3 Biotech (2021) 11:114
https://doi.org/10.1007/s13205-021-02653-7

ORIGINAL ARTICLE

RNAi activation with homologous and heterologous sequences that induce resistance against the begomovirus *Pepper golden mosaic virus* (PepGMV)

Mayela Vargas-Salinas[1] · Diana Medina-Hernández[1] · Guadalupe Fabiola Arcos-Ortega[1] · Irasema Elizabeth Luis-Villaseñor[2] · Ramón Jaime Holguín-Peña[1]

Received: 21 July 2020 / Accepted: 9 January 2021

Abstract

This study compared the transcriptional changes in *Nicotiana benthamiana* plants treated with homologous sequences derived from *Pepper golden mosaic virus* (PepGMV) and heterologous sequences that derived from another begomovirus, *Tomato chino La Paz virus* (ToChLPV) prior to infection by PepGMV. The results of microarray analyses identified upregulated genes associated with RNAi such as DCL2, DCL4, AGO3, AGO7, AGO10, NRPD2B (Pol IV), DRB3, CMT3, RDR6. The components that participate in different RNAi pathways were identified, including methylation induced by both constructs, as well as the code of these genes in *Arabidopsis thaliana* and its counterpart in *N. benthamiana* through different genome assembly. The expression of these genes was validated by quantitative reverse transcription polymerase chain reaction (RT-qPCR), where DCL3, DCL4, AGO1-1, AGO2, RDR6 and PPR1 showed increased expression during plant protection with the heterologous construct compared to those protected with the homologous construct. The results of this study confirmed the activation of the gene silencing mechanism at the transcriptional level with both constructs and established the possibility of their use as a protection system for both homologous and heterologous sequences.

Keywords Tomato chino La Paz virus (ToChLPV) · Gene silencing · Microarray · dsRNA

Introduction

Begomoviruses (family *Geminiviridae*) are plant pathogenic viruses that are widely disseminated by whiteflies (De Barro et al. 2011). These viruses are commonly found in mixed infections with other members of this genus, favoring their plasticity, emergence of new viruses of pathological importance, and even widen their host range (Navas-Castillo et al. 2011; García-Arenal and Zerbini 2019). Mixed infections are common in nature as it was observed for *Pepper golden mosaic virus* (PepGMV) with *Pepper huasteco yellow vein virus* (PHYVV), which can even exhibit a triple infection with *Tomato yellow leaf curl virus* (TYLCV) (Méndez-Lozano et al. 2003; Morales-Aguilar et al. 2019), or also TYLCV co-infecting with *Tomato chino La Paz virus* (ToChLPV) (Cárdenas-Conejo et al. 2010). One of the conserved mechanisms that plants use against viral infections is RNA interference (RNAi) (Lin et al. 2011). Some research has focused on evaluating the efficiency of RNAi as a protection system when the viral sequence has high homology to disease-causing begomoviruses. For example,

✉ Ramón Jaime Holguín-Peña
jholguin04@cibnor.mx

Mayela Vargas-Salinas
msalinas@pg.cibnor.mx

Diana Medina-Hernández
dmedina@cibnor.mx

Guadalupe Fabiola Arcos-Ortega
farcos04@cibnor.mx

Irasema Elizabeth Luis-Villaseñor
irasemaluis@uas.edu.mx

[1] Centro de Investigaciones Biológicas del Noroeste, Instituto Politécnico Nacional 195, Col. Playa Palo de Santa Rita, 23096 La Paz, Baja California Sur, Mexico

[2] Facultad de Ciencias del Mar, Universidad Autónoma de Sinaloa (UAS) Mazatlán, 82000 Sinaloa, Mexico

Published online: 03 February 2021

Chellappan et al. (2004) used the sequence of the Rep (AC1) gene, which encodes the replication associated protein of the *African cassava mosaic virus* (ACMV) in *Manihot esculenta* plants in challenge experiments with *East African cassava mosaic Cameroon virus* (EACMCV) and *Sri Lankan cassava mosaic virus* (SLCMV). These authors reported strong protection through an activating gene silencing mechanism. On the other hand, a sequence obtained from non-coding conserved regions of five different TYLCVs was used to design a construct capable of activating the gene silencing mechanism against three of these viruses and generate resistant *Solanum lycopersicum* and *Nicotiana benthamiana* plants (Abhary et al. 2006). The efficacy of this strategy was also demonstrated using other intron-hairpin constructs (ihp-RNA) from the CP (AV1) gene, which encodes the capsid protein, and Rep from the *Tomato yellow leaf curl Sardinia virus* (Sicily strain; TYLCSV-[Sic]). However, in viral challenge assays, the plants only showed resistance against viruses with homologous sequences and not against those with heterologous ones (Gharsallah-Chouchane et al. 2008). The gene silencing mechanism has been activated using fragments from DNA-A or DNA-B from begomoviruses. However, the best results were obtained using DNA-A fragments (Taha et al. 2016). In contrast to previous reports, a previous study observed extremely low efficiency of RNAi in the complex of the begomoviruses *Cotton leaf curl Multan virus* (CLCuMV) and Cotton leaf curl Multan β-satellite (CLCuMB) (Mubin et al. 2011). Based on their results, they argued that high efficiency is achieved when the target sequence is highly similar to the constructed sequence, which would allow resistance to be achieved to begomovirus complexes. In addition, in a previous report, the efficiency of these constructs was evaluated against infection by PepGMV, observing decreases in viral loads from 95.6 to 99.5% for the heterologous and homologous constructs, respectively (Medina-Hernández et al. 2013). Although the silencing mechanism was activated in these studies, as evidenced by the observed decreases in symptoms and viral load, the participating components and how they interact when homologous and heterologous sequences are used remains unclear.

RNA interference (RNAi) is gene regulation mechanism conserved in eukaryotic organisms that also plays important roles in the control of basic cellular processes in plants, such as gene expression, heterochromatin formation, and adaptive antiviral defense (Herr and Baulcombe 2004). RNAi has been used as a method to study antiviral defense in plants. It is activated in the presence of double-stranded RNA molecules (dsRNAs) or small interfering RNAs (siRNAs) and acts at different levels, such as in the degradation of homologous RNAs in a sequence-specific manner (homologous sequence) or DNA methylation and chromatin modification (Voinnet 2001; Tenllado et al. 2004; Matzke and Mosher 2014). With respect to the mechanism of RNAi, it is known that DICER cuts dsRNAs that may then generate siRNAs of different sizes, including primary and secondary siRNAs (Wang et al. 2011). Dicer-like 2 (DCL2) acts directly in viral control by participating in the generation of sRNAs and activating the production of a secondary series of siRNAs called secondary or transitive siRNAs. DCL2, DCL3 and DCL4 are known to process endogenous siRNAs in plants, which are categorized into secondary siRNAs. In addition, DCL4 also participates in the defense against viruses by inducing the production of primary siRNAs (Yoshikawa et al. 2005; Axtell et al. 2006; Borges and Martienssen 2015). These siRNAs then bind to the argonaute (AGO) protein to form the RNA-induced silencing complex RISC (Pumplin and Voinnet 2013).

However, the studies have shown that transgenes with heterologous sequences can also initiate the RNAi process (Lin et al. 2011; Mubin et al. 2011). Thus, to achieve effective control strategies against mixed begomoviral infections, it is necessary to understand the various interactions in a heterologous RNAi system. This study analyzed a differential gene expression using microarrays and quantitative real time reverse transcription polymerase chain reaction (RT-qPCR) to evaluate the key components that interact during RNAi activation in *N. benthamiana* plants, which can be over- or under-expressed using homologous and heterologous dsRNA constructs to PepGMV. The results obtained will allow implementing effective infection control strategies by begomoviruses with homologous and heterologous sequences.

Materials and methods

Plants growth and constructs

Nicotiana benthamiana plants were grown at 28 °C, with a photoperiod of 16 h light and 8 h dark. Plants at the 3–4-leaf stage were used in four treatments. To evaluate the key components that interact during RNA interference activation when heterologous constructs derived from ToChLPV and homologous to PepGMV are used, because viral loads decreased in (95.6 and 99.5%, respectively), as previously described by Medina-Hernández et al. (2013). The constructs comprised inverted repeat sequence fragments of these pathogens and were denominated as homologous CIRP (construct of the intergenic region of PepGMV) and heterologous CIRT (construct of the intergenic region of ToChLPV) and transformed into *Agrobacterium tumefaciens* GV2260. These constructs express an intron-hairpin comprising 146 nt of the 5′ end of the AV1 gene; the entire 326 nt intergenic region (IR) and 714 nt of the 5′ end of the AC1 gene; the AC1-IR-AV1 total segment comprising 1186 nt

with sequence identity of 50% and six regions longer than 22 nt of both viruses, which displays the highest identity from 82 to 100%. CIRP and CIRT contained inverted repeat sequences of the AC1-IR-AV1 region of each virus, including an intron as a spacer with an *Xba*I restriction digestion to confirm the presence of the sense and antisense arms and orientation of the inverted repeat sequences. Four treatments were performed: (1) CIRP, agro-infiltrated two leaves of *N. benthamiana* with CIRP and inoculated with PepGMV four days after; (2) CIRT, agro-infiltrated two leaves of *N. benthamiana* with CIRT and inoculated with PepGMV four days after; (3) positive control, agro-infiltrated two leaves of *N. benthamiana* with the empty vector (pH7GWIWG2 II) and inoculated with PepGMV four days after; and (4) negative control, agro-infiltrated two leaves of *N. benthamiana* with the empty vector but not inoculated with PepGMV.

Microarray and data analysis of *Nicotiana benthamiana* plants protected with homologous and heterologous constructs

Arabidopsis thaliana V.3.0.3 microarrays were used to analyze and compare global gene expression profiles of *N. benthamiana* experimental plants. Three leaves from each plant were collected at 10 days after inoculation (dpi), immediately frozen in liquid nitrogen and stored at -80 °C until used for RNA extraction. Total RNA was extracted using TRIzol (Invitrogen, CA, USA), and 10 µg of RNA was used for complementary DNA (cDNA) synthesis, incorporating fluorophores dUTP-Alexa Fluor 555 or dITP-Alexa Fluor 647. Incorporation of the fluorophore was analyzed by measuring absorbance at 555 nm for Alexa555 and 650 nm for Alexa647.

