documented by the MiniBIS Pro Bio-Imaging Systems, with the aid of the GelCapture™ software. The band intensity was analyzed using the GelAnalyzer software.

5.2.11 Mature miRNA and target gene expression analysis by qRT-PCR

qRT-PCR was performed to validate the 4 predicted miRNAs (vun-miRNAs) from the genome of *V. unguiculata* and their respective target genes. Stem-loop primers were used for conversion of miRNA to specific cDNA (Chen et al., 2005) that consisted of 44 conserved and 6 variables nucleotides that encompassed the first 6 nucleotides at the 5' end of the reverse complement of the respective miRNA (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACNNNNNN-3'), which was designed to anneal specifically with the 3' end of the mature miRNA sequence. Mature vun-miRNAs cDNA was synthesized in two steps. A 500 ng sample of total RNA obtained from the cowpea secondary leaves from V and MI plant groups was added to 1.0 µL of the 4 stem-loop primer mix (2 µM) and RNAse-free water added to a 10.0 µL final volume. The mixture was incubated at 70 °C for 5 min and immediately cooling to 4 °C for 5 minutes. Next, 6.0 µL [5×] enzyme buffer, 1.0 µL ImProm-II™ Reverse Transcription System (200 U/µL, Promega Madison, WI, USA), 1.0 µL dNTP (10 mM), 0.5 µL RNase inhibitor (40 U/µL) and RNase free water were added to 30.0 µL final volume. The cDNA synthesis was carried out at 40 °C for 60 min

and an additional step at 4 °C for 5 min. The RT-qPCR reactions were performed in 96-well microtiter plates (Eppendorf, Hamburg, Germany) and conducted in a RealPlex 4S thermocycler (Eppendorf, Hamburg, Germany) using GoTaq® qPCR [2x] Master Mix (PCR Master Mix) (Promega). The RT-qPCR mix consisted of 1 µg cDNA, 0.4 µM primer mix, 10 µL of the PCR Master Mix and RNAse-free water to reach 30.0 µL final volume. The RT-qPCR conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 10 s. Then, the mix was heated from 60 to 95 °C to acquire the cDNA melting curves of the amplified products. All reactions were run in triplicate.

To synthesize cDNA for target gene amplification, 500 ng of total RNA, 1.0 µL oligo $(dT)^{15}$ (10 µM), and RNase free water to reach 11.6 µL final volume were combined and incubated at 70 °C for 5 minutes. After ice cooling at 4 °C for 5 min, 4.0 µL [5×] enzyme buffer, 2.4 µL $MgCl_2$ (25 mM),1.0 µL ImProm-II™ Reverse Transcription System (200 U/µL, Promega), and 1.0 µL dNTP (10 mM) were added. The synthesis of cDNA was conducted 25 °C for 5 min, 42 °C for 60 min, 75 °C for 15 min, and an additional step at 4 °C for 5 minutes. RT-qPCR was performed in a 20 µL final volume mixture consisting of 4.0 µL cDNA (100 ng total), 10 µL PCR Master Mix, 1 µL of each primer at 300 nM final concentration, and 4 µL RNAse-free water. Thermocycling conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, annealing at the specific temperature of each gene (Table 2) for 15 s, and 60 °C for 20 s. This was followed by heating from 60 to 95 °C to acquire the melt curves. Cycle threshold (Ct) value for each sample was automatically determined using the Mastercycler® ep realplex 2.2 software from Eppendorf using default parameters. All reactions were run in triplicate.

5.2.12 Statistical analysis

All experiments were conducted using a completely randomized design. Three independent trials were conducted in which the uninoculated (MI) and CPSMV-inoculated (V) groups consisted of three independent triplicates, each comprising three plants per pot totalizing 9 plants per each treatment and time point (0, 1, 2- and 6-days after inoculation). The RT-qPCR analyses were performed with biological triplicate. Data were analyzed using the ASSISTAT 7.7 program (Silva and Azevedo, 2016). One-way analysis of variance (ANOVA) was used to evaluate whether there was any evidence of differences between means of MI and V plant groups and the significant differences amongst means were evaluated using the Tukey multiple comparison test ($p \leq 0.05$).

5.3 Results and discussion

5.3.1 Identification and stability of cowpea miRNAs

To understand the role of miRNAs in the defense response of a resistant cowpea genotype (BRS-Marataoã) to CPSMV, 1,345 mature plant miRNA sequences deposited in the miRBase database (http://www.mirbase.org/) were selected to search for exact or near-exact matches in the *V. unguiculata* genome. The miRNA-homologous sequences in the *V. unguiculata* genome were identified and ~200 nucleotide long fragment flanking the putative mature miRNA sequence were used to generate and evaluate the secondary structure of selected miRNA sequences. Table 3 shows the 4 miRNAs that were chosen for further analysis together with the expression of their respective target genes. We chose these 4 miRNAs because their

target genes encode classes of proteins previously shown to be responsive to CPSMV challenge (Paiva et al., 2016; Varela et al., 2017).

The prediction pipeline workflow and the main miRNA search results are summarized in Figure *2*.

Table 3 – Properties of four predicted precursors of *V. unguiculata* miRNAs

miRNAs	Sequence ID*	GC (%)**	PL***	MFE**** (kal/mol)	MFEI***** (kal/mol)	vun-miRNA precursor sequence
miR171	MIMAT0023200	41.66	120	-42.9	-0.858	UAUGAAUUGCACAAGCCAAGGUAUUGGCGCGCCUCAAUUUGAAGACAUGGUUAACAAGAAAACCAACCAUGUGGUUUUAAUUGAGCCGCGUCAAUAUCUCAUCUUGCACUUCUUUCCAUC
miR396	MATU01062931.1	45.76	118	-46.9	-0.869	CCTCAATTCTCAAGTCCTGGTCATGCTTTTCCACAGCTTTCTTGAACTTCTTCTGCACCTTCGCCATTTTAAGCCCTAGAAGCTCAAGAAAGCTGTGGGAGAATATGGCAATTCAGGC
miR2111	CP039354.1	36.00	100	-43.0	-1.194	GUAUUGGUUUGGGAUCAGAUAAUCUGCAUCCUGAGGUUUAGAAACAAGAUUUUGAUUGGAUCUAAUCCUUGGGAUGUAGAUUAUCACUUCCUUUUGUCAU
miR156	CP039355.1	45.00	100	-47.8	-1.062	UCAAAGGGUAAAGAGGAGGUGACAGAAGAGAGUGAGCACACAUGGUGUUUCUUGCAGAUAACAUGCUUGAAGCUAUGCGUGCUUACUCUCUAUCUGUCAC

Source: the author.

An underlined sequence represents the cowpea mature miRNA.

* Sequence accession identifier. A unique alphanumeric character string that unambiguously identifies a sequence record in a database.

