applied sciences

MDPI

Article

A Method to Produce vsiRNAs in Plants with Cross-Kingdom Gene Silencing Capacity

Hernán de Jesús Villanueva-Alonzo [1], Ana Paulina Haro-Álvarez [2], Arturo A. Alvarado-Segura [3], Raúl Enrique Valle-Gough [4], Juan Gualberto Collí-Mull [5], Alberto Cal-Torres [6], Víctor Ermilo Arana-Argáez [7], Julio César Torres-Romero [7], Oscar Alberto Moreno-Valenzuela [8], Geovanny Nic-Can [9], Benjamín Abraham Ayil-Gutiérrez [10] and Karla Y. Acosta-Viana [6,*]

1 Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", CONACyT-Universidad Autónoma de Yucatán, Av. Itzáes, núm. 490 x calle 59, col. Centro, Mérida 97000, Yucatán, Mexico; hernan.villanueva@correo.uady.mx
2 Instituto de Investigaciones en Ciencias Veterinarias, Universidad Autónoma de Baja California, Carretera, Mexicali-San Felipe Km 3.5, Laguna Campestre, Mexicali 21705, Baja California, Mexico; paulina.haro@uabc.edu.mx
3 Instituto Tecnológico Superior del Sur del Estado de Yucatán, Tecnológico Nacional de México, Carretera Muna-Felipe Carrillo Puerto Tramo Oxkutzcab-Akil km 41+400, Oxkutzcab 97880, Yucatán, Mexico; aalvarado@suryucatan.tecnm.mx
4 Instituto de Ciencias Agrícolas, Universidad Autónoma de Baja California, sin núm., Ejido Nuevo León, Mexicali 21705, Baja California, Mexico; raulvalle18@hotmail.com
5 Instituto Tecnológico Superior de Irapuato, Tecnológico Nacional de México, Carretera Irapuato—Silao km 12.5 Colonia El Copal, Irapuato 36580, Guanajuato, Mexico; juan.cm@irapuato.tecnm.mx
6 Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán, Av. Itzáes, núm. 490 x calle 59, col. Centro, Mérida 97000, Yucatán, Mexico; alberto19-95@hotmail.com
7 Facultad de Química, Universidad Autónoma de Yucatán, Calle 43 No. 613 x Calle 90, Col. Inalámbrica, Mérida 97069, Yucatán, Mexico; victor.arana@correo.uady.mx (V.E.A.-A.); julio.torres@correo.uady.mx (J.C.T.-R.)
8 Unidad de Bioquímica, Centro de Investigación Científica de Yucatán, A.C., Calle 43 No. 130. Col. Chuburná de Hidalgo, Mérida 97000, Yucatán, Mexico; oamv@cicy.mx
9 Facultad de Ingeniería Química, CONACyT-Universidad Autónoma de Yucatán, Periférico Norte, Km. 33.5. Tablaje Catastral 13615. Col. Chuburná de Hidalgo Inn., Mérida 97203, Yucatán, Mexico; geovanny.nic@correo.uady.mx
10 Centro de Biotecnología Genómica, CONACyT-Instituto Politécnico Nacional, Blvd. del Maestro, sin núm. esquina, Reynosa 88710, Tamaulipas, Mexico; bayil@ipn.mx
* Correspondence: aviana@correo.uady.mx; Tel.: +52-999-924-5755

Abstract: Plants have evolved defense mechanisms to suppress viral transcription and replication by transcriptional and post-transcriptional gene silencing mediated by virus-derived small interfering RNAs (vsiRNAs). Based on this response, virus-induced gene silencing (VIGS)-based technology has been developed to silence target genes on either host plants or insect pests. This mechanism could also be used for the silencing of genes of interest in the medical field. We used the VIGS vector pEuMV-YP:Krt18, which was obtained by inserting the *Mus musculus* (*M. musculus*) Krt18 sequence into pEuMV-YP:ΔAV1. The objective was to evaluate the capacity of pEuMV-YP:Krt18 to induce *Nicotiana benthamiana* (*N. benthamiana*) production of vsiRNAs of a specific sequence that belongs to neither the plant genome nor the wild virus genome, which were used to induce cross-kingdom gene silencing between plants and mammals. The percentage of vsiRNA for each viral gene was calculated from an sRNA library of *N. benthamiana* plants infected by pEuMV-YP: Krt18. When the vsiRNAs were characterized, it was found that they corresponded to all the genes of the pEuMV-YP:Krt18 vector. These vsiRNAs induced the silencing of the Krt18 gene in *M. musculus* macrophages, supporting the ability to use VIGS vectors in plants as biofactories for the production of sRNAs that induce gene silencing in mammals.