An equal quantity of labeled cDNA from the positive control, CIRP or CIRT groups was hybridized with labeled cDNA from controls at 42 °C for 14 h using UniHyb hybridization solution (Arrayit Corporation, Silicon Valley CA, USA). For each spot, the Alexa555 and Alexa647 signal density and background mean values were calculated with Array-Pro Analyzer (Houston, TX, USA). Processing and quantification of the images obtained from the microarray hybridization were performed in an Array-Pro Analyzer 4000 with the corresponding software (Packard BioChips, IL, USA). To analyze the microarray data and identify genes with significant differential expression between sample classes, the genArise package developed by the Computational Unit of the Institute of Cellular Physiology (UNAM, CDMX, MX) was used.

For gene ontology (GO) analysis, the IDs obtained from the microarray data were loaded into the Arabidopsis Information Resource (TAIR) database to convert them to FASTA format. Then, each sequence was searched against the non-redundant (NR) protein database with the Blast2GO tool (Conesa et al. 2005) using the parameters 20 hits per sequence, expected value $< 1 \times 10^3$. When the names of all the genes were identified, evidence codes (EC) were obtained, which showed the reliability index values of the GO annotation, and each gene was mapped in the GO database. A one-tailed Fisher's exact test was used to remove double IDs, and a false discovery rate (FDR) cutoff of 0.01 was used to verify a significant overrepresentation of GO annotations. Enrichment of a GO term was considered significant at $p < 0.05$. After mapping, each gene was annotated, and all of the enzymes were labeled using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Then, the data were labeled with respect to InterPRO protein families (Conesa and Götz 2008).

The TAIR (Arabidopsis.org) annotation data were also obtained from the different databases with available genome and transcriptome assemblies for *N. benthamiana*, including the draft genomes Niben0.4.4 (NbS000) and Niben1.0.1 generated by the Boyce Thompson Institute for Plant Research (Bombarely et al. 2012), Nbv3K, Nbv6.1 (Nakasugi et al. 2013), and the re-annotated NbD (Kourelis et al. 2018).

Differential gene expression analysis

First, upregulated genes associated with defense response, stress, and systemic acquired resistance were selected. Second, the overexpressed (upregulated) genes obtained in the microarray analysis with a Z score $\leq$ 1.50–3.0 were selected. To analyze the differential expression of the genes involved in the RNAi activation response by homologous and heterologous sequences to PepGMV, a search was performed for key genes involved in RNA silencing pathways, such as AGO, DCL-type proteins and RNA-dependent RNA polymerases (RDRs) using the tools described above (see the GO analysis section) with the TAIR database. To determine the effects of differential gene expression and interpret the results in the context of a protein–protein interaction network, an analysis was performed using the STRING version 9.1 database, which is a valuable tool for searching protein/protein interactions. The STRING network results were restricted to those genes that are known to be associated with RNAi (Franceschini et al. 2012; Szklarczyk et al. 2016).

RT-qPCR analysis of differential gene expression

For RT-qPCR analysis, RNA from experimental plants was treated with DNase I (1 U/µL; Thermo Fisher Scientific, Inc. MA, USA) to remove genomic DNA. Then, first strand cDNA was synthesized from 5 µg of total RNA with Super Script III Reverse Transcriptase (Thermo Fisher Scientific, Inc. MA, USA) according to the manufacturer's instructions. Actin (ACT) was used as an internal control for normalization due to its similar expression level in all viral infections

and its efficiency in PCR amplification. According to Liu et al. (2012) and Wu et al. (2014), genes associated with RNAi were selected for gene expression analysis, including DCL1, DCL2, DCL3, DCL4, AGO1, AGO2, RDR6, and pentatricopeptide repeat (PPR) proteins using the primers shown in Table 1. This study analyzed the primers previously described for AGO1 with available sequences in the NCBI database for AGO1a, AGO1b, AGO1-1 and AGO1-2 from *N. benthamiana*, observing alignment with AGO1a and AGO1-1 but not AGO1b or AGO1-2. RT-qPCR was performed in triplicate in a 96-well thermocycler (CFX-96, Bio-Rad, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad) in a final volume of 20 µL per reaction. The PCR conditions were as follows: one cycle at 95 °C/30 s followed by 39 cycles of 95 °C/0.05 s and 60 °C/0.05 s. After amplification, melting curve analyses were performed to verify the products amplified by their specific melting temperatures (T_m) from 65 to 95 °C with increments of 0.5 °C/0.05 s. A sample without DNA was used as control in each analysis. The amplification efficiency (E), $E = 10\ (-1/\text{slope}) \times 100\%$ was determined in accordance with the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines. The RT-qPCR data were normalized with respect to the CT reference gene (Table 1). Relative expression was determined with the 2-ΔΔCT method, where ΔΔCT = ([CT1Target-C1TReference] - [CT0Target-CT0Reference]) (Livak and Schmittgen 2001), using the results from three independent biological replicates for each treatment ($n = 3$). One-way analysis of variance (ANOVA) and statistical significance was determined using IBM SPSS Statistics (version 22.0, IBM Corp. NY, USA) software.

Table 1 Primer sequences used for quantitative gene expression analysis

Primer	Sequence (5′-3′)	Reference
DCL2F	CGGGATCCCCGGGATTTATTCGTAAT	
DCL2R	CCCTCGAGAATGACAAAGCCGCTACT	Wu et al. (2014)
DCL3F	ACTTGTTGAATGCGGTGAAG	
DCL3R	CCCCTGTCGTTCTAGCTCAT	Wu et al. (2014)
DCL4F	CGTCCGTGCCCAGAAATCT	
DCL4R	AATGCAATTGCCGCTTTGA	Wu et al. (2014)
AGO1F	GCTCTAGAAGATCTGTACAAGACTTGGC	
AGO1R	CGAATTCTTATTGGCAAACAACCTAGT	Wu et al. (2014)
AGO2F	CATTTGAACCTCCTTTCTATCGAC	
AGO2R	CATACCTCTAGAAGTGAGGATCAC	Liu et al. (2012)
RDR6F	TTCAGGAATGTCTTCTTCGAGCG	
RDR6R	AGTGATCTAGCAACCCAATGAG	Liu et al. (2012)
PPR1	ATGAGGGTCCATTTGAGTGAC	
PPR1	AGGCTGATGTTGGAATCTGG	Liu et al. (2012)
ACT	TCCTGATGGGCAAGTGATTAC	
ACT	TTGTATGTGGTCTCGTGGATTC	Liu et al. (2012)

This table shows primer sequences used for quantitative reverse transcription polymerase chain reaction (RT-qPCR) gene expression analysis of treatments of *Nicotiana benthamiana* plants protected with CIRP (homologous construct of the intergenic region of PepGMV) or CIRT (heterologous construct of the intergenic region of ToChLPV) and challenged with PepGMV

Results

Microarray based gene expression analysis of plants previously protected

A total of 27,342 and 27,218 genes were obtained in the samples of plants protected with homologous CIRP and heterologous CIRT constructs. Figure 1a shows the microarray results where the data and colors are associated with the differential gene expression, and the color of each point depends on the absolute value of -3 to 3 *Z*-score associated with the differential expression of down-or upregulated genes. Functional characterization of the differentially expressed genes was performed using TAIR and the GO program Blast2GO, with 1473 upregulated genes were identified in plants with CIRP and 2169 identified in plants with CIRT (Fig. 1b).

Each identified gene was assigned to one of the three primary GO categories (Fig. 2): (1) biological process (BP); (2) molecular function (MF); and (3) cellular components (CC). BP (GO level 2) was represented by transcripts associated with cellular processes, such as differentiation, organization, growth, cycle, cell–cell signaling (16 and 17% in plants with CIRP and CIRT, respectively), metabolic processes of protein, lipid, carbohydrate, nucleobase-containing compound, DNA (15 and 16% in plants with CIRP and CIRT, respectively) and primary and secondary metabolites (generation of precursor metabolites and energy), as well as response to stimuli (biotic, abiotic, endogenous) or defense (9 and 10% in plants with CIRP and CIRT, respectively). In plants protected by CIRP, these transcripts were associated with response to stress; abiotic, biotic, endogenous and external stimuli mediated through mitogen-activated protein kinase (MAPK) cascades; gene expression regulation and epigenetics (histones methylation and maintenance of DNA methylation). In CIRT, these transcripts were associated with (MAPK cascades) oxide reduction and transcription regulation, response to abscisic acid, acquired systemic resistance, signaling pathways mediated by salicylic acid, hypersensitive response,