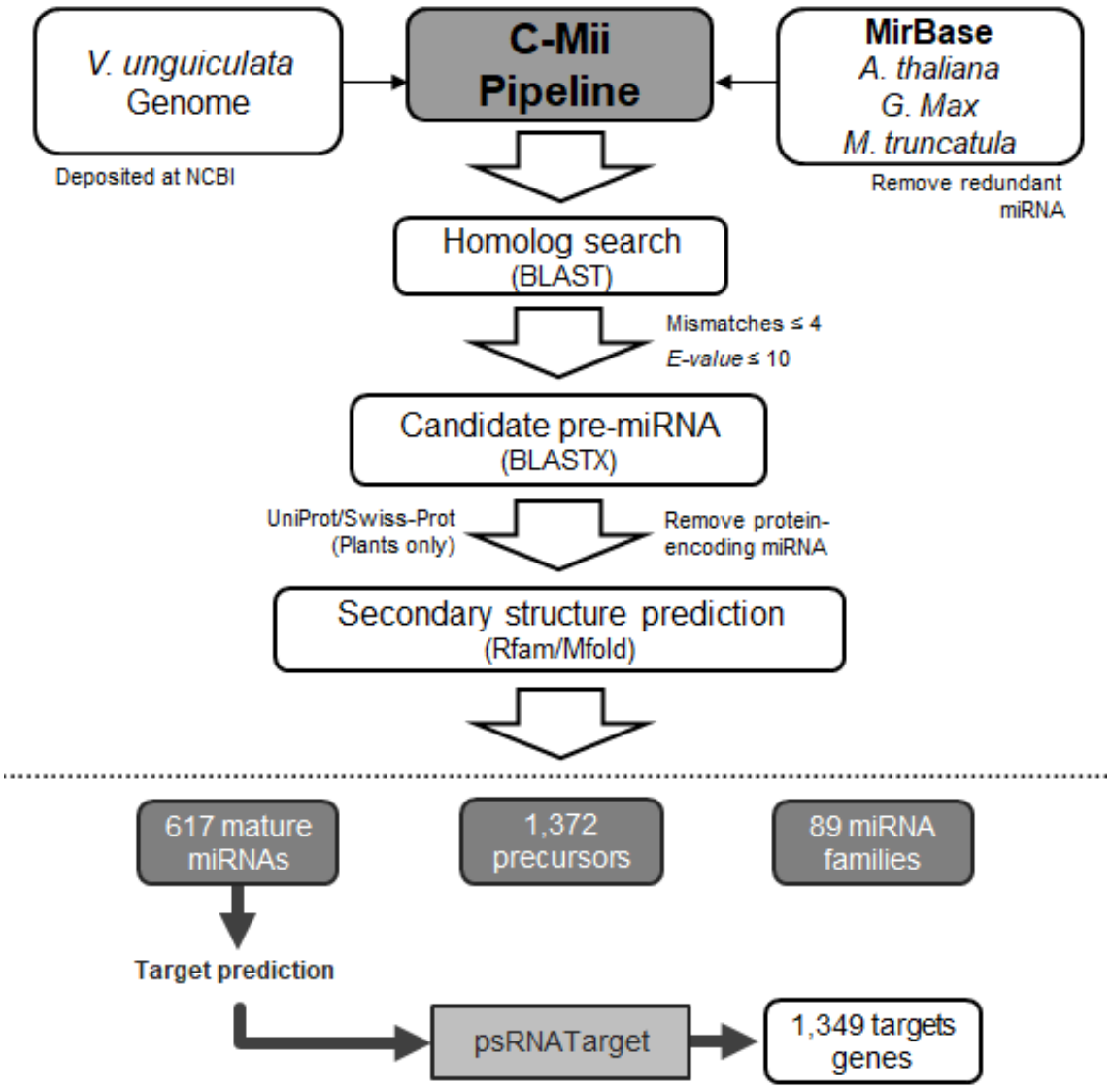
** Percentage of *guanine* + *cytosine* content.

*** Precursor length (PL).

**** Minimum free energy (MFE) of folding.

***** *Minimal folding free energy* index (MFEI).

Figure 2 – Pipeline workflow for identification of miRNAs and putative number of specific target genes in the genoma of *V. unguiculata*. Mature miRNA sequences of *A. thaliana*, *G. max,* and *M. truncatula* were chosen as reference miRNA sets for homology-based miRNA identification. The sequences of the putative *V. unguiculata* miRNAs were used for prediction of specific target genes.

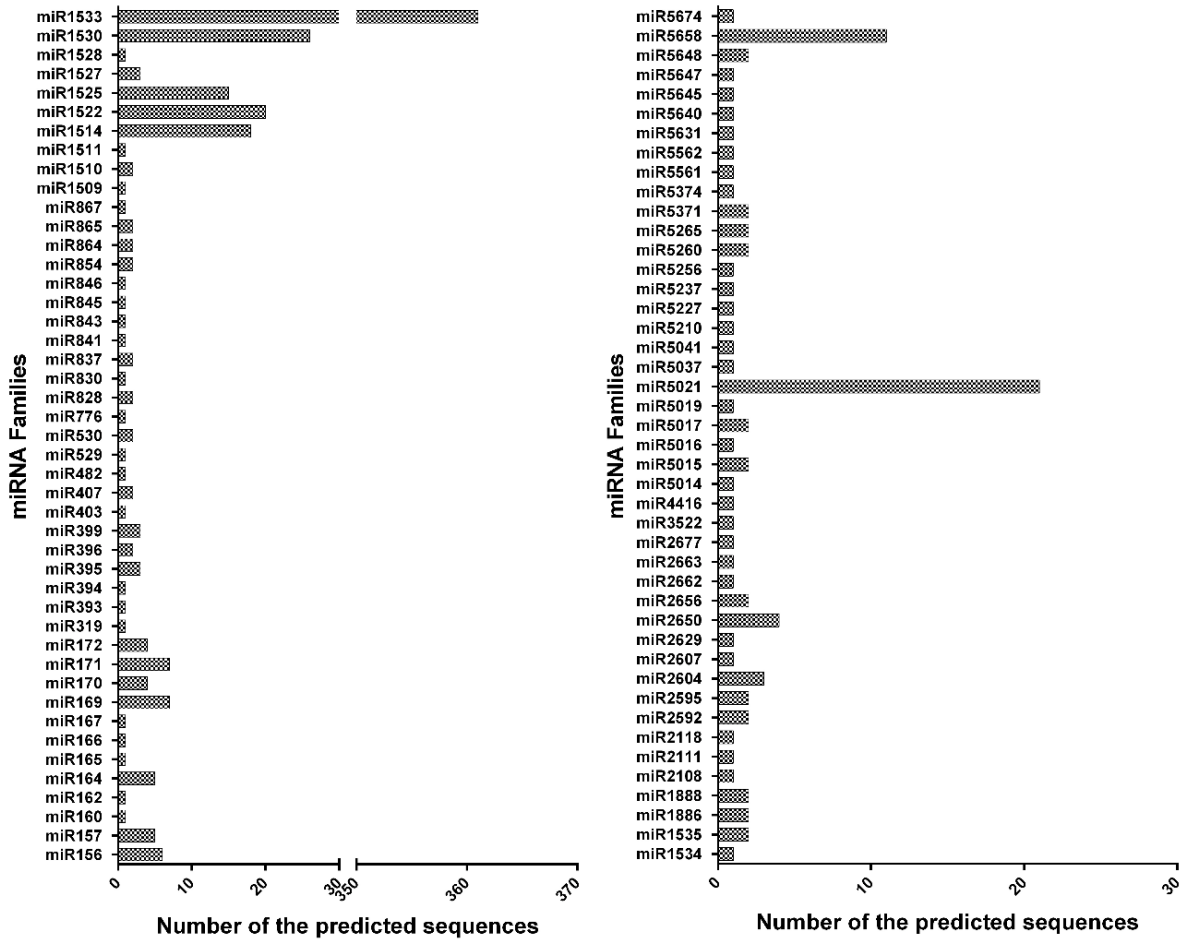


Source: the author.