Keywords: vsiRNA; cross-kingdom; VIGS; gene silencing

Citation: Villanueva-Alonzo, H.d.J.; Haro-Álvarez, A.P.; Alvarado-Segura, A.A.; Valle-Gough, R.E.; Collí-Mull, J.G.; Cal-Torres, A.; Arana-Argáez, V.E.; Torres-Romero, J.C.; Moreno-Valenzuela, O.A.; Nic-Can, G.; et al. A Method to Produce vsiRNAs in Plants with Cross-Kingdom Gene Silencing Capacity. *Appl. Sci.* **2022**, *12*, 5329. https://doi.org/10.3390/app12115329

Academic Editors: Michal Sobkowski, Adam Kraszewski and Joanna Romanowska

Received: 6 April 2022
Accepted: 20 May 2022
Published: 25 May 2022

1. Introduction

RNA interference (RNAi) is a sequence-specific gene silencing process mediated by small RNAs (sRNAs) of 18 to 24 nucleotides (nt). Depending on their biogenesis, small RNAs can be classified into small interfering RNAs (siRNAs), microRNAs (miRNAs), or virus-derived interfering RNAs (vsiRNAs) [1–3]. RNAi regulates endogenous and exogenous gene expression at the transcriptional and post-transcriptional levels [1,4]. Briefly, the gene silencing mechanism consists of (i) the formation of double-stranded RNA (dsRNA), (ii) cutting of dsRNA into sRNA of 18–25 nt by the DICER enzyme family, (iii) methylation of sRNA (in plants), (iv) sRNA incorporation into the RNA-induced silencing complex (RISC), (v) recognition of target sequence, and (vi) mRNA degradation or inhibition of transcription or translation [5]. This mechanism occurs in most eukaryotic cells, as has been shown in pioneering studies in animals [6], plants [7], and fungi [8]. This process has an impact on cell differentiation and division and defense against the stress of biotic and abiotic origin [9,10].

The ability of sRNAs to mediate gene silencing is not limited to the cell that produces it but can be transported to other cells and even among organisms from different kingdoms. miRNA168a present in rice has been reported to be found in the blood serum of people who consume rice daily. On the other hand, it has been shown that when miRNA168a is included in the mouse diet, silencing of the gene that codes for the low-density lipoprotein receptor is induced [11]. Other plant miRNAs have therapeutic potential; for example, the honeysuckle plant (*Lonicera japonica*) is used in traditional Chinese medicine, whose active substance is miRNA2911, which can inhibit the replication of influenza A and SARS-CoV-2 viruses [12,13]. Other therapeutic properties attributed to plant miRNAs are anticarcinogenic [14–16], anti-inflammatory, and immunomodulatory properties of the immune system [17].

Therefore, the use of plant miRNAs is a promising alternative to combat diseases in mammals. However, these applications are limited to only some plant miRNAs, which naturally have a target with therapeutic potential; alternatively, transgenic plants can be used to generate artificial miRNA (amiRNA) with therapeutic use. Another possibility, which does not require the use of transgenic plants and is technically less complex, is based on the use of the plants' antiviral system, which is the approach addressed in the present investigation.

The vsiRNAs are chemically similar to miRNAs. It has been shown that in some viral infections in plants, vsiRNAs are produced to trigger gene-silencing mechanisms against viral genomes [4,18–20]. From this defense mechanism of plants, virus-induced gene silencing (VIGS) vectors have been designed to induce the silencing of host genes, substituting part of the viral genome for the sequence of the host gene that is intended to be silenced [21].

Unlike the use of the VIGS vector previously described, we explored the possibility of inducing specific vsiRNAs in *Nicotiana benthamiana* (*N. benthamiana*) for a mammalian gene and using them to silence the gene in *Mus musculus* (*M. musculus*). For this, we used the VIGS vector pEuMV-YP:ΔAV1 based on the *Euphorbia Mosaic Virus* (Euphorbia Mosaic Virus Yucatan Peninsula; EuMV-YP) [22], to which, as a study model, we included a fragment of the Cytokeratin-18 (Krt18) gene from *M. musculus*, which is expressed in macrophages and is not present in *N. benthamiana*. The resulting vsiRNA profile showed that these are distributed throughout the pEuMV-YP:Krt18 genome with greater accumulations for derivatives of the BC1, AC3, AC2, and AC1 genes. The production of Krt18 gene-derived vsiRNA was detected in new tissues, including leaves that develop after pruning the plant. We demonstrated the ability of VIGS using pEuMV-YP:Krt18 to yield vsiRNA, which enables the induction of Krt18 gene silencing in cultures of *M. musculus* macrophages (cross-kingdom silencing).

2. Materials and Methods

2.1. VIGS Vectors

The pEuMV-YP:Krt18 vector was constructed with a fragment of the *M. musculus* Krt18 gene (GenBank: NM_010664) that was amplified using the primers forward 5′ACTCGCTCCACCACCTTCTC3′ and reverse 5′AGACAGAAATCGAGGCACTCAAGG3′, generating a 589 bp amplicon. This amplicon was digested with *Pst* I and *EcoR* V enzymes, generating a 432 pb fragment. This allows the Krt18 fragment to be ligated to pEuMV-YP:ΔAV1, which contains these two enzyme cutting sites (*Pst* I and *EcoR* V) in its multiple cloning site (MCS) [22], which had been digested previously with the same pair of enzymes (Figure 1). To verify that similar sequences of Krt18 are not present in the *N. benthamiana* genome, BLAST was used as a search engine in GenBank. pEuMV-YP:ΔAV1:chlI, which contains a 403 bp DNA fragment from the *Nicotiana tabacum* (*N. tabacum*) Magnesium-chelatase subunit I (ChlI) gene [22], was used as a negative control for the silencing assay.