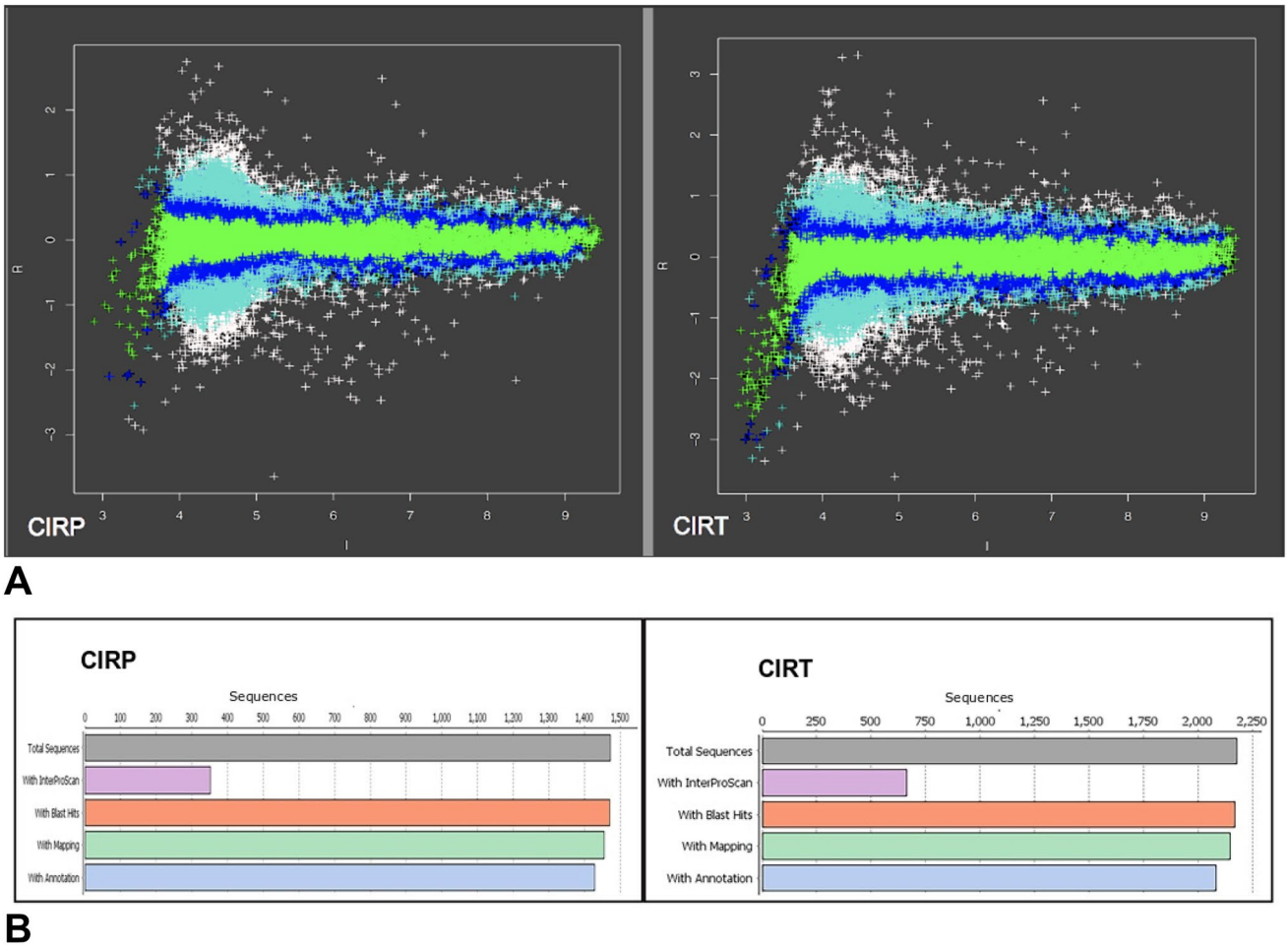


Fig. 1 **a** Graphic results of the evaluation of *Z*-scores from the microarrays analysis from *Nicotiana benthamiana* plants protected with CIRP (homologous construct of the intergenic region of PepGMV) or CIRT (heterologous construct of the intergenic region of ToChLPV) and challenged with PepGMV, where 'up" and "down" values are indicated by *green*, *blue*, *cyan* and *white*. **b** Results of the analysis of annotated sequences

even positive regulation transcription dependent on DNA, and protein ubiquitination. MF was represented by transcripts associated with binding of proteins, DNA, RNA, nucleotide, lipid, chromatin, signaling receptor, DNA-binding transcription factor activity (51% in plants with both constructs), catalytic activity (41 and 40% in plants with CIRP and CIRT, respectively) and transcription regulator activity (8 and 9% in plants with CIRP and CIRT, respectively). CC was represented in different percentages for each construct. For example, in plants protected by CIRP, transcripts were associated with cell part (27%), cell (27%), organelles (23%), membrane (10%), organelle part (6%), and membrane part (3%). Differences were also observed with respect to the extracellular region (3 and 8% in plants with CIRP and CIRT, respectively), for plants protected by CIRT transcripts associated with nucleus (25%), chloroplast (13%), intracellular organelle (13%), protein-containing complex (13%), plasma membrane (11%), mitochondrion (9%), and cytosol (8%) (Fig. 2).

Upregulated genes associated with defense response, stress, systemic acquired resistance and hypersensitive response in plants protected with CIRP and CIRT are shown in Table 2. The data were obtained from different databases with available genome and transcriptome assemblies and have the names of the proteins and biological functions derived from TAIR. The genes were also associated with IDs that correspond to the assembly version and code that identify genome and transcriptome assemblies (Bombarely et al. 2012; Nakasugi et al. 2013; Kourelis et al. 2018).

Differential expression of genes associated with RNAi and methylation in plants protected by CIRP and CIRT

Regarding the genes associated with RNAi (Table 3), upregulated genes with high identity with genes annotated from *N. benthamiana* transcriptomes (Bombarely et al.

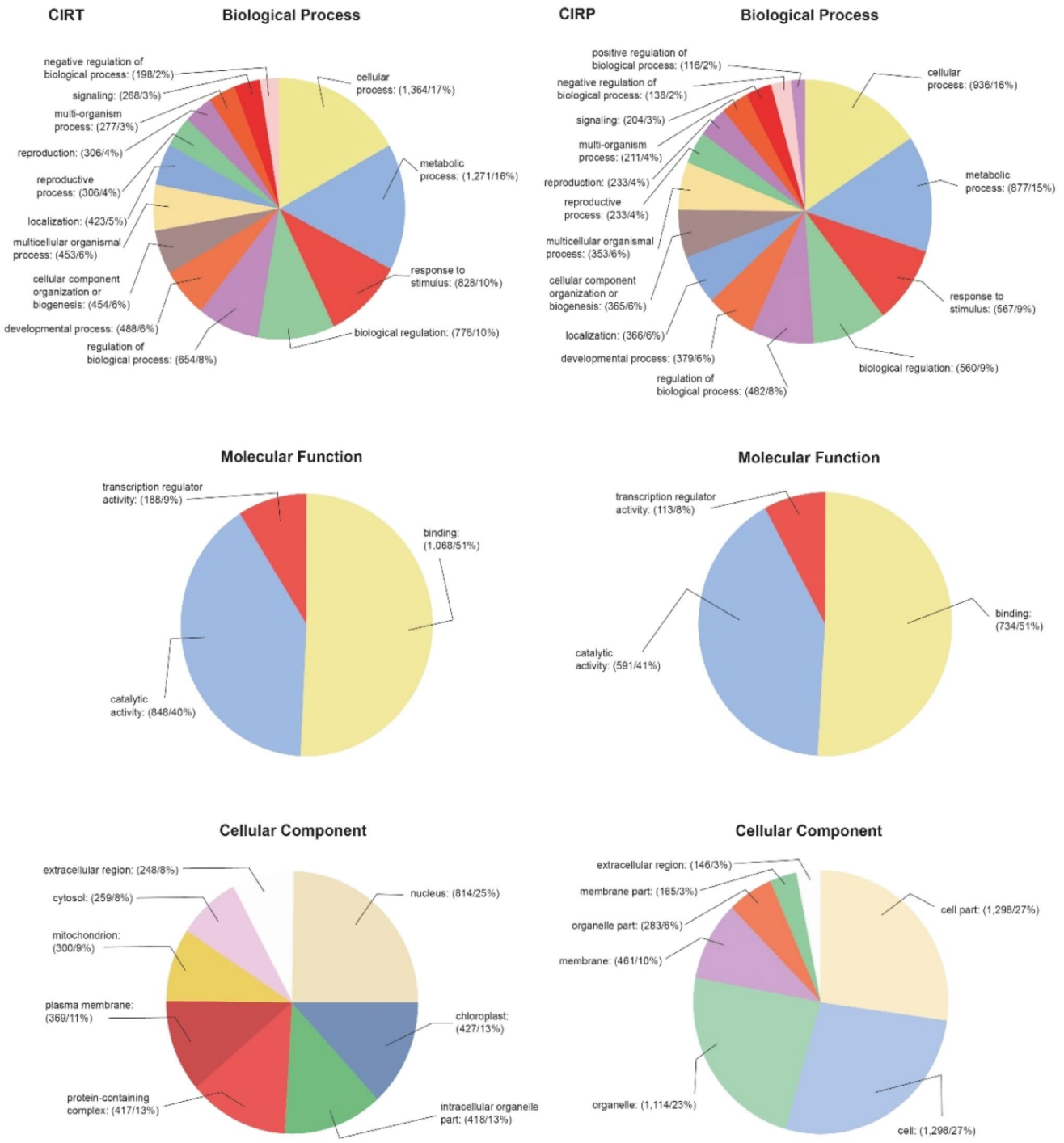


Fig. 2 Gene ontology annotation obtained by microarray analysis from *Nicotiana benthamiana* plants protected with CIRP (homologous construct of the intergenic region of PepGMV) or CIRT (heterologous construct of the intergenic region of ToChLPV) and challenged with PepGMV. Transcripts were grouped into functional Blast2GO groups: biological process, molecular function and cellular component categories with *Z*-scores 2 and 1.5. The percentages of differentially expressed genes during infection in each category are shown within the parentheses

2012; Nakasugi et al. 2013) were observed in the plants protected with homologous CIRP and heterologous CIRT constructs; as DCL2, DCL4, AGO3, AGO5, AGO6, AGO7, AGO10, nuclear RNA polymerase D2B (NRPD2B/POL IV), NRPD1A, Dawdle (DDL), defective in meristem silencing 3 (DMS3), dsRNA-binding protein 3 (DRB3), DRB4, DRB5, domains rearranged methylase 3 (DRM3), RDR6, chromo-methylase 3 (CMT3), PPR, SU(VAR)3–9 homolog

Table 2 List of upregulated genes in the transcriptome of protected *Nicotiana benthamiana* plants, associated with defense response, stress, and systemic acquired resistance

Gene model	Protein name	GO term	*Nicotiana benthamiana*	GO or product	CIRP 25	CIRT 26
AT2G13360	AGT	Response to stress	AGT1 NbD006757 NbS00019758g0008.1 Niben101Scf00797g18009.1	Serine-glyoxylate aminotransferase	1.5	2
AT2G14170	ALDH6B2	Oxidation–reduction	mmsA NbD000775 ALDH6B2 NbS00024307g0010.1	Response to oxidative stress	2	2
AT2G19560	EER5	Response to stress	Niben101Scf01633g00029.1 Niben101Scf07229g00011.1 EER5 NbS00028917g0001.1	Response to stress	1.5	1.5
AT1G60940	SNRK2.10	Response to stress	Niben101Scf01993g08016.1 Niben101Scf01970g 08001.1 SRK2B NbS00005219g0010.1	Response to stress	1.5	1.5
AT2G24850	TAT3	Response to stress	Nf		1.5	1.5
AT2G30360	CIPK11	Response to stress	WNK4 NbD010417 CIPK11 NbS00028941g0005.1	Response to stress	2	1.5
AT2G38730	RCA	Systemic acquired resistance	Niben101Scf04098g00017.1 NbS00029897g0007.1	Systemic acquired resistance	1.5	2
AT3G11840	PUB24	Hypersensitive response	PUB24 NbD001850 NbS00032295g0001.1	Defense response	2	1.5
AT3G11930	AT3G11930	Response to stress	Niben101Scf09610g00017.1 NbS00029709g0002.1	Response to stress	1.5	2
AT3G23250	MYB15	Defense response	MYB4-like NbD008259 Niben101Scf01013g01003.1 NbS00011517g0003.1	Defense response	2	2
AT3G32920	CSNSA	Defense response	Nf		2	1.5
AT3G58620	TTL4	Response to stress	Nf	Nf	2	2
AT4G08500	MAPK MEKK1	Response to stress	STK10 Niben101Scf03906g03013.1 MEKK1 NbS00011174g0009.1	Response to stress	1.5	1.5
AT4G19530	AT4G19530	Defense response	Nf	Nf	2	2
AT4G19840	ATPP2-A1	Hypersensitive response	Niben101Scf03867g02041.1 Niben101Scf02174g03009.1 ATPP2-A1 NbS00041802g0007.1	Protein phloem protein 2-LIKE A1	2	1.5
AT4G29100	AT4G29100	Defense response	bHLH68-like NbD002325 NbS00049384g0003.1	Defense response	2	2
AT4G30480	AT4G30480	Hsp90 TPR1	TTC1 Niben101Scf03964g04005.1 NbS00020723g0011.1	TTC1	1.5	1.5
AT4G31550	WRKY11	Response to stress	WRK22 NbD013410 Niben101Scf01721g08007.1 NbS00042305g0004.1	Response to stress	2	1.5