The homology-based miRNA identification allowed detection of 617 mature miRNA sequences in the cowpea genome. These predicted miRNAs were classified into 89 miRNA families (Figures 2 and 3). Previously, 47 novel miRNAs belonging to

13 miRNA families were identified from the expressed sequence tags (EST) and Genomic Survey Sequence (GSS) databases of *V. unguiculata* by Lu and Yang (2010) and, later, Gul et al. (2017) identified and functionally annotated other 46 novel miRNAs belonging to 45 families from *V. unguiculata* EST database. Barrera-Figueroa et al. (2011), based on the similarities of mature miRNA sequences in *V. unguiculata*, clustered the miRNA genes into 89 families, from which 27 families (93 miRNAs) matched to miRNAs from other plants in the miRBase (release 16).

Figure 3 – Predicted number of mature sequences for each miRNA family identified in the genoma of *V. unguiculata*



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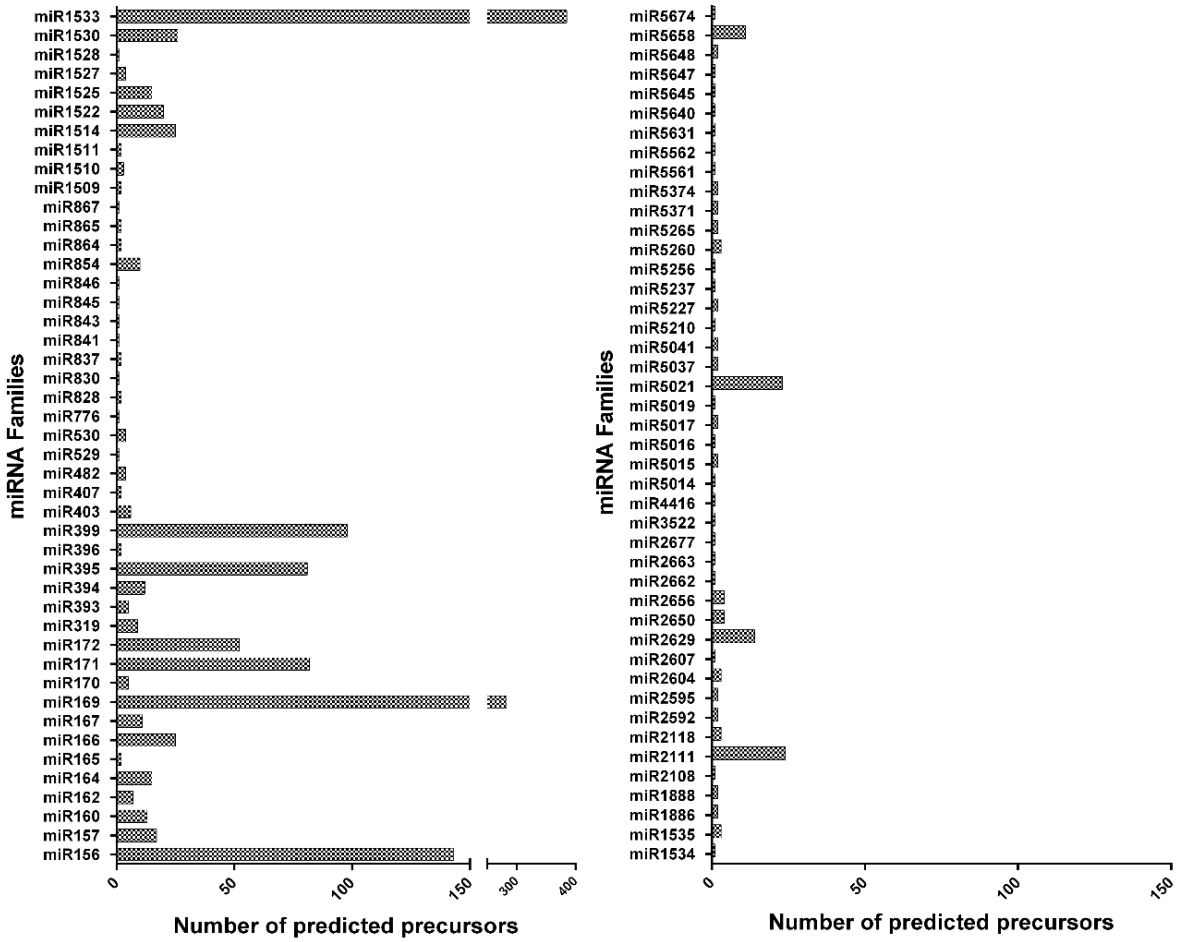
Within these 27 miRNA families, 18 were found by *in silico* searches conducted in this present study. The supplementary tables S1 and S2 show the mature miRNA sequences predicted for *V. unguiculata* and 1,526 precursor sequences, respectively, that reached the criteria described in the pipeline (Figure 2). Most predicted mature plant miRNA have a sequence length of 21 nt (Alptekin et al. 2017). The length of the mature cowpea miRNAs identified herein ranged from 19 to 24 nt with an average of 21 nt. Only 3 *V. unguiculata* miRNAs analyzed in this present study contain 21 nt. The length of the cowpea miRNA precursors ranged from 31 to 547 nt,

and the number of precursors per family was different. For example, the miRNA family 1533 presented 385 members, but 30 other families have only one predicted precursor each (Figure 4). The miRNA family 1533 was the largest among the 89 miRNA families described herein for *V. unguiculata*. Similar results in *Brassica rapa* were reported by Wang et al. (2011) who found mature miRNA with 19 to 24 nt and pre-miRNA ranging from 45 to 602 nt.

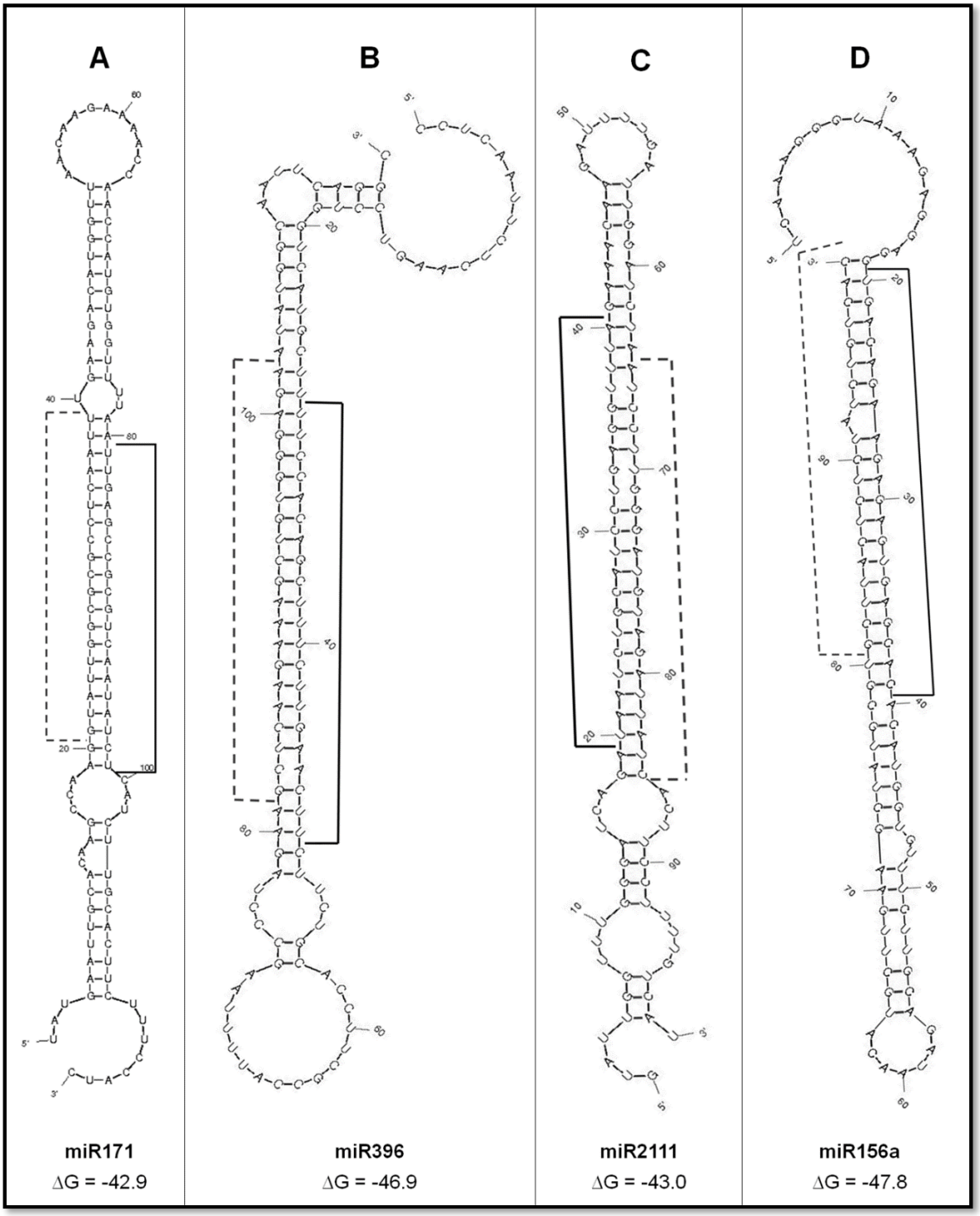
Figure 4 – Predicted number of precursors (pre-miRNA) for each miRNA belong to the 89 miRNA families identified in the genoma of *V. unguiculata*