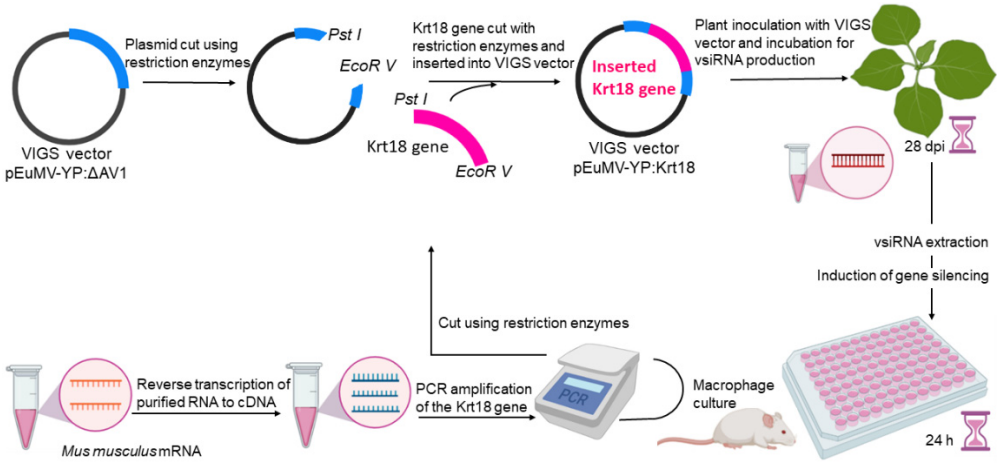

Figure 1. Scheme of the construction of pEuMV-YP:Krt18 vector and production of virus-derived small interfering RNAs (vsiRNAs) in plants with cross-kingdom gene silencing potential (created in biorender.com).

2.2. Nicotiana Benthamiana Culture and VIGS Inoculation

N. benthamiana plants were grown in pots (120 cm^3) containing an Agrolite/Peat moss mixture, cultured at 25 °C under a 16/8 light/dark photoperiod, and fertilized (Ultra Fol™; 1 mL/L) every two weeks. *N. benthamiana* is a widely used experimental host in VIGS vectors studies, and the vector pEuMV-YP:ΔAV1 works efficiently. Furthermore, the production of leaf area generated under culture conditions is adequate for this type of study [22,23].

Plants with four true leaves were inoculated by bombardment with 10 µL of a prepared mixture (1 µm gold; 50 µL 2.5 M $CaCl_2$; 20 µL 0.1 M spermidine; 10 µg DNA from pEuMV-YP:Krt18, or pEuMV-YP:ΔAV1:chlI) [24]. Using a custom-built device, the bombardment was applied at 30 psi at 2 cm from the plant surface using pressurized He as a propellant.

2.3. cDNA Synthesis from sRNAs and Amplification of Krt18 vsiRNA by PCR

The sRNA cDNA (including the vsiRNAs) were synthesized using the microScript microRNA cDNA Synthesis Kit (Norgen Biotek Corp, Thorold, ON, Canada) following the manufacturer's instructions, starting with 1 µg of total RNA from plants with 28 days postinoculation (dpi) with pEuMV-YP:Krt18 (Figure 1). When plants defend themselves against viruses through the gene silencing system, they produce vsiRNAs that can cover the entire viral genome [25], so it is expected that vsiRNAs covering the entire 432 bp sequence of Krt18 will be generated. Therefore, to quantify the concentration of generated vsiRNAs, a region of the Krt18 sequence with a Tm of 60 °C was randomly chosen. Starting with 25 ng of cDNA, the amplification of the Krt18 vsiRNA was performed using the primer

Forward 5′-CCAGACCGAGAAGGAGACCATG-3′ and Universal PCR Reverse Primer through Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Wilmington, NC, USA). The amplification protocol consisted of 1 cycle at 94 °C for 3 min and 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 45 s, using a thermal cycler CFX96 Real-Time System (BioRad Laboratories Inc., Singapore).

For quantification, the PCR product of the Krt18 vsiRNA cDNA was used as the DNA standard. Dilutions of 1.5×10^7, 1.5×10^6, 1.5×10^5, 1.5×10^4, and 1.5×10^3 fM were performed. PCR was performed as described in the previous section. The logarithmic values of vsiRNA concentrations (x-axis) were plotted against the CT values (y-axis). From the fitted linear equation, the vsiRNA concentrations were estimated.