Table 2 (continued)

Gene model	Protein name	GO term	*Nicotiana benthamiana*	GO or product	CIRP 25	CIRT 26
AT5G09420	ATTOC64-V	Hypersensitive response	OM64 NbD010896 Niben101Scf01370g00007.1 NbS00018282g0024.1	Hypersensitive response	1.5	2
AT5G37930	AT5G37930	Ubiquitin-protein	NbS00011136g0002.1		2	2
AT5G40010	AATP1	Systemic acquired resistance	AATP1 NbD010669.1 NbS00033104g0001.1	AATP1	1.5	1.5
AT5G40990	GLIP1	Systemic acquired resistance	Nf	Nf	1.5	1.5
AT5G42020	BIP2	Response to stress	BIP5 Niben101Scf04126g01011.1 NbS00020855g0001.1	BIP5	2	2
AT5G45800	MEE62	Systemic acquired resistance	Probable LRR NbS00022774g0009.1 NbS00016395g0015.1	LRR	2	2
AT5G50720	HVA22E	Response to stress	HVA22-like protein Niben101Scf03886g04003.1 NbS00031130g0003.1	Response to stress	1.5	1.5
AT5G55990	CBL2	Hypersensitive response	CBL2 NbS00029252g0013.1	CBL2	1.5	1.5

The following table shows a list of upregulated genes in the transcriptome of *Nicotiana benthamiana* plants protected with CIRP (homologous construct of the intergenic region of PepGMV) or CIRT (heterologous construct of the intergenic region of ToChLPV) constructs and challenged with PepGMV that are associated with defense response, stress, and systemic acquired resistance. Annotation data obtained from different databases genome and transcriptome assemblies available for *N. benthamiana* Niben0.4.4 (NbS000) and Niben1.0.1 (Bombarely et al. 2012). Nbv3K, Nbv6.1 (Nakasugi et al. 2013). NbD (Kourelis et al. 2018). *Nf* not found

1 (SUVH1), SUVH5 and HUA enhancer 1 (HEN1). These upregulated genes, which were activated by both constructs, participate in interwoven pathways of gene regulation and plant defense that generate and use different types of sRNAs (siRNA, miRNA, ta-siRNA, nat-siRNA and hc-siRNA) to participate in viral defense, developmental regulation, stress response and transcriptional silencing (Eamens et al. 2008; Nakasugi et al. 2013; Borges and Martienssen 2015). In plants protected with the homologous CIRP construct upregulated genes included AGO4a because AGO9 from *A. thaliana* is homologous to AGO4 of *N. benthamiana* AGO4b (Nakasugi et al. 2013), as well as DRM2, variant in methylation 1 (VIM1) and SUVH2. In contrast, in plants protected with the heterologous CIRT construct, upregulated genes included RDR2 and SUVH4. In addition, genes upregulated by CIRP but not CIRT and vice versa were associated with the synthesis of different siRNAs and methylation (Eamens et al. 2008).

Downregulated genes observed in both plants protected with homologous CIRP and heterologous CIRT constructs included DCL1, DCL3, AGO1, AGO2, RNA Pol II (NRPB1), DRB1, DRB2, DRM1, SUVH3 and SUVH6. Genes that were only downregulated in the plants protected with the homologous CIRP construct included RDR2 and SUVH4, whereas those only observed in the plants protected with the heterologous CIRT construct included AGO4a, DRM2, VIM1 and SUVH2.

In addition, the protein network analysis using STRING allowed the molecular interactions to be visualized among the differentially expressed genes identified from the microarray. The results of this analysis identified groups of proteins involved in RNAi-activated pathways by CIRT (Fig. 3a) and CIRP (Fig. 3b) constructs, such as the major proteins involved in biogenesis of different small RNAs (sRNAs), including secondary siRNAs and other core components that participate in initiation and maintenance of RNA-directed DNA methylation (RdDM) (Eamens et al. 2008; Nakasugi et al. 2013; Borges and Martienssen 2015).

RT-qPCR gene expression analysis

To validate the reference genes, the relative expression level of target genes was analyzed, and the Actin (ACT) gene was used for data normalization. Figure 4 shows the RT-qPCR results for seven RNAi-associated genes, previously identified as being differentially expressed by microarray analyses of *N. benthamiana* plants protected with homologous CIRP and heterologous CIRT constructs and inoculated with PepGMV. These genes include DCL2, DCL3, DCL4, AGO1-1, AGO2, RDR6, and PPR1, all of which exhibited significant differences in expression ($p < 0.05$).

Table 3 List of transcripts associated with RNAi obtained of protected *Nicotiana benthamiana* plants

TAIR locus	Protein	Description	Locus in *Nicotiana benthamiana*	CIRP up or down regulation	CIRT up or down regulation
AT1G01040	DCL1 (DICER-LIKE 1)	Dicer is an RNA helicase involved in miRNA and siRNA processing and virus-induced gene silencing	DCL1 Nbv3K605750463 Nbv6.1trP38701 Niben101Scf03304g01025.1 NbS00015538g0006.1	-0.33	-0.41
AT3G03300	DCL2 (DICER-LIKE 2)	Functions in the antiviral silencing response. Involved in the production of ta-siRNAs	DCL2 Nbv3K725833766 Nbv6.1trP4604 Niben101Scf08272g00021.1 Niben101Scf06666g01011.1	0.70	0.53
AT3G43920	DCL3 (DICER-LIKE 3)	Required for endogenous RDR2-dependent siRNA (but not miRNA) formation	DCL3 Nbv3K585704110 Nbv6.1trP10636	-0.43	-0.53
AT5G20320	DCL4 (DICER-LIKE 4)	Catalyzes processing of ta-siRNA precursors in a distinct sRNA biogenesis pathway. Involved in the production of 21-nt primary siRNAs from both inverted repeat constructs and endogenous sequences as well as the RDR6-dependent 21-nt secondary siRNAs involved in long-range cell-to-cell signaling. Binds DRB4, a dsRNA-binding protein	DCL4 Nbv3K725837175 Nbv3K625768999 Nbv6.1trP61740	0.829	0.93
AT1G48410	AGO1 (ARGONAUTE 1)	Encodes an RNA slicer that selectively recruits micro-RNAs and siRNAs	AGO1a Nbv3K705826800 Nbv6.trA73469 Niben101Scf12841g03019.1 AGO1b Niben101Scf08137g02022.1 Niben101Scf05146g06007.1	-1.1	-1.06
AT1G31280	AGO2 (ARGONAUTE 2)	Encodes an argonaute protein that binds viral siRNAs and is involved in antiviral defense response	Nbv3K585706870 Nbv3K785652117 Nbv3K705830082	-1.05	-1.7
AT1G31290	AGO3 (ARGONAUTE 3)	Functions in the defense response to viruses, gene silencing by RNA the regulation of DNA-templated translations and transcription	AGO2 Nbv3K585706870	0.82	1.193
AT2G27040	AGO4 (ARGONAUTE 4)	Protein involved in siRNA-mediated gene silencing. Loss-of-function mutations reduce site-specific CpNpG and CpHpH methylation and increase susceptibility to bacterial pathogens	AGO4a Nbv3K585737054 AtAGO9—AGO4b Nbv3K5745626388 Niben101Scf05519g01007.1	0.18	-0.123
AT2G27880	AGO5 (ARGONAUTE 5)	Required for antiviral RNA silencing. confers resistance to Potato virus X	AGO5 Nbv3K585731374 Nbv6.1trP59647 Niben101Scf04371g04008.1	2.13	1.05

Table 3 (continued)

TAIR locus	Protein	Description	Locus in *Nicotiana benthamiana*	CIRP up or down regulation	CIRT up or down regulation
AT2G32940	AGO6 (ARGONAUTE 6)	Encodes a nuclear-localized 879-amino-acid protein that contains conserved PAZ and PIWI domains and is important for the accumulation of specific heterochromatin-related siRNAs and for DNA methylation and TGS	AGO6 Nbv3K705827462 Nbv6.1trP35996	0.49	0.693
AT1G69440	AGO7 (ARGONAUTE 7)	Required for the accumulation of TAS3 ta-siRNAs but not miR171, miR173, miR390 or mi391	AGO7 Nbv3K585720936 Nbv6.1trP11465 Niben101Scf20224g00004.1 Niben101Scf00272g02007.1 NbD002754.1	0.063	0.59
AT5G43810	AGO10 (ARGONAUTE 10)	Function in the defense response to viruses, gene silencing by RNA and miRNA metabolic process	AGO10 Nbv3K585734208 Nbv6.1trP33879 Niben101Scf01240g11006.1 NbD010099.1	0.961	1.954
AT3G18090	Pol IV NRPD2B	Encodes a subunit of RNA polymerase IV (RNA polymerase D). NRPD2b is closely associated with NRPD2a but has lower levels of transcription and does not affect endogenous siRNA when mutated	NRPD2b NbS00016929g0017.1	0.69	1.74
AT1G63020	Pol IV NRPD1A SDE4	Encodes one of the two alternative largest subunits of putative plant-specific RNA polymerase IV (RNA polymerase D). Required for PTGS	NRPD1A Nbv3K585708997	0.84	1.17
AT4G35800	RNA Pol II (NRPB1)	Encodes the unique largest subunit of nuclear DNA-dependent RNA polymerase II	NRPB1 NbC23346514g0001.1	-0.81	-0.74
AT3G20550	DDL (DAWDLE)	Participates in the production of miRNAs involved in miRNA gene silencing	Nf	0.614	1.05
AT3G49250	DMS3 (DEFECTIVE IN MERISTEM SILENCING 3)	Can potentially link nucleic acids in facilitating RNA1-mediated epigenetic modification involving secondary siRNA and spreading of DNA methylation	DMS3 NbS00047856g0005.1 Niben101Scf08455g00014.1	1.48	1.26
AT1G09700	DRB1 (DSRNA-BINDING PROTEIN 1)	Encodes a nuclear dsRNA-binding protein. Involved in mRNA cleavage	DRB1 Nbv3K605753726 DRB1501 NbD009575.1 Niben101Scf01181g02012.1	-2.44	-2.036
AT2G28380	DRB2 (DSRNA-BINDING PROTEIN 2)	Encodes a cytoplasmic dsRNA-binding protein. A maternally expressed imprinted gene. DRB2 and DRB4 have antagonistic impacts on polymerase IV-dependent siRNA levels	DRB2 Nbv3K585718488 Niben101Scf02669g05009.1 NbD013708	-0.46	-0.58