Source: the author.

MFE values of -42.9, -46.9, -43.0, and -47.8 kcal mol^{-1} were observed for the predicted secondary structures of the identified precursors of miR171.2, miR396, miR2111, and miR156a, whereas MFEI values were -0.858, -0.869, -1.194, and -1.062 kcal mol^{-1}, respectively (Table 3). These data confirm their thermodynamic stability (Figure 5).

Figure 5 – Predicted secondary structures (hairpin) of some selected *V. unguiculata* miRNAs identified in this study. Mature miRNA sequence (guide strand) is labeled with solid line and its complementary sequence (miRNA*, passenger strand) with dotted line



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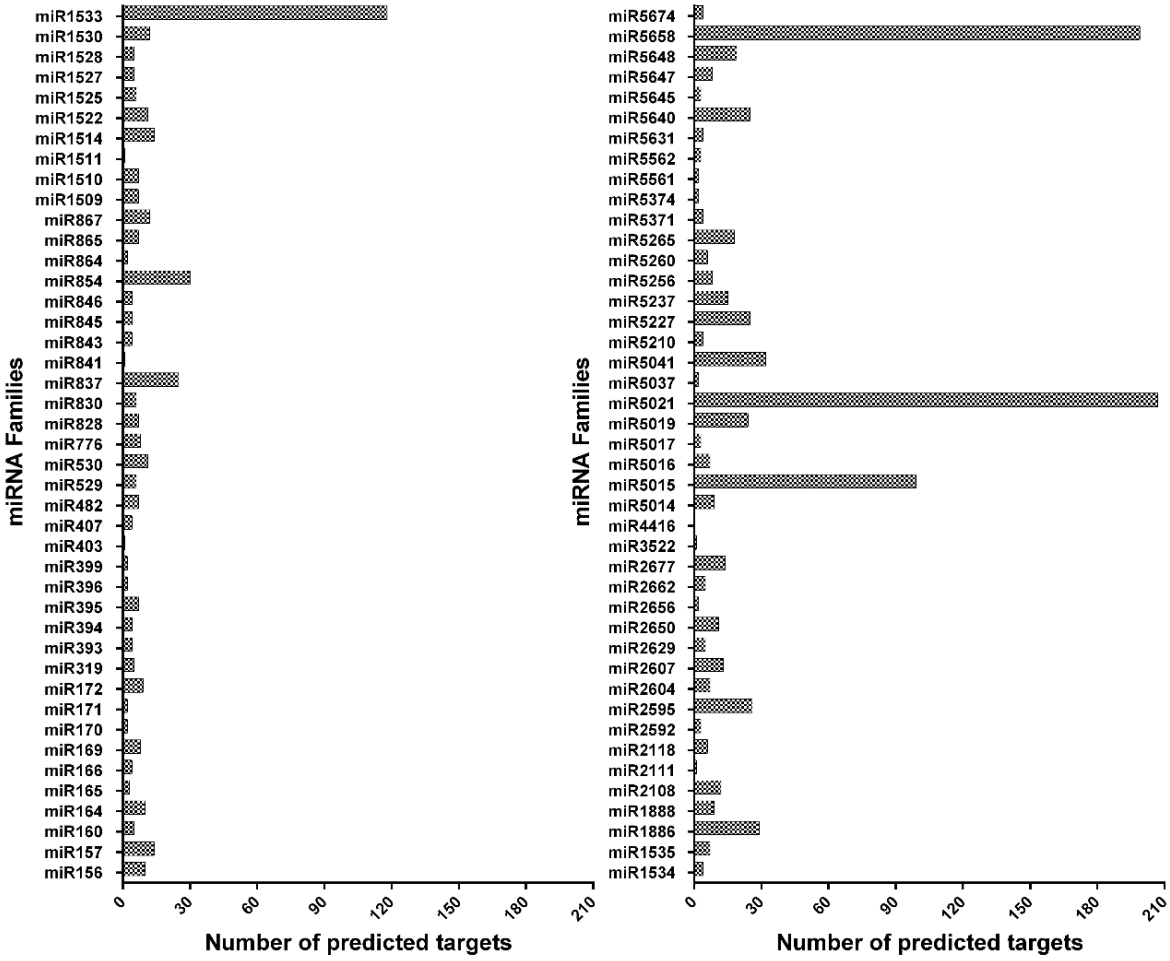
MFE Values of -19.70, -19.10, -17.10, -29.40, and -17.20 kcal mol^{-1} and MFEI of -0.52, -0.44, -0.36, -0.60, and -0.66 kcal mol^{-1} were found for miR169i-3p, miR1214, miR3695, miR9666b-3p, and miRR8182, respectively, in *Cajan cajun* (Hussain, 2018). Singh and Sharma (2014) reported similar MFE values for *Ocimum basilicum* miRNA precursors ranging from -9.20 to -81.34 kcal mol^{-1} and MFEI varying from -0.34 to 0.81 kcal mol^{-1}.

Distribution analysis of nucleotides in the sequence of the identified *V. unguiculata* miRNAs was carried out in reference to the 5' end. Most miRNAs (68.5%) had uridine (U) at the 5' end. Takeda et al. (2008) showed in *A. thaliana* that small 5' end uridine-containing RNAs possess preference for the AGO1 protein. The 89 *V. unguiculata* miRNAs families identified in this study regulate 1.349 predicted target transcripts. It was observed that miR5021 possesses multiple predicted targets, which suggests multiple regulatory functions (Figure 6 and Table S3).