2.4. RNA Extraction and sRNA Enrichment

Total RNA was extracted from the *N. benthamiana* plants at 28 dpi with pEuMV-YP:Krt18. Briefly, leaf tissues (100 mg) were macerated with 1 mL TRIzol (Invitrogen, Waltham, MA, USA) and purified with a Direct-zol™ RNA MiniPrep Plus kit (Zymo Research Corp., Irvine, CA, USA) according to the manufacturer's instructions. The sRNA enrichment was performed using the protocol of Fulneček et al. (2007) [26] as follows: total RNA, previously dissolved in 250 µL of TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA), was heated at 65 °C for 5 min and mixed with 10% polyethylene glycol 8000 (PEG 8000) and 1 M NaCl, followed by incubation for 30 min at 4 °C and centrifuged at 15,000× g for 15 min. This step allows the precipitation of high-molecular-weight RNA, leaving low-molecular-weight RNA in the supernatant. Finally, the supernatant (transferred to a new tube) was precipitated with two volumes of 100% ethanol and one-tenth volume of 3 M sodium acetate pH 5.2 and centrifuged at 15,000× g for 15 min at 4 °C. The sRNA was dissolved in 25 µL of RNase-free water.

2.5. sRNA Sequencing

Total RNAs from five plants were pooled. The pooled samples were quantified by spectrophotometry (Nanodrop, Thermo Scientific), and the quality was verified by agarose gel electrophoresis. After purification and verification, the samples were sent to the Genomic Services Laboratory (Labsergen, Irapuato, Guanajuato, Mexico) for library construction (TruSeq Small RNA) and sequencing (Illumina NextSeq-500 platform; SE, 1 × 36, 10 Mb). The read quality control was performed in the Galaxy platform (usegalaxy.org) with the "FastQC" program, and sequencing adapters (small RNA) were removed with "Trim Galore". The resulting sequences were filtered by "Manipulate Fastq" based on an 18–24 nt size using regular expressions. The resulting files were used for the abundance estimation by single ends in "Salmon" with the coding sequences of the EuMV-YP viral components (Component A, No. DQ318937; component B, No. DQ318938) in sense and antisense.

The siRNAs were mapped by HISAT2 on the Galaxy platform using components A and B of EuMV-YP as the reference genome. The alignment was carried out with the default parameters of the platform, and the resulting file was converted to SAM format for further processing and visualization. The number of reads of each sequence was arranged in a Microsoft Excel® sheet to generate the graphs and the heatmap.

Clusters containing more than 700 vsiRNAs were deemed vsiRNA hotspots. The guanine and cytosine contents of each sequence were calculated with ApE software. A two-tailed t-test was performed to compare the percentage of guanine and cytosine between the vsiRNAs.

2.6. Extraction of Peritoneal Macrophages from BALB/c Mice

Male mice of the BALB/c strain were anesthetized with chloroform in a closed container for 5 min and sacrificed by cervical dislocation according to the Mexican Official Standard NOM-062-ZOO-1999 (technical specifications for production, care, and use of laboratory animals). Cold PBS was introduced into the peritoneal area. After a few seconds, the peritoneal fluid was removed, two washes were performed with PBS, and the obtained

pellet was resuspended in DMEM supplemented with 10% FBS. Finally, the number of viable cells was counted by trypan blue exclusion in a Neubauer chamber, and they were seeded in plates, as required for each experiment. For assays, 48 h cultured macrophages at 37 °C and 5% CO_2 were used.

2.7. Transfection of sRNAs into Macrophages of BALB/c Mice

Following the instructions of the Xfect™ siRNA Transfection Reagent kit (Clontech, Takara Bio. Co., Ltd., Dalian, China), 10 µg of sRNAs were transfected with 4 µL of the Xfect transfection polymer into 2×10^5 macrophages in 24-well plates to assess the level of Krt18 gene silencing. The plates were incubated for 24 h at 37 °C and 5% CO_2 (Figure 1).

2.8. Determination of the Relative Expression of Krt18 in Macrophages

Twenty-four hours after transfection, the relative expression level of the Krt18 gene in macrophages from BALB/c mice was determined. Three mouse macrophage cultures were used: the first one was only treated with the transfecting agent. A mouse macrophage culture was transfected with 10 µg of sRNA from plants infected with pEuMV-YP:Krt18 and, as a negative control, 10 µg of sRNA from plants infected with pEuMV-YP:ΔAV1:chlI were used. Total RNA was extracted in the presence of TRIzol reagent™ (Invitrogen), followed by RNA purification using the Direct-zol™ miniprep Plus kit (Zymo Research). After treatment with DNase (Ambion Inc., Austin, TX, USA), reverse transcription of total RNA was performed using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific), following the manufacturer's instructions. Real-time PCR was performed using 5 ng of cDNA and the primers Krt18-F 5′-TTGCCGCCGATGACTTTAGAG-3′ and Krt18-R 5′-GATTCCACCCATTCCCGCCA-3′. All quantifications were normalized to the housekeeping gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH), whose primers are gapdh-R 5′-GGCATGGACTGTGGTCATGA-3′ and gapdh-F 5′-TTCACCACCATGGAGAAGGC-3′, using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific). The relative expression was determined by the $2^{-\Delta\Delta Ct}$ method. Data are expressed as the mean of three biological replicates with three technical replicates, and the significance was analyzed with a two-tailed Student's *t*-test.