Table 3 (continued)

TAIR locus	Protein	Description	Locus in *Nicotiana benthamiana*	CIRP up or down regulation	CIRT up or down regulation
AT3G26932	DRB3 (DSRNA-BINDING PROTEIN 3)	dsRNA-binding protein 3; methylation-mediated antiviral defense	DRB3 Nbv3K725608215	1.24	2.12
AT3G62800	DRB4 (DSRNA-BINDING PROTEIN 4)	Encodes a nuclear dsRNA-binding protein that specifically interacts with DCL4. May regulate DCL4 function and thereby affect miRNA biogenesis and also has an impact on polymerase IV-dependent siRNA levels	DRB4 Nbv3K725839976 Niben101Scf05841g01021.1	0.63	1.45
AT5G41070	DRB5 (DSRNA-BINDING PROTEIN 5)	Encodes a dsRNA-binding protein	DRB5 Nbv3K585684100	1.44	1.46
AT5G15380	DRM1 (DOMAINS REARRANGED METHYLASE 1)	Encodes a MTase involved in de novo DNA methylation and the maintenance of asymmetric methylation of DNA sequences	DRM1 NbS00047903g0006.1	-0.88	-1.56
AT5G14620	DRM2 (DOMAINS REARRANGED METHYLASE 2)	Functions in DNA methylation, the defense response to fungus, gene silencing, and histone H3K9 methylation	DRM2 NbS00009663g0003.1	3.11	-1.37
AT3G17310	DRM3 (DOMAINS REARRANGED METHYLASE3)	Required for normal maintenance of non-CG DNA methylation, establishment of RNA-directed DNA methylation triggered by repeat sequences and accumulation of repeat-associated sRNAs	DRM3 Nbv3K745624408 DRM2a NbD010724.1 Niben101Scf01334g06016.1	0.27	1.26
AT4G11130	RDR2 (RNA-DEPENDENT POLYMERASE 2)	Encodes an RNA-dependent RNA polymerase required for endogenous siRNA (but not miRNA) formation	RDR2 Nbv3K625766705 Niben101Scf04296g00019.1	-1.55	0.569
AT3G49500	RDR6 (RNA-DEPENDENT POLYMERASE 6) SDE1	Encodes an RNA-dependent RNA polymerase. Involved in the biogenesis of ta-siRNAs and other siRNAs. Required for PTGS and natural virus resistance	RDR6 Nbv3K585707928 Niben101Scf12609g01010.1	1.54	1.61
AT1G69770	CMT3 (CHROMO-METHYLASE 3)	Encodes a chromomethylase involved in methylating cytosine residues at non-CG sites. Involved in gene silencing	CMT3 Nbv3K625768297 Niben101Scf02006g04014.1	0.50	0.75
AT1G02420	PPR (PENTATRICOPEPTIDE REPEAT)	Function unknown	PPR NbS00000911g0002.1 Niben101Scf00151g05009.1 NbD001629.1	1.06	0.156
AT1G57820	VIM1 (VARIANT IN METHYLATION 1)	Functions in DNA methylation of cytosine, DNA methylation of cytosine within a CG sequence, and the maintenance of DNA methylation	VIM1 NbS00027916g0013.1 Niben101Scf01582g09001.1 ORTH2 NbD012430.1	1.29	-0.55

Table 3 (continued)

TAIR locus	Protein	Description	Locus in *Nicotiana benthamiana*	CIRP up or down regulation	CIRT up or down regulation
AT5G04940	SUVH1 (SU(VAR)3–9 HOMOLOG 1)	Involved in epigenetic control of gene expression and acts as a histone MTase	SUVH1 NbS00008825g0019.1	0.35	0.19
AT2G33290	SUVH2 (SU(VAR)3–9 HOMOLOG 2)	Involved in epigenetic control of gene expression and acts as a histone MTase	SUVH2 NbC23204936g0001.1	0.095	-0.23
AT1G73100	SUVH3 SU(VAR)3–9 HOMOLOG 3)	Involved in epigenetic control of gene expression and acts as a histone MTase	SUVH3 NbS00008825g0007.1	-0.091	-0.23
AT5G13960	SUVH4 SU(VAR)3–9 HOMOLOG 4)	Encodes an H3K9-specific MTase involved in the maintenance of DNA methylation	SUVH4 NbS00002211g0017.1 NbD040130.1	-0.17	0.13
AT2G35160	SUVH5 SU(VAR)3–9 HOMOLOG 5)	Exhibits histone MTase activity in vitro and contributes to the maintenance of H3-mK9 (methylation of histone H3 at Lys 9) and CMT3-mediated non-CG methylation in vivo	SUVH5 NbS00025360g0007.1 Niben101Scf00614g00025.1 NbD005519.1	1.83	1.89
AT2G22740	SUVH6	MTase involved in histone methylation	SUVH6 NbS00018104g0005.1 NbD001814.1	-0.5	-0.29
AT4G20910	HEN1 HUA ENHANCER 1	Involved in de novo methylation, methylates the terminal nucleotide of 24-nt sRNAs	HEN1 Nbv3K645785075 NbD011168.1	0.48	0.71
AT1G05950	AT1G05950	Methylation DNA repair protein	RECA NbD009068.1 Niben101Scf01116g01005.1 Unnamed protein NbS00029630g0016.1 NbS00029754g0004.1 NbS00043925g0028.1 Niben101Scf02044g06013.1	3.86	2.44
AT1G65660	SMP1	Gene silencing pre-mRNA-splicing factor SLU7-like	slu7 NbD028145 SMP1 NbS00031927g0008.1	2.78	2
AT2G04660	APC2	Gene silencing	APC2 Niben101Scf10322g01013.1 NbS00038059g0007.1	2	2
AT2G19930	RDR3 (RNA-DEPENDENT POLYMERASE 3)	Gene silencing by RNA	RDR3 NbD002592 Niben101Scf00257g00018.1 Niben101Scf04189g00002.1 RDR5 NbS00054825g0009.1	1.5	1.5

Table 3 (continued)

TAIR locus	Protein	Description	Locus in *Nicotiana benthamiana*	CIRP up or down regulation	CIRT up or down regulation
AT3G11440	MYB65 MYB DOMAIN PROTEIN 65	DNA binding Contains a binding site for miRNA159	MYB16 NbD007160 MYB65 NbS00013240g0015.1	2	2
AT3G12210	AT3G12210	DNA binding protein	Niben101Scf12783g01008.1 NbS00028950g0007.1	2	2
AT3G13682	LDL2 LSD1-LIKE2	Involved in H3K4 methylation	LDL2 Niben101Scf01976g00017.1 NbS00024949g0008.1	1.5	1.5
AT3G26932	DRB3 DSRNA- BINDING PROTEIN3	Gene silencing	DRB1 Niben101Scf09603g04002.1 DRB3 Nbv3K725608215	2	2
AT3G57300	INO80 ATINO80	Gene silencing DNA repair Genome stability maintenance	INO80 NbD012748 Niben101Scf01634g02020.1 NbS00008749g0009.1	1.5	1.5
AT4G16420	ADA2B	Gene silencing Chromatin remodeling	ADA2B Niben101Scf03129g01013.1 ADA2B NbS00013870g0004.1	2	2
AT4G29830	VIP3 VERNALIZATION INDEPENDENCE 3	Involved in histone H3-K36 methylation and histone H3-K4 methylation	Niben101Scf02790g03009.1 VIP3 NbS00040297g0001.1	1.5	2
AT5G23570	SGS3 SUPPRESSOR OF GENE SILENCING 3	Required for posttranscriptional gene silencing and natural virus resistance	SGS3 NbE44071533 Niben101Scf01326g11010.1 Niben101Scf05278g04003.1 Nbv3K785651293 NbS00001969g0002.1	2	2
AT5G55920	OLI2 OLIGOCELLULA 2	Methylation	OLI2 NbS00015992g0001.1	1.5	1.5

This table shows a list of transcripts obtained by microarray analysis of *Nicotiana benthamiana* plants protected with CIRP (homologous construct of the intergenic region of PepGMV) or CIRT (heterologous construct of the intergenic region of ToChLPV) and challenged with PepGMV. The numbers in the up- or down regulation columns indicate fold changes in the expression of the transcripts associated with RNAi. Annotation data obtained from different databases with genome and transcriptome assemblies available for *N. benthamiana* Niben0.4.4 (NbS000) and Niben1.0.1 (Bombarely et al. 2012). Nbv3K, Nbv6.1 (Nakasugi et al. 2013). NbD (Kourelis et al. 2018). *Nf* not found