Figure 6 – Predicted number of target sequences for each miRNA belong to the 89 miRNA families in the genoma of *V. unguiculata*



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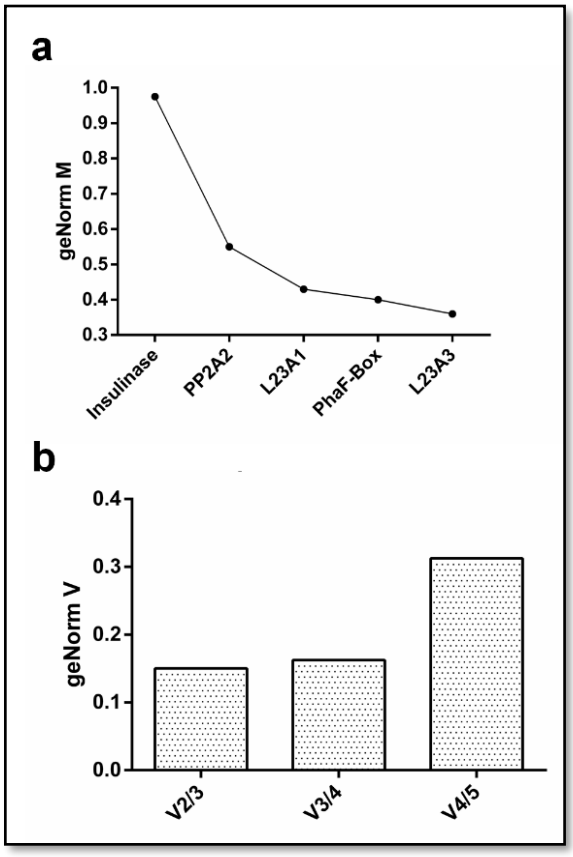
5.3.2 Reference gene normalization by RT-qPCR

Gene normalization experiments and analysis in the GeNorm program revealed L23A3 and PhaF-Box genes as the most stable in cowpea plants inoculated with CPSMV, with values for average expression stability (M) smaller than 0.15. The insulinase gene was the least stable one (Figure 7a). *PhaF-Box* and *L23A3* (ratio 2/3) genes presented low pair variation (V) smaller than 0.15, which is the ideal value for

gene normalization of miRNA expression data (Figure 7b). In this study, the use of these two most stable reference genes allowed normalization of gene expression.

Figure 7 – Average expression stability of *insulinase, fosfolipase A2, protein L23A1, L23A3,* and *PhaF-box* genes using cowpea cDNA from leaves mock-inoculated and inoculated with CPSMV (a). Average expression stability was determined by GeNorm. More stably expressed genes are positioned on the right side of the diagram and the less stably expressed on the right side. Determination of the optimal number of *V. unguiculata* reference genes for RT-qPCR data *normalization* by pairwise variation (geNorm V) was done using the GeNorm algorithm (b). A pairwise variation (Vn/Vn+1) below 0.15 indicates that the *optimum number* of *reference gene* for RT-qPCR data *normalization* is n



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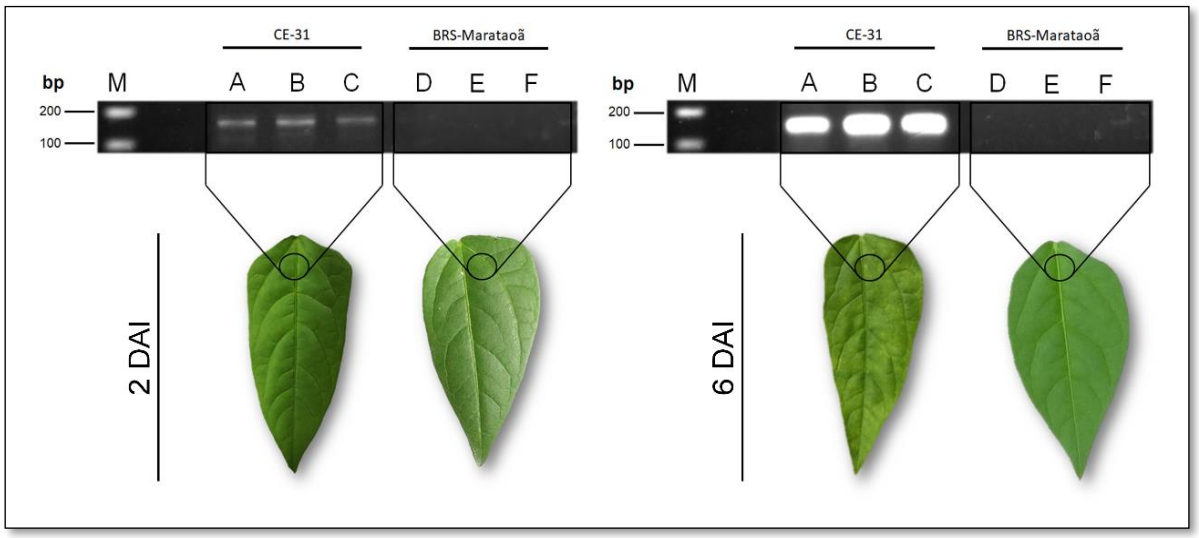
5.3.3 Viral Infection Symptoms

No disease symptoms were observed in plants of the resistant BRS-Marataoã cowpea genotype inoculated with CPSMV (Figure 8). Moreover, RT-PCR analysis did not revealed accumulation of viral particles (CPSMV-CP) in the inoculated leaves. These results are in agreement with previous studies conducted with this pathosystem by our research group, in which an incompatible interaction exists between the resistant cowpea genotype BRS-Marataoã and CPSMV (Varela et al. 2017, 2019). Contrary to this the characteristic visual symptoms of the viral disease and high accumulation of CPSMV particles (CPSMV-CP) was noticed in the highly susceptible cowpea genotype CE-31 inoculated with CPSMV (Figure 8). Accumulation of CPSMV particles in the CE-31 cowpea genotype was previously reported by our research group (Souza et al., 2017, 2020).

Figure 8 – Assessment of the presence of CPSMV by RT-PCR and morphological aspect of the cowpea leaves from the genotypes CE-31 (susceptible) and BRS-Marataoã (resistant) inoculated with CPSMV, at 2 and 6 days after inoculation (DPI). Agarose gel electrophoresis 1.5% (m/v) of the amplified PCR products was performed with 1.5 ng cDNA extracted from the secondary cowpea leaves: M - molecular marker; A, B, C - three biological replicates of CE-31 genotype inoculated with CPSMV; D, E, F - three biological replicates of BRS-Marataoã genotype inoculated with CPSMV.