3. Results

3.1. vsiRNA Production

A fragment of the Krt18 gene of *M. musculus* was isolated via PCR. This fragment was cloned into pEuMVYP:ΔAV1 [22] to produce the VIGS vector pEuMV-YP:Krt18. *N. benthamiana* plants were inoculated with pEuMV-YP:Krt18, and symptoms of infection were visible after the first week and remained throughout the plants' life cycle. The symptoms consisted of deformed leaves in the first week; after that, chlorotic mosaic leaves appeared (Figure 2a–c), and vsiRNAs derived from the Krt18 fragment were detected with an accumulation of 0.386 pmol/g by leaf (28 days postinoculation), quantified by RT–PCR.

3.2. vsiRNA Sequencing Analysis

A library of sRNA from plants 28 dpi was constructed and sequenced on the Illumina NextSeq-500 platform. Of 16 million reads, 7,499,184 corresponded to 18–24 nt sequences. From this total, 68% belonged to the *N. benthamiana* genome, and 32% belonged to the VIGS vector genome (Figure 2d). The VIGS vector genome vsiRNAs were distributed equally between DNA-A (including Krt18) and DNA-B. The distribution of the vsiRNAs in the DNA-A genes was as follows: AV1 gene remainder (0.275%); Krt18 (6.165%); AC3 (8.11%); AC2 (17.40%); AC1 (18.03%); and in the DNA-B genes: BV1 (6.11%); and BC1 (43.44%) (Figure 2e).

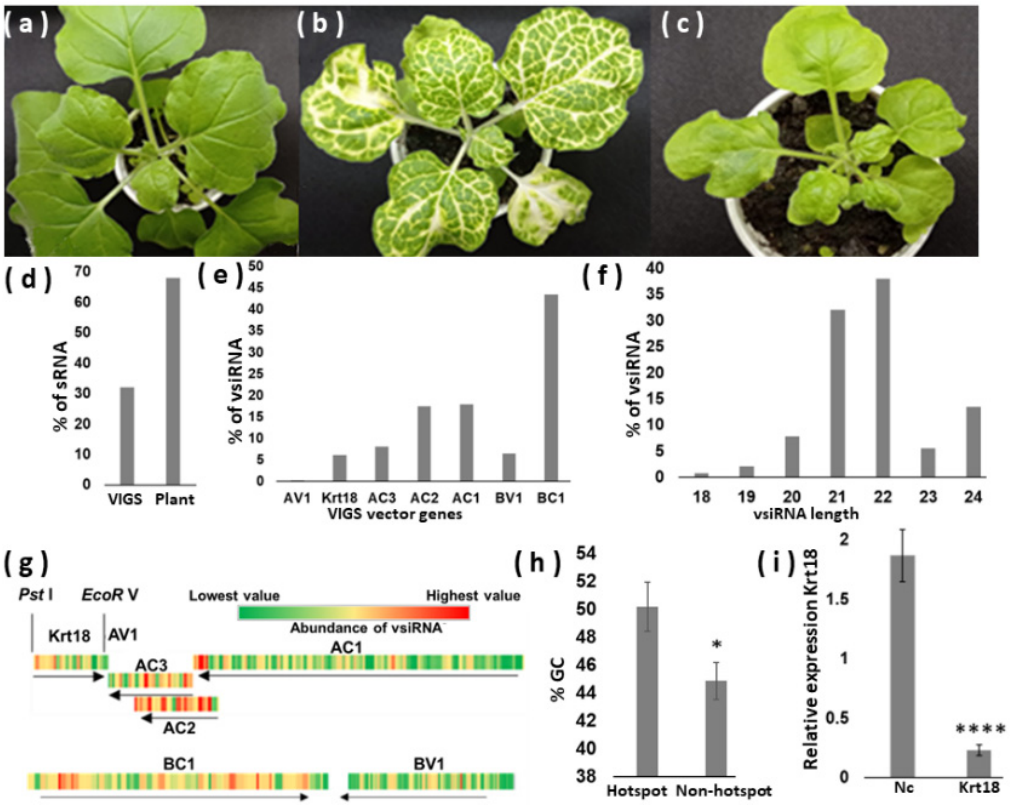

Figure 2. (**a**) *Nicotiana benthamiana* plant uninfected; (**b**) plant infected with pEuMV-YP: ΔAV1:chlI, the white regions are a consequence of the gene silencing of Magnesium-chelatase subunit I (ChlI); (**c**) plant infected with pEuMV-YP:Krt18 that shows deformations on the edges and slight yellow mosaics; (**d**) percentage of sRNA corresponding to VIGS vector (pEuMV-YP:Krt18) and the plant genes; (**e**) percentage of vsiRNAs derived from genes in pEuMV-YP:Krt18; (**f**) distribution by size of the vsiRNAs of plant infected with pEuMV-YP:Krt18; (**g**) heatmap of the pEuMV-YP:Krt18 genome, the red color represents a greater accumulation of vsiRNA, the gold color represents those of medium accumulation, while the green color represents less accumulation; (**h**) average GC content in VIGS vector regions with highest and lowest of vsiRNA accumulation (hotspots and nonhotspots, respectively); *t*-test * *p*-value < 0.01; (**i**) vsiRNA-induced silencing of the Krt18 gene in macrophages of *Mus musculus*, being Nc sRNA from plants 28 days postinoculation (dpi) with pEuMV-YP: ΔAV1:chlI, and Krt18 is sRNA from plants 28 dpi with pEuMV-YP: Krt18; *t*-test **** *p*-value < 0.00001.