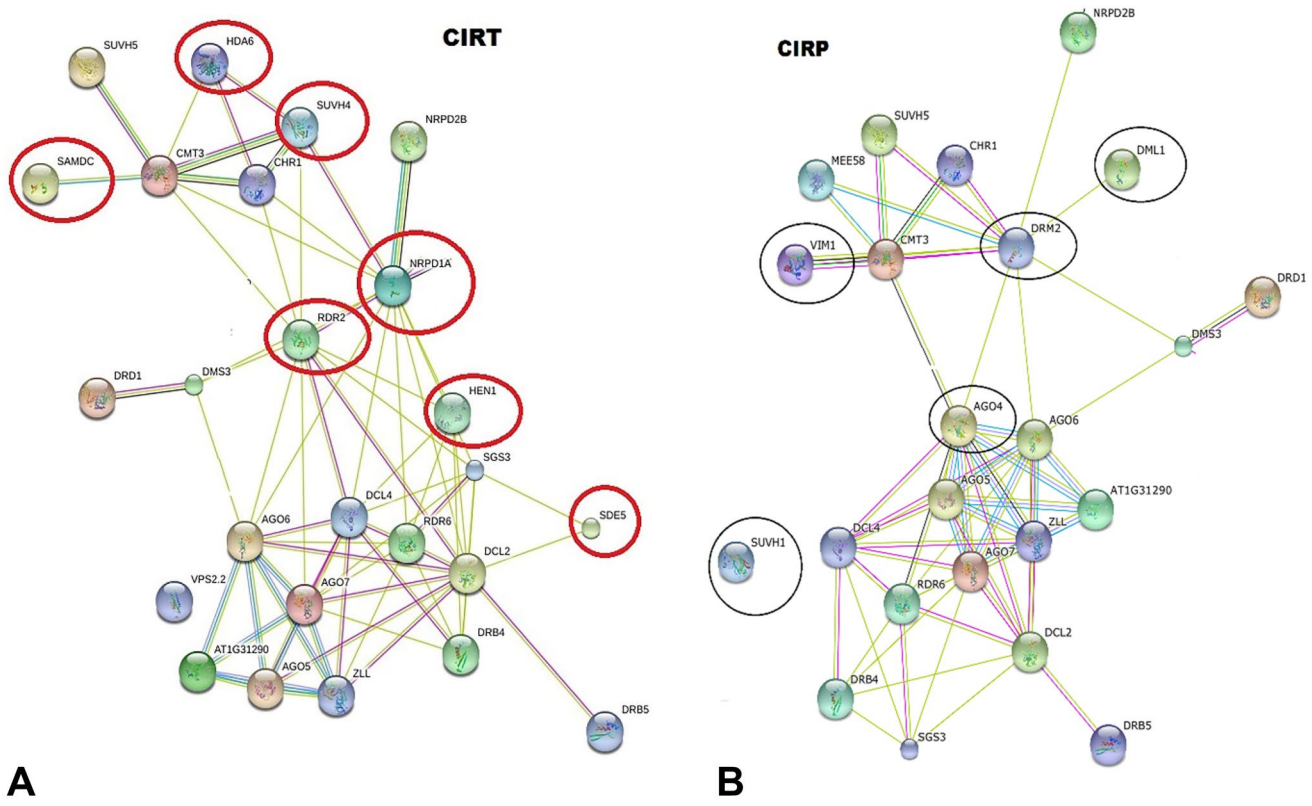


Fig. 3 STRING network of functional protein associations with gene silencing. **a** High connectivity is observed among the proteins associated with gene silencing and methylation in CIRT (construct of the intergenic region of ToChLPV), and **b** subset of proteins involved in gene silencing and methylation in CIRP (construct of the intergenic region of PepGMV). The colors of lines connecting the proteins indicate an interaction

The qPCR analysis showed that genes RDR6, AGO2, DCL3 and AGO1-1 had higher expression levels 55, 38, 35, and 31%, respectively when the plants were protected with heterologous CIRT constructs, whilst the same genes in plants protected with homologous CIRP constructs represented 8, 10, 8, and 17, respectively. Expression levels at the controls were close to zero. DCL4 had higher expression level 6% when the plants were protected with heterologous CIRT constructs than when the plants were protected with homologous CIRP constructs 2%, and in the control with virus, it had an expression level of 1.3%. However, PPR1 and DCL2 showed a similar expression pattern when both constructs were used for protection and similar in the controls. PPR1 and DCL2 had an expression level of 6 and 2%, respectively, when the plants were protected with heterologous CIRT constructs and when they were protected with homologous CIRP constructs, representing 40 and 27%, respectively. Whilst the control with virus cv PPR1 and DCL2 had an expression level of 10 and 11%, respectively, and in the controls without virus PPR1 and DCL2 had higher expression levels that represented 160 and 130%, respectively.

Discussion

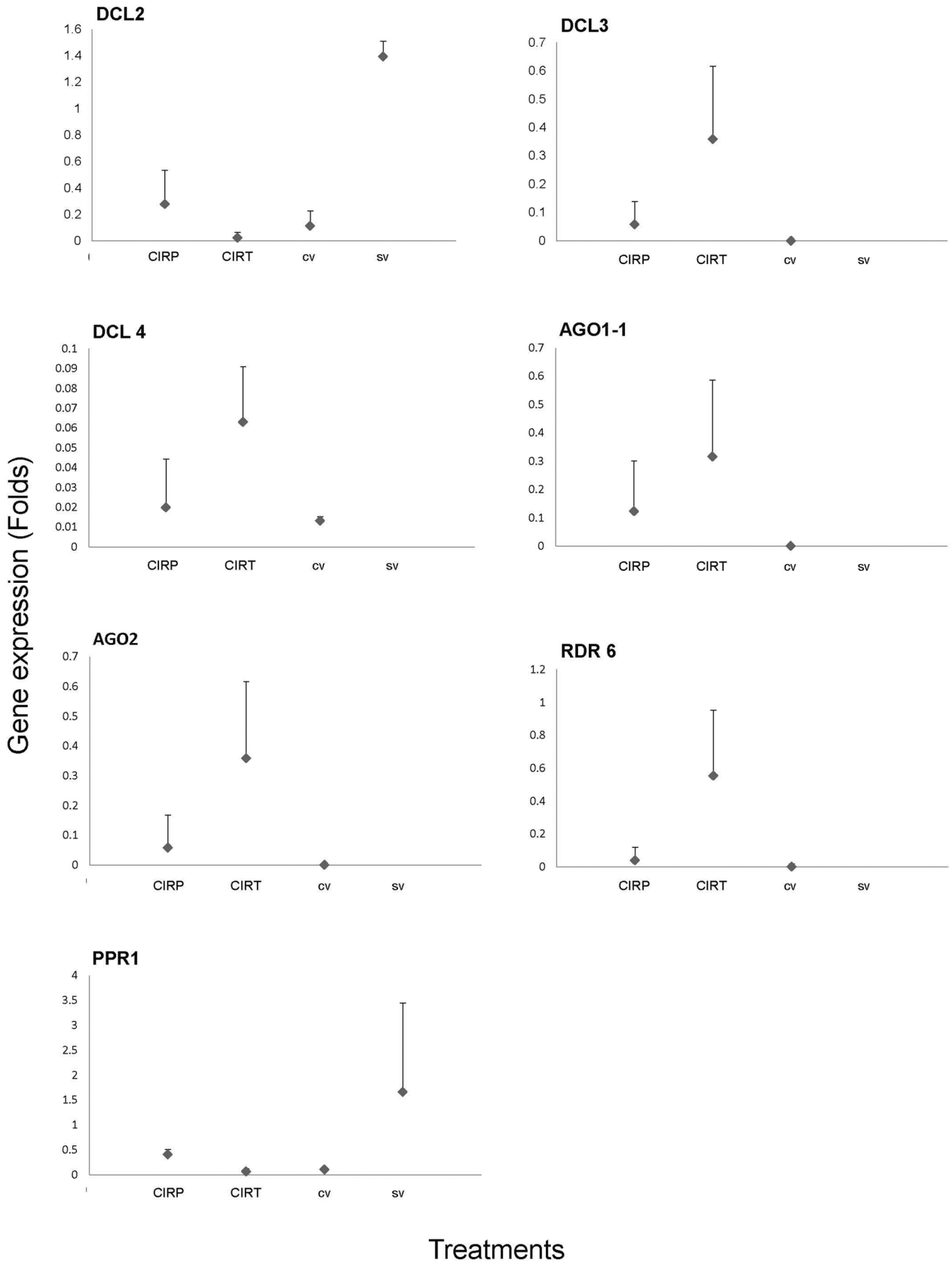
The results of this study indicate that homologous CIRP (construct of the intergenic region of PepGMV) and heterologous CIRT (construct of the intergenic region of ToChLPV) against infection of the PepGMV activate key components of the RNAi gene silencing mechanism. Global gene expression results demonstrated that the overexpression of some key RNAi and methylation-associated components could have been activated by heterologous dsRNA obtained from ToChLPV. Upregulated genes

activated by both constructs participated in interwoven pathways of gene regulation and plant defense that generate and use different types of sRNAs (siRNA, miRNA, ta-siRNA, nat-siRNA and hc-siRNA) to participate in viral defense, development regulation, stress response and activate transcriptional silencing (Eamens et al. 2008; Nakasugi et al. 2013). The results showed that the mechanisms activated in response to infection by PepGMV in *N. benthamiana* recognized the dsRNAs generated from CIRP and CIRT and caused the overexpression of DICER as DCL2, DCL3 and DCL4 (Fig. 5). DICER has been reported to cleave dsRNAs, and these dsRNAs may then generate siRNAs of different sizes, including primary and secondary siRNAs (Wang et al. 2011). Small siRNAs are assembled in the multiprotein complex RISC (Li et al. 2017), and the systemic movement of siRNAs among cells can occur through the phloem (Qin et al. 2017; Zhang et al. 2019). DCL2 acts directly in viral control in a hierarchical manner, participates in the generation of sRNAs, and activates the production of a second series of siRNAs called secondary or transitive siRNAs. In addition, DCL2, DCL3 and DCL4 are known to participate in the processing of endogenous siRNAs in plants, which are categorized as secondary siRNAs, while DCL4 also participates in the defense against viruses by inducing the production of primary siRNAs (Yoshikawa et al. 2005; Axtell et al. 2006; Borges and Martienssen 2015). In this study, microarray and RT-qPCR analyses confirmed that DCL2 and DCL4 were activated by the homologous CIRP and heterologous CIRT constructs. RDR6 is involved in the pathway that triggers the biogenesis of secondary RNAi in cell-to-cell signaling, and plants with mutated RDR6 have shown to be more susceptible to begomoviral infections than wild-type plants (Qu et al. 2005). A transcript associated with RDR6 is SDE5, which has RNA helicase activity and functions with RDR6 in the generation of dsRNA from specific dsRNA to produce trans-acting small-interfering RNA (ta-siRNA), which participates in RNA interference (Hernandez-Pinzon et al. 2007). Both CIRP and CIRT constructs activated SGS3, another transcript that directly interacts with RDR6 in the ta-siRNA production pathway (Xie et al. 2012; Li et al. 2017). HEN1, which is a crucial factor in biogenesis of siRNAs and miRNAs that methylates and protects against uridylation (Movahedi et al. 2018), was also activated by homologous CIRP and heterologous CIRT constructs. In addition, NRPD1A can maintain RNA silencing and transitivity through DNA methylation, participating in sRNA production and amplification together with NRPD2B/Pol IV (Eamens et al. 2008).