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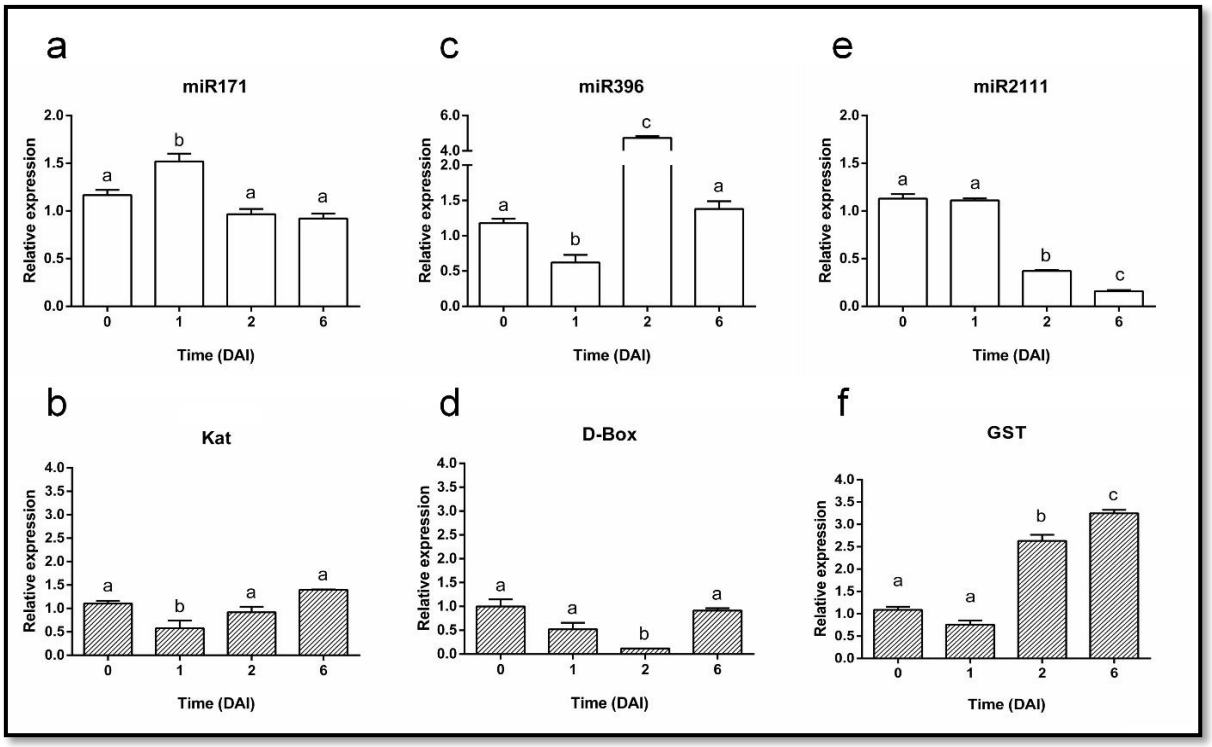
5.3.4 Comparative expression analysis of the predicted miRNAs in the incompatible interaction between BRS-Marataoã and CPSMV

To understand the role of miRNAs in the incompatible interaction between BRS-Marataoã and CPSMV, the transcript expression levels of 4 miRNAs (vun-miRNA171, vun-miRNA396, vun-miRNA2111, and vun-miRNA156) that target, respectively, the genes that encode the cowpea defense proteins Kat-p80, DEAD-Box, GST, and SPB9 were evaluated at 0, 1, 2, and 6 DAI by RT-qPCR (Table 3) in the

leaves of BRS-Marataoã inoculated with CPSMV in comparison to the transcript expression levels of the respective mock-inoculated control. Increased expression level of vun-miRNA171 was observed at 1 DAI, but returned to the same level at 0 DAI, 2 DAI, and 6 DAI (Figure 9a). In response to increased expression of vun-miRNA171 at 1 DAI (Figure 9a), its predicted target gene, which encodes Katanine p80 protein subunit (Kat-p80), showed decreased expression at the same time point (Figure 9b). The Kat-p80 protein is involved in the turnover of microtubules (Burk et al. 2007). Microtubules (MTs) together with actin filaments form the cell cytoskeleton that plays important roles in cell division, vesicle transport, and cell morphogenesis (Kost et al. 2002; Wasteneys and Yang 2004). However, in some plant-virus interactions, cytoskeleton proteins are associated with plant susceptibility by favoring virus replication and movement (Harries et al. 2009). Recently, Souza et al. (2020) showed enhanced accumulation of two actin isoforms in the susceptible cowpea genotype CE-31 inoculated with CPSMV, in comparison with the corresponding mock-inoculated control. However, when the seeds of the susceptible cowpea genotype CE-31 was treated with the mutagenic agent ethyl methane sulfonate (EMS), the generated mutagenized plants, which became resistant to CPSMV, decreased the levels of two actin isoforms upon CPSMV inoculation in comparison to CE-31, which was similarly inoculated with the virus (Souza et al. 2020). Association of actin filaments and microtubules form tracks along which the motor proteins myosin and kinesin may play a role in regulating the pore size of plasmodesmata (PD) that are tiny cell wall channels that connect the plasma membrane and cytoplasm of neighboring plant cells (White and Barton 2011). Plasmodesmata are structures used by viruses for efficient cell-to-cell movement to systematically invade plants (Diao et al. 2018; Reagan and Burch-Smith 2020). For example, the Cucumber mosaic virus movement protein remodels

the actin filaments to increase PD size exclusion limit in tobacco (Su et al. 2010). Microtubules (MTs) comprise the second major component of plant cytoskeletons. They are formed by the assembling of void cylinders of αβ-tubulin heterodimers, which contribute to the complexity and strength of microtubules (Gardiner et al. 2012). Some viruses need MTs to facilitate viral RNA transport from their intracellular sites of replication to PD. Therefore, virus- or host-induced rearrangement of the spatial organization and dynamics in MT regulatory proteins play a key role in the intracellular transport of virus particles and various other processes that are critical to the establishment of viral infection (Ferralli et al., 2006; Nick, 2013; Naghavi and Walsh, 2017). A proteomic study of CPSMV infection of a resistant cowpea genotype (BRS-Marataoã), detected that several actin and tubulin isoforms, involved in the plant cytoskeleton architecture, decreased in abundance at 2 and 6 DPI in the CPSMV inoculated plantlets compared with mock-controls (Varela et al. 2019). In this current study, we showed reduced expression of the gene (Figure 9a) that encodes the Kat-p80 protein, which may inhibit polymerization of new microtubules, by miRNA silencing, after CPSMV inoculation of the resistant cowpea (BRS-Marataoã) plants. Therefore, similar to Varela et al. (2019), these results strongly suggest that decrease in abundance of tubulin and actin proteins is related to the resistance of the cowpea genotype BRS-Marataoã to CPSMV replications and disease establishment.

Figure 9 – Relative expression levels (RT-qPCR) of vun-miR171 (a), vun-miR396 (c), vun-miR2111 (e), and the respective target genes *Kat-p80* (b), *DEAD-Box* (d) and *GST* (f) in leaves of BRS-Marataoã inoculated with CPSMV. Expression was calculated at 0, 1, 2, and 6 DAI in relation to reference genes. Comparison within each miRNA and gene was done between the values obtained at 1, 2, and 6 DAI in relation to 0 DAI. Comparison of the transcript and gene expression levels was done between cowpea plants inoculated with CPSMV in relation to the respective mock-inoculated control



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The vun-miRNA396 decreased significantly in expression level at 1 DAI, but a 500% increased expression was verified at 2 DAI, in comparison with 0 DAI (Figure 9c). At 2 DAI, expression of its target gene, which encode the DEAD-box ATP-dependent RNA helicase 3, decreased by 90% (Figure 9d). DEAD-box ATP-dependent RNA helicase proteins are plant host factors that enhance viral replication of many