Sequencing analysis showed that 21, 22, and 24 nt RNAs were the most abundant (83%) in the VIGS vector genome (Figure 2f). The vsiRNA sequences were differentially accumulated throughout the VIGS vector genome, as shown in the heatmap in Figure 2g. The red color indicates that in that region of the VIGS vector, there was a high accumulation of vsiRNA (hotspot), the gold color, an intermediate accumulation, and the green color, a low accumulation (nonhotspot). When analyzing hotspots, we found that the average GC content is higher than nonhotspots, as shown in Figure 2h.

3.3. Induction of Gene Silencing in Macrophages of Mus musculus

To evaluate whether the sRNAs of plants infected with pEuMV-YP:Krt18 can induce gene silencing in mammalian cells, three mouse macrophage cultures were used: the first one was only treated with the transfecting agent; a mouse macrophage culture was transfected with 10 μg of sRNA from plants infected with pEuMV-YP:Krt18 and, as a negative control, 10 μg of sRNA from plants infected with pEuMV-YP:ΔAV1:chlI were used. After 24 h of incubation, the relative expression of the krt18 gene was more than

eightfold lower than when transfected with the sRNAs derived from pEuMV-YP:ΔAV1:chlI (p-value < 0.0001) (Figure 2i).

4. Discussion

The VIGS vector remained infective in *N. benthamiana* after adding the Krt18 fragment, observing that plant symptoms were similar to those previously reported [22]. It has been reported that the VIGS method can induce gene silencing in host and insect pests using vsiRNA. This is achieved by replacing part of the viral genome with the sequence of interest while preventing any loss of the infective capacity [22]. Gene silencing level is due to the nature of the host and the virus, the stem-like structures that form their mRNA, and the sequence length. For instance, in Begomoviruses, this needs to be between 90 and 800 bp for optimal results [27]. In this study, the vsiRNAs of interest were those derived from a 432 bp fragment of the *M. musculus* Krt18 gene. The dsRNA generated during viral infection [19,28] is subsequently processed by RNAse III, called Dicer (DCL 1–4), to generate siRNAs mainly from 18–24 nt in size. The siRNA derived from pEuMV-YP:Krt18 were mostly 21, 22, and 24 nt, which is consistent with reports for Begomovirus and other viral infections, in which DCL4, DCL2, and DCL3 activity produce 21, 22, and 24 nt siRNA, respectively [29,30].

Begomovirus-derived vsiRNAs can be distributed evenly between the two components (DNA-A and DNA-B) of the viral genome, although, on occasion, the accumulation of one component can be greater [19]. In the present results, the vsiRNA derived from pEuMV-YP:Krt18 accumulated equally between the two components. This is potentially useful in increasing the production of vsiRNA since the B component can be used for VIGS vector modification [27].

Generally, vsiRNAs are derived from all viral genes [25]. In infections with a Begomovirus such as the African Cassava Mosaic Virus, the greatest vsiRNA accumulation corresponds to gene overlap regions [31]. We found that the vsiRNA distribution in the viral genome was similar to what was previously reported [25,31]. Our results agree that the vsiRNAs had greater accumulation in the overlapping genes (Figure 2g), and the expression level of viral genes can influence the greater accumulation of derived vsiRNAs. Additionally, another factor that influenced the greater accumulation of vsiRNA (hotspots) was the % GC (Figure 2h), which coincides with what was reported [3].

In order to estimate the concentration of vsiRNA generated, a region of the Krt18 sequence with a Tm of 60 °C was randomly chosen. The accumulation of vsiRNA derived from the Krt18 sequence at 28 dpi was 0.386 pmol/g from fresh tissue. This quantification corresponded to vsiRNA 5′-CCAGACCGAGAAGGAGACCAUG-3′. However, other vsiRNAs derived from the Krt18 sequence were also found (Figure 2g), so the total concentration of vsiRNA was higher. In addition, a diverse group of vsiRNA sequences can be produced for the same gene, which is more effective for gene silencing than a single sequence [32]. pEuMV-YP:Krt18-derived vsiRNAs efficiently induced Krt18 gene silencing in *Mus musculus* macrophages. This suggests that plants infected with a VIGS vector could be used for the production of vsiRNA for some therapeutic purposes, similar to how some medicinal plants are used, whose active substance is miRNA [12,13,16]. VIGS vectors can be applied to edible plants so that decoctions or consumption of these plants through the diet can be used for therapeutic purposes since mammals can absorb plant miRNA through the gastrointestinal tract and, at the same time, maintain their ability to induce gene silencing in the different organs of the animal [16,33]. However, in vivo studies are still needed. Because of the diversity and quantity of vsiRNAs that are produced by this strategy, it would be interesting to investigate if there is a silencing of additional genes. This could be performed by mRNA sequence analysis of vsiRNA-treated and untreated cells.