Genome and transcriptome microarray data from *A. thaliana* vs different genome and transcriptome assembly versions of *N. benthamiana* were compared, resulting in the identification of genes homologous to transcripts associated with those involved in defense response, stress, systemic acquired resistance; even some genes that participate in MAPK cascades involved in signaling multiple defense responses as the defense gene activation- were identified (Ichimura et al. 2002; Meng and Zhang, 2013) (Table 2), and RNAi (Table 3). The results indicated that AGO3 from *A. thaliana* is similar to AGO2 of *N. benthamiana*, AGO1 is homologous to AGO1a or AGO1-1 from *N. benthamiana* and that AGO9 from *A. thaliana* is homologous to AGO4b from *N. benthamiana* (Bombarely et al. 2012; Nakasugi et al. 2013; Kourelis et al. 2018). All of those components were activated by the heterologous CIRT construct, suggesting that CIRT can activate transcriptional silencing.

The protein network analysis using STRING allowed visualizing molecular interactions among the differentially expressed genes induced by the CIRT (Fig. 3a) and CIRP (Fig. 3b) constructs. In both cases, the network of proteins was involved in pathways associated with RNAi, including proteins involved in biogenesis of different small RNAs, secondary siRNAs, other core components that participate in the initialization and maintenance of RNA-directed DNA methylation (RdDM) in *N. benthamiana* and other dicots (Eamens et al. 2008; Nakasugi et al. 2013; Borges and Martienssen 2015). Furthermore, both constructs could activate PTGS and TGS.

The RT-qPCR results (Fig. 4) for genes associated with RNAi showed that PPR1 was expressed in protected plants with homologous CIRP and heterologous CIRT constructs and in the controls, with higher expression levels in sv. PPR1 is known to participle in growth and developmental stages in plant structure and plant genomes have up to five hundred PPR genes per genome whereas non-plant genomes encode only two to six PPR proteins (Klepikova et al. 2016). High expression levels in sv might be normal in plants, and they were affected by exogenous material in the other treatments. PPR1 belongs to the clade of genes that encode PPR proteins, which have been documented to play important roles in antiviral defense in plants (Wu et al. 2016). In addition, PPR1 participates in the reaction cascade by which gene silencing is propagated via the SGS3/RDR6/DCL4 pathway, and their transcription depends on trans-acting siRNAs (tasiRNAs) (Li et al. 2017). An alternate RNAi route involves small RNAs called ct-siRNAs derived from transcription encoders, which have been shown to function as a regulatory system to maintain controlled protection systems and prevent cells from being overloaded by the overexpression of foreign genes (Zhang et al. 2015). This route solely focuses on the defense against the transcription of invasive DNA (Alvarez et al. 2010), and these small RNAs as well as ta-siRNAs depend on interactions with RDR6, SGS3, DCL2, DCL4 and AGO1 (Li and Wang 2018). The microarray and RT-qPCR analysis results were congruent, indicating that these transcripts were expressed in the assayed plants, when


DCL2
DCL3
DCL 4
AGO1-1
AGO2
RDR 6
PPR1
CIRP
CIRT
cv
sv
Gene expression (Folds)
Treatments

◂**Fig. 4** RT-qPCR expression analysis of DCL2, DCL4, AGO1, AGO2, RDR6 and PPR1 in *Nicotiana benthamiana* plants protected with CIRP (homologous construct of the intergenic region of PepGMV) or CIRT (heterologous construct of the intergenic region of ToChLPV) and infected with PepGMV. Means indicate significant difference ($p < 0.05$) among treatments CIRP, CIRT, cv (positive control) and sv (negative control)

heterologous constructs derived from ToChLPV and homologous to PepGMV are used, because viral loads decreased in (95.6 and 99.5%, respectively), as previously described by Medina-Hernández et al. (2013). DCL2 has been reported to be involved in processing endogenous siRNAs in plants as well in the defense against viruses but it also has specific roles in regulation of genome expression and maintenance (Vazquez and Hohn 2013), which may explain its high expression levels observed in negative control (sv) plants.

The high expression levels of AGO1-1 and AGO2 provide evidence for the activation of RNAi in plants protected with homologous CIRP and heterologous CIRT constructs and infected with PepGMV. Furthermore, several studies have documented the participation of NbAGO1 and NbAGO2 in defense mechanisms against viruses (Ghoshal and Sanfaçon 2014; Kontra et al. 2016; Odokonyero et al. 2017; Paudel et al. 2018).

The results of this study indicate that plants protected with homologous and heterologous constructs against PepGMV activate key components of the RNAi gene silencing mechanism (RNAi). Despite the sequence identity of DNA-A of PepGMV with ToChLPV was 52% and that of the fragments used was approximately 50% with six regions longer than 22 nt of both viruses, with highest identity. This result contributes to understanding cross protection using RNAi, and might be efficient for controlling these pathogens, even in mixed infections (Robinson et al. 2014; Yousaf et al. 2015; Chen et al. 2016; Rasool et al. 2016). Evaluating heterologous dsRNAs is important due to the occurrence of mixed infections in nature and may result in the development of strategies that focus on diseases with symptoms that are difficult to diagnose and control. However, to our knowledge the majority of published studies have not compared transcripts between homologous and heterologous sequences to the challenged virus. Such comparisons may increase our understanding of virus-virus interactions and provide information to confront unknown etiologies. The results in this study were consistent with those that have demonstrated that each type of dsRNA produces a different type of small RNA that functions in diverse pathways involved in gene silencing or DNA methylation activation (Matzke and Mosher 2014). The increased expression of some key factors involved in the TGS and PTGS responses play a crucial role in silencing and epigenetic regulation (Ding 2010; Saze et al. 2012). In this study, different specialized pathways were activated

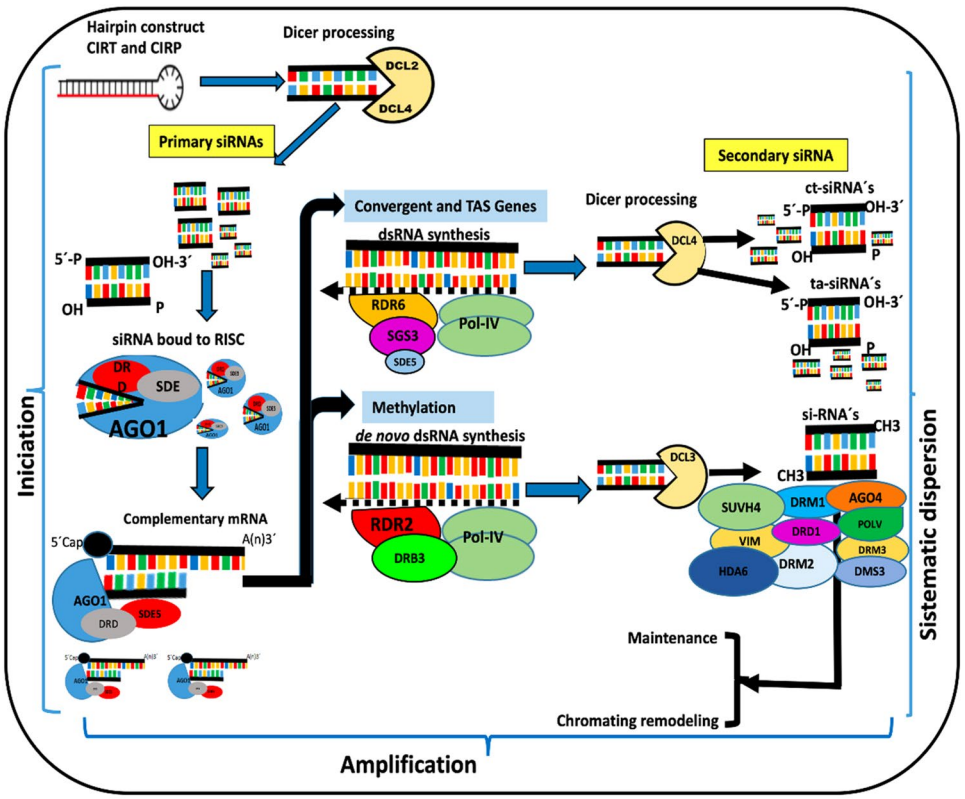


Fig. 5 Schematic representation of the proposed model for RNAi-mediated protection of *Nicotiana benthamiana* against PepGMV infection with CIRP (homologous construct of the intergenic region of PepGMV) and CIRT (heterologous construct of the intergenic region of ToChLPV) based on the results of microarrays and RT-qPCR analysis from the this study and those of several other studies on this topic

and involved in the production and function of different classes of sRNAs that are required to trigger and propagate the PTGS and TGS responses.

Acknowledgements This research was financed by the Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico) through projects SEP2007-84777 and SEP2015-253828 "Analysis of the regulatory function of sRNAs in a gene silencing system as a mechanism of protection against mixed begomoviral infections" granted to R.J. Holguín-Peña. The authors are grateful to Gerardo Rafael Hernández García for his support in figure editing, the Laboratory of Phytopathology, CIBNOR, México, and Diana Fischer for editorial services.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Diana Medina Hernández, Mayela Vargas Salinas, Guadalupe Fabiola Arcos Ortega and Irasema Elizabeth Luis Villaseñor. The first draft of the manuscript was written by Diana Medina Hernández, Mayela Vargas Salinas, Guadalupe Fabiola Arcos Ortega and all authors commented on previous versions of the manuscript. Review and editing: Mayela Vargas Salinas and Guadalupe Fabiola Arcos Ortega. Funding acquisition: Ramón Jaime Holguín Peña. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

References

Abhary MK, Anfoka GH, Nakhla MK, Maxwell DP (2006) Post-transcriptional gene silencing in controlling viruses of the *Tomato yellow leaf curl virus* complex. Arch Virol 151:2349–2363. https://doi.org/10.1007/s00705-006-0819-7

Alvarez ME, Nota F, Cambiagno DA (2010) Epigenetic control of plant immunity. Mol Plant Pathol 11:563–576. https://doi.org/10.1111/j.1364-3703.2010.00621.x

Axtell MJ, Jan C, Rajagopalan R, Bartel DP (2006) A two-hit trigger for siRNA biogenesis in plants. Cell 127:565–577. https://doi.org/10.1016/j.cell.2006.09.032

Bombarely A, Rosli HG, Vrebalov J et al (2012) A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. Mol Plant-Microbe Interact 25:1523–1530

Borges F, Martienssen RA (2015) The expanding world of small RNAs in plants. Nat Rev Mol cell Biol 16:727–741