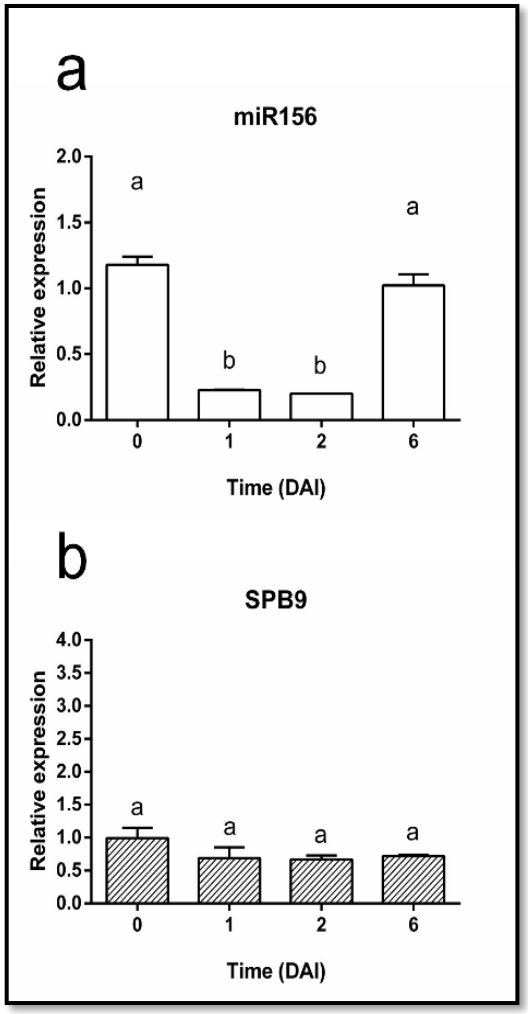
viruses such as Bromovirus, Potyvirus, Tombusvirus, and Comovirus (Noueiry et al., 2000; Huang et al., 2010; Varela et al., 2019; Souza et al., 2020). Other classes of plant RNA helicase are recruited by viruses to stabilize their RNA genome and induce correct replication and translation (Huang et al., 2010; Kovalev and Nagy, 2014; Wu and Nagy, 2019). In the proteomic study conducted by Varela et al. (2017) it was observed that at 2 and 6 DAI, the CPSMV-resistant cowpea genotype BRS-Marataoã decreased the levels of DEAD-box ATP-dependent RNA helicase 3 (DDX). In addition, in a compatible interaction between the susceptible cowpea genotype CE-31 and CPSMV, compared to mock- inoculated controls, an 100% increase in Dead-box protein accumulation was noticed (Souza et al. 2020). These results suggest that the Dead-box protein is important for CPSMV infection, and the reduction of its accumulation in CPSMV-resistant genotype is important in preventing CPSMV infection

Significant down-regulation of vun-miR2111 was observed at 2 and 6 DAI in the resistant cowpea genotype BRS-Marataoã inoculated with CPSMV (Figure 9e). As expected, its possible target gene that encodes for glutathione-s-transferases (GSTs) significantly increased its expression at 2 and 6 DAI (Figure 9f). It is known that some plant *GST* genes are specifically up-regulated by pathogens. GST is a ubiquitous ROS-scavenging enzyme that in conjunction with glutathione mitigates oxidative stress and can detoxify toxic lipid hydroperoxides, which could accumulate during infections and damage the cellular machinery (Gullner et al., 2018). Our study, which suggests a positive correlation between increased *GST* gene expression and plant resistance, is in agreement with previous studies in which *GST* gene expression increases were observed in *A. thaliana* (Ishihara et al. 2004) and *Capsicum annuum* (Gamage et al. 2016) resistant plant lines infected with Cucumber mosaic virus (CMV) and with

Capsicum chlorosis virus (CaCV), respectively. Our result on GST is also supported by the study of Paiva et al. (2016) in which decreased levels of GST were observed in the leaves of the highly susceptible cowpea genotype CE-31, after CPSMV inoculation.

Expression of vun-miR156 decreased at 1 and 2 DAI in comparison to that at 0 DAI (Figure 10). In spite of this, vun-miR156 did not alter the expression of the predicted target gene that encodes SBP9 (Figure 10). Presently, we do not can explain why vun-miR156 did not promote alteration in this gene expression. Perhaps, it is not the right target gene for vun-miR156.

Figure 10 – Relative expression levels (RT-qPCR) of vun-miR156 (a) and its respective target gene *SPB9* (b) in leaves of BRS-Marataoã inoculated with CPSMV. Expression levels were calculated at 0, 1, 2, and 6 DAI in relation to reference genes. Comparison of the transcript and gene expression levels was done between cowpea plants inoculated with CPSMV in relation to the respective mock-inoculated control



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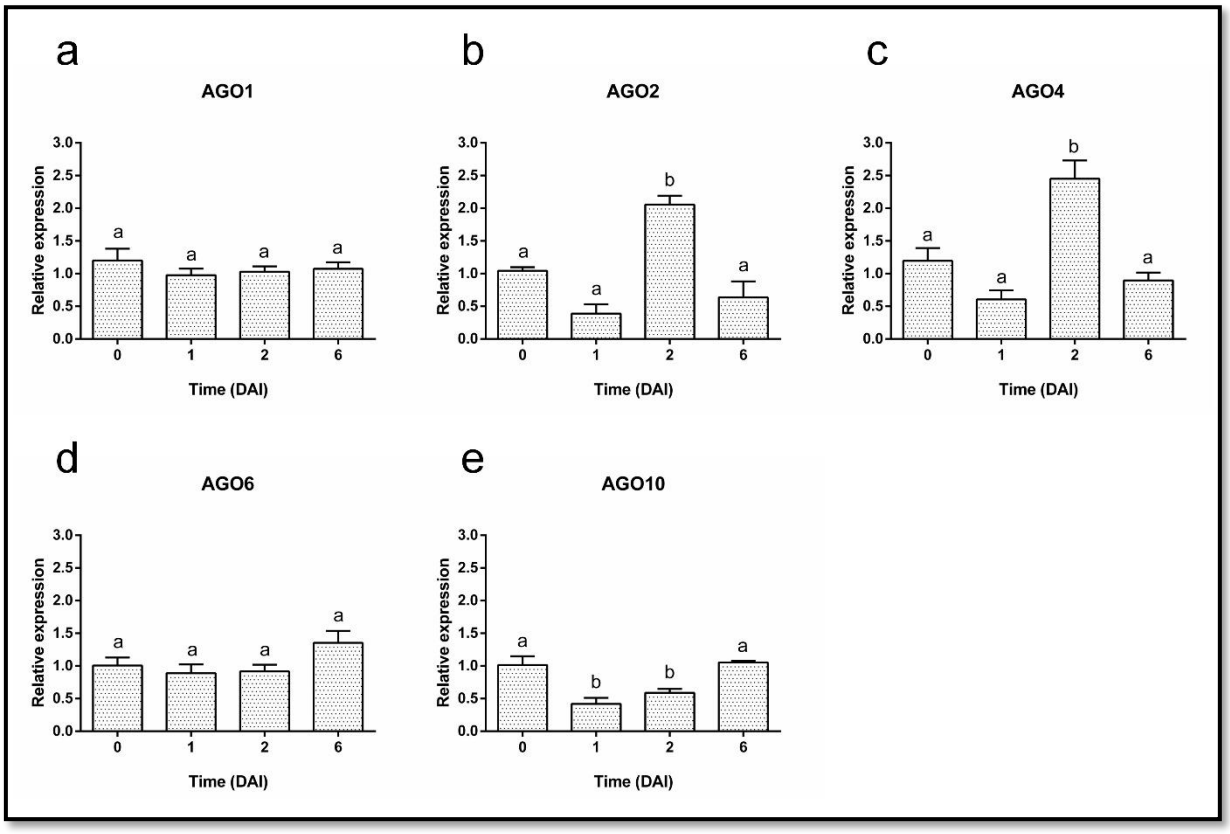
5.3.5 Differential expression of Vigna unguiculata AGO genes responsive to CPSMV inoculation

In plants, at least 10 genes encode Argonaute (AGO) proteins. They are associated with various physiological processes and plant defense, and are required for RNA interference-mediated gene silencing process (Rodríguez-Leal et al., 2016). Although widely studied in plant-virus interaction, there is no information on the *AGO* gene responsiveness to CPSMV challenge in cowpea plants. This work also aimed to analyze differential expression of 5 *AGO* genes (*AGO1, AGO2, AGO4, AGO 6*, and *AGO10*) associated with the resistance of cowpea to CPSMV (Figure 11). Gene expression analysis revealed that *AGO2* (Figure 11b) and *AGO4* (Figure 11c) are responsive to CPSMV infection as they showed 100 and 150% increased levels, respectively, at 2 DAI, in relation to that at 0 DPI, whereas AGO*1* (Figure 11a) and *AGO6* (Figure 11d) did not change, and *AGO10* presented reduced expression levels at 1 and 2 DAI (Figure 11e). Although *AGO1* and *AGO10* have been associated with plant immunity against viruses (Garcia-Ruiz et al., 2015; Minoia et al. 2014), this study did not find they are important to cowpea immunity toward CPSMV.