5. Conclusions

pEuMV-YP:Krt18 induces *Nicotiana benthamiana* to produce vsiRNAs, which are distributed differentially for all vector genes, including the *Mus musculus* gene sequence. The

antiviral capacity of plants can be used to produce vsiRNAs with a specific sequence, which can induce cross-kingdom gene silencing. Therefore, the plant–VIGS system has the potential to be used as a vsiRNA biofactory for a diversity of applications, such as the health area.

Author Contributions: All authors contributed to the study design. Conceptualization, H.d.J.V.-A. and K.Y.A.-V.; methodology, H.d.J.V.-A., A.C.-T., G.N.-C., V.E.A.-A. and J.C.T.-R.; software, R.E.V.-G., J.G.C.-M. and O.A.M.-V.; formal analysis, H.d.J.V.-A. and A.A.A.-S.; writing—original draft preparation, H.d.J.V.-A. and A.A.A.-S.; writing—review and editing, A.P.H.-Á., B.A.A.-G. and G.N.-C.; supervision, K.Y.A.-V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of the Campus of Biological and Agricultural Sciences of the Universidad Autónoma de Yucatán (protocol code CB-CCBA—2016-005).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Guo, H.; Ingolia, N.T.; Weissman, J.S.; Bartel, D.P. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* **2010**, *466*, 835–840. [CrossRef] [PubMed]
2. Yoshikawa, M.; Han, Y.W.; Fujii, H.; Aizawa, S.; Nishino, T.; Ishikawa, M. Cooperative recruitment of RDR6 by SGS3 and SDE5 during small interfering RNA amplification in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **2021**, *118*, e2102885118. [CrossRef] [PubMed]
3. Ho, T.; Wang, H.; Pallett, D.; Dalmay, T. Evidence for targeting common siRNA hotspots and GC preference by plant Dicer-like proteins. *FEBS Lett.* **2007**, *581*, 3267–3272. [CrossRef] [PubMed]
4. Fu, L.Y.; Liu, Z.Q.; Bai, Y.L. Advances in the RNA-directed DNA methylation in plants. *Chin. J. Biotechnol.* **2006**, *22*, 891.
5. Ruiz-Ferrer, V.; Voinnet, O. Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.* **2009**, *60*, 485–510. [CrossRef]
6. Fire, A.; Xu, S.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E.; Mello, C.C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **1998**, *391*, 806–811. [CrossRef]
7. Napoli, C.; Lemieux, C.; Jorgensen, R. Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* **1990**, *2*, 279–289. [CrossRef]
8. Romano, N.; Macino, G. Quelling: Transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol. Microbiol.* **1992**, *6*, 3343–3353. [CrossRef]
9. Bartel, D.P. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **2004**, *116*, 281–297. [CrossRef]
10. Baulcombe, D. RNA silencing in plants. *Nature* **2004**, *431*, 356–363. [CrossRef]
11. Zhang, L.; Hou, D.; Chen, X.; Li, D.; Zhu, L.; Zhang, Y.; Li, J.; Bian, Z.; Liang, X.; Cai, X.; et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: Evidence of cross-kingdom regulation by microRNA. *Cell Res.* **2012**, *22*, 107–126. [CrossRef]
12. Zhou, Z.; Li, X.; Liu, J.; Dongxia, H.; Chen, Q.; Liu, J.; Kong, H.; Zhang, Q.; Qianyi, Z.; Hou, D.; et al. Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses. *Cell Res.* **2015**, *25*, 39–49. [CrossRef]
13. Zhou, L.K.; Zhou, Z.; Jiang, X.M.; Zheng, Y.; Chen, X.; Fu, Z.; Xiao, G.; Zhang, C.Y.; Zhang, L.K.; Yi, Y. Absorbed plant MIR2911 in honeysuckle decoction inhibits SARS-CoV-2 replication and accelerates the negative conversion of infected patients. *Cell Discov.* **2020**, *6*, 54. [CrossRef]
14. Kumar, D.; Kumar, S.; Ayachit, G.; Bhairappanavar, S.B.; Ansari, A.; Sharma, P. Cross-Kingdom Regulation of Putative miRNAs Derived from Happy Tree in Cancer Pathway: A Systems Biology Approach. *Int. J. Mol. Sci.* **2017**, *18*, 1191. [CrossRef]
15. Stefano, P.; Letizia, Z.; Maurice, K.; Carla, M.; Antonella, M.; Marina, P. MicroRNA from Moringa oleifera: Identification by High Throughput Sequencing and Their Potential Contribution to Plant Medicinal Value. *PLoS ONE* **2016**, *11*, e0149495. [CrossRef]
16. Chin, A.R.; Fong, M.Y.; Somlo, G.; Wu, J.; Swiderski, P.; Wu, X. Cross-kingdom inhibition of breast cancer growth by plant miR159. *Cell Res.* **2016**, *26*, 217–228. [CrossRef]
17. Cavalieri, D.; Rizzetto, L.; Tocci, N.; Rivero, D.; Asquini, E.; Si-Ammour, A. Plant microRNAs as novel immunomodulatory agents. *Sci. Rep.* **2016**, *6*, 25761. [CrossRef]
18. Rodríguez-Negrete, E.A.; Carrillo-Tripp, J.; Rivera-Bustamante, R.F. RNA silencing against geminivirus: Complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *J. Virol.* **2008**, *83*, 1332–1340. [CrossRef]
19. Chellappan, P.; Vanitharani, R.; Fauquet, C.M. Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol.* **2004**, *78*, 7465–7477. [CrossRef]