Cárdenas-Conejo Y, Arguello-Astorga G, Poghosyan A et al (2010) First report of *Tomato yellow leaf curl virus* co-infecting Pepper with *Tomato chino La Paz virus* in Baja California Sur. Mexico Plant Dis 94:1266. https://doi.org/10.1094/PDIS-06-10-0444

Chellappan P, Masona MV, Vanitharani R et al (2004) Broad spectrum resistance to ssDNA viruses associated with transgene-induced gene silencing in cassava. Plant Mol Biol 56:601–611. https://doi.org/10.1007/s11103-004-0147-9

Chen H, Lin C, Tsai W et al (2016) Resistance to viral yellow leaf curl in tomato through RNAi targeting two Begomovirus species strains. J Plant Biochem Biotechnol 25:199–207. https://doi.org/10.1007/s13562-015-0325-7

Conesa A, Götz S (2008) Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics 2008:619832

Conesa A, Götz S, García-Gómez JM et al (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676

De Barro PJ, Liu S-S, Boykin LM, Dinsdale AB (2011) *Bemisia tabaci*: a statement of species status. Annu Rev Entomol 56:1–19

Ding S-W (2010) RNA-based antiviral immunity. Nat Rev Immunol 10:632

Eamens A, Vaistij FE, Jones L (2008) NRPD1a and NRPD1b are required to maintain post-transcriptional RNA silencing and RNA-directed DNA methylation in *Arabidopsis*. Plant J 55:596–606. https://doi.org/10.1111/j.1365-313X.2008.03525.x

Franceschini A, Szklarczyk D, Frankild S et al (2012) STRING v9.1: protein–protein interaction networks, with increased coverage and integration. Nucleic Acids Res 41:D808–D815

García-Arenal F, Zerbini FM (2019) Life on the edge: geminiviruses at the interface between crops and wild plant hosts. Annu Rev Virol 6(1):411–433

Gharsallah-Chouchane S, Gorsane F, Nakhla MK et al (2008) Evaluation of two gene-silencing constructs for resistance to tomato yellow leaf curl viruses in *Nicotiana benthamiana* plants. Acta Virol 52:143–149

Ghoshal B, Sanfaçon H (2014) Temperature-dependent symptom recovery in *Nicotiana benthamiana* plants infected with tomato ringspot virus is associated with reduced translation of viral RNA2 and requires argonaute 1. Virology 456–457:188–197. https://doi.org/10.1016/j.virol.2014.03.026

Hernandez-Pinzon I, Yelina NE, Schwach F et al (2007) SDE5, the putative homologue of a human mRNA export factor, is required for transgene silencing and accumulation of trans-acting endogenous siRNA. Plant J 50:140–148

Herr AJ, Baulcombe DC (2004) RNA silencing pathways in plants. In: Cold Spring Harbor symposia on quantitative biology. Cold Spring Harbor Laboratory Press, pp 363–370

Ichimura K, Shinozaki K, Tena G et al (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci 7:301–308

Klepikova AV, Kasianov AS, Gerasimov ES et al (2016) A high resolution map of the *Arabidopsis thaliana* developmental transcriptome based on RNA-seq profiling. Plant J 88:1058–1070

Kontra L, Csorba T, Tavazza M et al (2016) Distinct effects of p19 RNA silencing suppressor on small RNA mediated pathways in plants. PLoS Pathog 12:e1005935

Kourelis J, Kaschani F, Grosse-Holz FM et al (2018) Re-annotated *Nicotiana benthamiana* gene models for enhanced proteomics and reverse genetics. bioRxiv 373506

Li F, Wang A (2018) RNA decay is an antiviral defense in plants that is counteracted by viral RNA silencing suppressors. PLoS Pathog 14:e1007228

Li F, Wang Y, Zhou X (2017) SGS3 cooperates with RDR6 in triggering geminivirus-induced gene silencing and in suppressing geminivirus infection in *Nicotiana benthamiana*. Viruses 9:247

Lin CY, Ku HM, Tsai WS et al (2011) Resistance to a DNA and a RNA virus in transgenic plants by using a single chimeric transgene construct. Transgenic Res 20:261–270. https://doi.org/10.1007/s11248-010-9412-7

Liu D, Shi L, Han C et al (2012) Validation of reference genes for gene expression studies in virus-infected *Nicotiana benthamiana* using quantitative real-time PCR. PLoS One 7:e46451

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25:402–408

Matzke MA, Mosher RA (2014) RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. Nat Rev Genet 15:394

Medina-Hernández D, Rivera-Bustamante R, Tenllado F, Holguín-Peña RJ (2013) Effects and effectiveness of two RNAi constructs for

resistance to *Pepper golden mosaic virus* in *Nicotiana benthamiana* plants. Viruses 5:2931–2945

Méndez-Lozano J, Torres-Pacheco I, Fauquet CM, Rivera-Bustamante RF (2003) Interactions between geminiviruses in a naturally occurring mixture: *Pepper huasteco virus* and *Pepper golden mosaic virus*. Phytopathology 93:270–277

Meng X, Zhang S (2013) MAPK cascades in plant disease resistance signaling. Annu Rev Phytopathol 51:245–266

Morales-Aguilar JJ, Rodríguez-Negrete EA, Camacho-Beltrán E et al (2019) Identification of *Tomato yellow leaf curl virus*, *Pepper huasteco yellow vein virus* and *Pepper golden mosaic virus* associated with pepper diseases in northern Mexico. Can J Plant Pathol 41:544–550

Movahedi A, Zhang J, Sun W et al (2018) Plant small RNAs: definition, classification and response against stresses. Biologia (Bratisl) 73:285–294. https://doi.org/10.2478/s11756-018-0034-5

Mubin M, Hussain M, Briddon RW, Mansoor S (2011) Selection of target sequences as well as sequence identity determine the outcome of RNAi approach for resistance against cotton leaf curl geminivirus complex. Virol J 8:122

Nakasugi K, Crowhurst RN, Bally J et al (2013) De novo transcriptome sequence assembly and analysis of RNA silencing genes of *Nicotiana benthamiana*. PLoS One 8:e59534. https://doi.org/10.1371/journal.pone.0059534

Navas-Castillo J, Fiallo-Olivé E, Sánchez-Campos S (2011) Emerging virus diseases transmitted by whiteflies. Annu Rev Phytopathol 49:219–248. https://doi.org/10.1146/annurev-phyto-072910-095235

Odokonyero D, Mendoza MR, Moffett P, Scholthof HB (2017) *Tobacco rattle virus* (TRV)-mediated silencing of *Nicotiana benthamiana* Argonautes (NbAGOs) reveals new antiviral candidates and dominant effects of TRV-NbAGO1. Phytopathology 107:977–987. https://doi.org/10.1094/PHYTO-02-17-0049-R

Paudel DB, Ghoshal B, Jossey S et al (2018) Expression and antiviral function of Argonaute 2 in *Nicotiana benthamiana* plants infected with two isolates of *Tomato ringspot virus* with varying degrees of virulence. Virology 524:127–139. https://doi.org/10.1016/j.virol.2018.08.016

Pumplin N, Voinnet O (2013) RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. Nat Rev Microbiol 11:745–760

Qin C, Li B, Fan Y et al (2017) Roles of dicer-like proteins 2 and 4 in intra- and intercellular antiviral silencing. Plant Physiol 174:1067. https://doi.org/10.1104/pp.17.00475

Qu F, Ye X, Hou G et al (2005) RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*. J Virol 79:15209. https://doi.org/10.1128/JVI.79.24.15209-15217.2005

Rasool G, Yousaf S, Amin I et al (2016) Transient expression of synthetic coat protein gene of *Cotton leaf curl Burewala virus* in tobacco (*Nicotiana benthamiana*). J Agric Res 54:21–34

Robinson KE, Worrall EA, Mitter N (2014) Double stranded RNA expression and its topical application for non-transgenic resistance to plant viruses. J plant Biochem Biotechnol 23:231–237

Saze H, Tsugane K, Kanno T, Nishimura T (2012) DNA methylation in plants: relationship to small RNAs and histone modifications, and functions in transposon inactivation. Plant Cell Physiol 53:766–784

Szklarczyk D, Morris JH, Cook H et al (2016) The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. Nucleic Acids Res 45:D362–D368. https://doi.org/10.1093/nar/gkw937

Taha O, Farouk I, Abdallah A, Abdallah NA (2016) Use of posttranscription gene silencing in squash to induce resistance against the Egyptian isolate of the *Squash leaf curl virus*. Int J Genomics 2016:1–9

Tenllado F, Llave C, Díaz-Ruíz JR (2004) RNA interference as a new biotechnological tool for the control of virus diseases in plants. Virus Res 102:85–96. https://doi.org/10.1016/j.virusres.2004.01.019

Vazquez F, Hohn T (2013) Biogenesis and biological activity of secondary siRNAs in plants. Scientifica (Cairo) 2013:783253

Voinnet O (2001) RNA silencing as a plant immune system against viruses. Trends Genet 17:449–459. https://doi.org/10.1016/S0168-9525(01)02367-8

Wang X-B, Jovel J, Udomporn P et al (2011) The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative Argonautes in *Arabidopsis thaliana*. Plant Cell 23:1625–1638. https://doi.org/10.1105/tpc.110.082305

Wu WQ, Fan HY, Jiang N et al (2014) Infection of *Beet necrotic yellow vein virus* with RNA4-encoded P31 specifically up-regulates pathogenesis-related protein 10 in *Nicotiana benthamiana*. Virol J 11:118

Wu C, Li X, Guo S, Wong S-M (2016) Analyses of RNA-Seq and sRNA-Seq data reveal a complex network of anti-viral defense in TCV-infected *Arabidopsis thaliana*. Sci Rep 6:36007

Xie M, Ren G, Costa-Nunes P et al (2012) A subgroup of SGS3-like proteins act redundantly in RNA-directed DNA methylation. Nucleic Acids Res 40:4422–4431

Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. Genes Dev 19:2164–2175

Yousaf S, Rasool G, Amin I et al (2015) Evaluation of the resistance against begomoviruses imparted by the single-stranded DNA binding protein VirE2. Pak J Agri Sci 52:887–893

Zhang X, Zhu Y, Liu X et al (2015) Suppression of endogenous gene silencing by bidirectional cytoplasmic RNA decay in *Arabidopsis*. Science 80(348):120–123

Zhang X, Lai T, Zhang P et al (2019) Mini review: revisiting mobile RNA silencing in plants. Plant Sci 278:113–117. https://doi.org/10.1016/j.plantsci.2018.10.025