Figure 11 – Relative expression levels (RT-qPCR) of the Argonaute genes *AGO1* (a), *AGO2* (b), *AGO4* (c), *AGO6* (d), and *AGO10* (e) in leaves of BRS-Marataoã inoculated with CPSMV. Expression levels were calculated at 0, 1, 2, and 6 DAI in relation to reference genes. Comparison of the transcript and gene expression levels was done between cowpea plants inoculated with CPSMV in relation to the respective mock-inoculated control



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However, other AGO proteins such as AGO2 (Carbonell et al. 2012) and AGO4 (Brosseau et al., 2016) play relevant roles in the antiviral defense mechanism (Minoia et al. 2016; Paudel et al. 2018). Overexpression of *AGO2* in tobacco leaves repress Potato virus X (PVX) replication (Brosseau and Moffett 2015). AGO2 is

fundamental for the resistance of *Nicotiana benthamiana* to Turnip mosaic potyvirus (TuMV) (Carbonell et al. 2012). In *A. thaliana*, AGO2 mediates immunity against Turnip crinkle virus, Potato virus X, Cucumber mosaic virus, and Turnip mosaic virus (Harvey et al. 2011; Jaubert et al. 2011; Dzianott et al. 2012; Minoia et al. 2014). Garcia-Ruiz et al. (2015) showed the importance of AGO2 for antiviral defense in *A. thaliana* leaves. They verified that TuMV-AS9-GFP did not infect *A. thaliana* wild type, whereas ago2-silenced *A. thaliana* plants inoculated with TuMV-AS9-GFP presented symptoms of infection. These above studies suggest the importance of AGO2 protein to virus defense.

AGO4 has been associated with Arabidopsis defense against Plantago asiatica mosaic virus (Brosseau et al. 2016) and Tobacco rattle virus (Ma et al. 2015), in addition to its role in plant defense to DNA viruses like Beet curly top virus (Raja et al. 2008). Hamera et al. (2012) reported that Cucumber mosaic virus (CMV) silencing suppressor 2b targeted *AGO4* and reduced its accumulation leading to enhanced CMV infection. Nevertheless, differential expression of AGO encoding genes (Figure 11b-c) suggests the involvement of AGO2 and AGO4 in the resistance of the resistant genotype BRS-Marataoã to CPSMV infection. As shown in Figure 8, the CPSMV-susceptible genotype CE-31 accumulated CPSMV-CP at 2 DAI. Most likely, increased expression levels of *AGO2* and *AGO4* at 2 DAI upon CPSMV inoculation are important to inhibit CPSMV replication in the resistant BRS-Marataoã cowpea genotype. To the best of our knowledge, this is the first report on the involvement of AGO protein in cowpea resistance toward CPSMV infection.

Other relevant result of this study was the negative regulation of *AGO10* at 1 and 2 DAI (Figure 11e). A very interesting information on *AGO10* is that its miRNA trapping function prevents its association with *AGO1* leading to suppression of gene

silencing (Manavella et al. 2011). The AGO10 protein has high affinity for miR165/166 and regulates the HD-ZIP III transcription factor expression, which is related to secondary cell wall (SCW) synthesis in the formation of conductive channels (Zhu et al. 2011). Thus, the resistance of the cowpea genotype BRS-Marataoã to CPSMV may be also related to decreased expression of *AGO10*, which, consequently, may favor down regulation of genes associated with metabolic pathways linked to plant defense.

5.4 Conclusion

The data reported in this work provide new information on cowpea resistance to CPSMV infection. The resistance of the cowpea genotype BRS-Marataoã to CPSMV is very likely also linked to down regulation of genes that encode proteins that favor CPSMV infection, like D-BOX, Kat-p80 and other proteins also important in resistance/susceptibility as revealed in previous studies conducted by our research team (Paiva et al. 2016; Silva et al. 2016; Souza et al. 2017, 2020; Varela et al. 2017, 2019). In addition, it was observed for the first time upregulation of *AGO2* and *AGO4* genes and downregulation of *AGO10* in a resistant cowpea genotype in response to CPSMV challenge. This work adds toward a better understanding of the cowpea defense mechanisms against CPSMV infection.

5.5 Author contribution statement

JTAO and IMV conceived and designed the research, critically revised the paper, contributed to the analysis of the results, and to the writing of the manuscript. PFNS analyzed the data, and actively participated in the writing and correction of the first

draft of the manuscript. FDAS performed the selection and expression stability analysis of the candidate cowpea reference genes. MRA participated in the maintenance of plants in a greenhouse, extraction, and analysis of the integrity of RNAs.

6 CONCLUSÃO

Os dados relatados neste trabalho fornecem novas informações sobre a resistência do feijão-DE-CORDA à infecção pelo CPSMV. A resistência do genótipo BRS-Marataoã ao CPSMV provavelmente também está ligada à regulação negativa de genes que codificam proteínas que favorecem a infecção pelo CPSMV, como D-BOX, Kat-p80 e outras proteínas também importantes na resistência/suscetibilidade, conforme revelado anteriormente em estudos realizados por nossa equipe de pesquisa (Paiva et al. 2016; Silva et al. 2016; Souza et al. 2017, 2020; Varela et al. 2017, 2019). Além disso, foi observado pela primeira vez a regulação positiva dos genes AGO2 e AGO4 e a regulação negativa da AGO10 em um genótipo resistente do feijão-de-corda em resposta ao desafio do CPSMV. Este trabalho contribui para uma melhor compreensão dos mecanismos de defesa do feijão-de-corda contra a infecção pelo CPSMV.

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Conflict of interest

The authors hereby declare no conflicts of interest.

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APÊNDICE A – TAB S1

Predicted potential mature miRNAs identified in the genoma of *V. unguiculata* after using the C-mii software.

Link:

https://drive.google.com/open?id=1GZnxvQF4MKZzbczF4Yi1F_zPB8R5qyK9

QR CODE:

APÊNDICE B – TAB S2

Predicted miRNA precursors (pre-miRNAs) identified in the genoma of *V. unguiculata* after using the C-mii software.

Link:

https://drive.google.com/open?id=1pyd2-VoHuD3JUx1Jtyr0SfOtPo9Mq_78

QR CODE:

APÊNDICE C – TAB S3

Predicted potential target candidate genes of miRNA families identified in the genoma of *V. unguiculata*.

Link:

https://drive.google.com/open?id=17-Q7DI54ijha2TqhtBekwoe37Xe9DxNa

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