20. Yan, H.; Bombarely, A.; Xu, B.; Frazier, T.P.; Wang, C.; Chen, P.; Chen, J.; Hasing, T.; Cui, C.; Zhang, X.; et al. siRNAs regulate DNA methylation and interfere with gene and lncRNA expression in the heterozygous polyploidy switchgrass. *Biotechnol. Biofuels* **2018**, *11*, 208. [CrossRef]
21. Robertson, D. VIGS vectors for gene silencing: Many targets, many tools. *Annu. Rev. Plant Biol.* **2004**, *55*, 495–519. [CrossRef]
22. Villanueva-Alonzo, H.J.; Us-Camas, R.Y.; López-Ochoa, L.A.; Robertson, D.; Guerra-Peraza, O.; Minero-García, Y.; Moreno-Valenzuela, O.A. A new virus-induced gene silencing vector based on *Euphorbia mosaic virus*-Yucatan peninsula for NPR1 silencing in *Nicotiana benthamiana* and *Capsicum annuum* var. Anaheim. *Biotechnol. Lett.* **2013**, *35*, 811–823. [CrossRef]
23. Goodin, M.M.; Zaitlin, D.; Naidu, R.A.; Lommel, S.A. *Nicotiana benthamiana*: Its history and future as a model for plant-pathogen interactions. *Mol. Plant-Microbe Interact.* **2008**, *21*, 1015–1026. [CrossRef]
24. Kjemtrup, S.; Sampson, K.S.; Peele, C.; Nguyen, L.V.; Conkling, M.A.; Thompson, W.F.; Robertson, D. Gene silencing from plant DNA carried by a geminivirus. *Plant J.* **1998**, *14*, 91–100. [CrossRef] [PubMed]
25. Liu, C.; Li, F.; Sun, Y.; Zhang, X.; Yuan, J.; Yang, H.; Xiang, J. Virus-derived small RNAs in the penaeid shrimp Fenneropenaeus chinensis during acute infection of the DNA virus WSSV. *Sci. Rep.* **2016**, *28*, 28678. [CrossRef] [PubMed]
26. Fulnecek, J. Isolation and detection of small RNA molecules. *Plant Soil Environ.* **2007**, *53*, 451–455. [CrossRef]
27. Peele, C.; Jordan, C.V.; Muangsan, N.; Turnage, M.; Egelkrout, E.; Eagle, P.; Hanley-Bowdoin, L.; Robertson, D. Silencing of a meristematic gene using geminivirus-derived vectors. *Plant J.* **2001**, *27*, 357–366. [CrossRef] [PubMed]
28. Dalmay, T.; Hamilton, A.; Rudd, S.; Angell, S.; Baulcombe, D.C. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **2000**, *101*, 543–553. [CrossRef]
29. Axtell, M.J. Classification and comparison of small RNAs from plants. *Annu. Rev. Plant Biol.* **2013**, *64*, 137–159. [CrossRef]
30. Deleris, A.; Gallego-Bartolome, J.; Bao, J.; Kasschau, K.D.; Carrington, J.C.; Voinnet, O. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **2006**, *313*, 68–71. [CrossRef]
31. Chellappan, P.; Vanitharani, R.; Ogbe, F.; Fauquet, C.M. Effect of temperature on geminivirus-induced RNA silencing in plants. *Plant Physiol.* **2005**, *138*, 1828–1841. [CrossRef]
32. Parashar, D.; Paingankar, M.S.; Kumar, S.; Gokhale, M.D.; Sudeep, A.B.; Shinde, S.B.; Arankalle, V.A. Administration of E2 and NS1 siRNAs inhibit chikungunya virus replication in vitro and protects mice infected with the virus. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2405. [CrossRef]
33. Zeng, J.; Gupta, V.K.; Jiang, Y.; Yang, B.; Gong, L.; Zhu, H. Cross-kingdom small RNAs among animals, plants and microbes. *Cells* **2019**, *8*, 371. [CrossRef]