Chapter 13

Design, Synthesis, and Functional Analysis of Highly Specific Artificial Small RNAs with Antiviral Activity in Plants

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Abstract

Artificial microRNAs (amiRNAs) and synthetic trans-acting small interfering RNAs (syn-tasiRNAs) are two classes of artificial small RNAs (sRNAs) that have been broadly used to confer antiviral resistance in plants. However, methods for designing, synthesizing and functionally analyzing antiviral artificial sRNAs have not been optimized for time and cost-effectiveness and high-throughput applicability since recently. Here we present a systematic methodology for the simple and fast-forward design, generation, and functional analysis of large numbers of artificial sRNA constructs engineered to induce high levels of antiviral resistance in plants. Artificial sRNA constructs are transiently expressed in *Nicotiana benthamiana* plants, which are subsequently inoculated with the virus of interest. The antiviral activity of each artificial sRNA construct is assessed by monitoring viral symptom appearance, and through molecular analysis of virus accumulation in plant tissues. This approach is aimed to easily identify artificial sRNAs with high antiviral activity that could be expressed in transgenic plants for highly durable antiviral resistance.

Key words Plant virus, Antiviral resistance, Artificial small RNAs, AmiRNAs, Syn-tasiRNAs

1 Introduction

Artificial microRNAs (amiRNAs) and synthetic trans-acting small interfering RNAs (syn-tasiRNAs) are two classes of artificial small RNAs (sRNAs) engineered to selectively silence transcripts in plants, including viral RNAs [1]. AmiRNAs and syn-tasiRNAs are produced in planta by expressing a functional miRNA or tasiRNA precursor with modified miRNA or tasiRNA sequences, respectively. AmiRNAs are generated from DICER-LIKE1 (DCL1) cleavage of precursors with foldback structures, while syn-tasiRNAs are produced after cleavage of a *TAS* precursor by an miRNA/ ARGONAUTE (AGO) complex, RNA-DEPENDENT RNA POLYMERASE 6 conversion of one of the cleavage products to

Kappei Kobayashi and Masamichi Nishiguchi (eds.), *Antiviral Resistance in Plants: Methods and Protocols*,
Methods in Molecular Biology, vol. 2028, https://doi.org/10.1007/978-1-4939-9635-3_13,

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double-stranded RNA, and DLC4-mediated processing of the dsRNA into 21-nucleotide phased syn-tasiRNAs in register with the miRNA-guided cleavage site. Despite their biogenesis pathways are different, both classes of artificial sRNAs associate with an AGO protein, generally AGO1, to target and cleave highly sequence complementary transcripts [2].

AmiRNAs have been extensively used to selectively confer antiviral resistance in transgenic plants [3]. However, because antiviral amiRNAs typically target a single viral RNA site, this resistance can be overcome if virus variants accumulate mutations in the amiRNA target site [4, 5]. To avoid these limitations, multiple amiRNAs have been expressed from different precursors or from a single polycistronic precursor to target several regions within a single viral RNA [6–9]. Syn-tasiRNAs have recently emerged as an alternative to amiRNA to induce antiviral resistance in plants. This is another possibility of expressing multiple artificial sRNAs from a single precursor, which should also allow the targeting of multiple sites within a viral RNA or of multiple sequence unrelated viruses, and therefore could induce more durable antiviral resistance [10]. Indeed, syn-tasiRNAs have already been reported to induce simultaneous resistance to two different viruses [11], and to interfere with viroid infections as well [12].

Despite both classes of artificial sRNAs have been used to confer antiviral resistance, methods for designing, synthesizing and functionally analyzing artificial sRNAs have not been optimized for time and cost-effectiveness and high-throughput applicability since recently [13–15]. In this chapter, we describe a detailed methodology for the automated design, high-throughput generation and functional analysis of large number of artificial sRNA constructs with antiviral activity in plants. First, we detail the design process of highly specific antiviral amiRNAs with the P-SAMS ("Plant Small RNA Maker Suite") web tool [15] and the molecular methods for their cloning into *Bsa*I/*ccd*B "B/c" vectors, a recently reported new generation of amiRNA vectors for efficient gene silencing in plants [13, 14]. Next, the experimental plan to analyze the antiviral activity of large numbers of amiRNA constructs is described. AmiRNA constructs are transiently expressed in *Nicotiana benthamiana* plants, which are subsequently inoculated with the virus of interest. The antiviral activity of each amiRNA construct is assessed by monitoring viral symptoms, and through molecular analysis of virus accumulation in plant tissues. Finally, we describe the design, generation, and functional analysis of syn-tasiRNA constructs that include several artificial sRNA sequences with high antiviral activity, which were previously selected from the initial amiRNA screening. This methodology has been successfully applied to identify highly active artificial sRNAs against *Potato spindle tuber viroid* [12] and *Tomato spotted wilt virus* [16].

2 Materials

2.1 Antiviral AmiRNA Design

1. Computer with Internet connection.
2. Web browser (e.g., Google Chrome, Safari, Mozilla Firefox, Internet Explorer).

2.2 Antiviral AmiRNA Cloning

1. Oligo Annealing buffer: 60 mM Tris–HCl (pH 7.5), 500 mM NaCl, 60 mM $MgCl_2$, 10 mM DTT (*see* **Note 1**).
2. Thermocycler.
3. Water bath.
4. Sterile H_2O.
5. T4 DNA ligase (5 U/μL, Thermo Fisher Scientific).
6. *Bsa*I (10 U/μL, New England Biolabs).
7. Competent cells of *Escherichia coli* (*E. coli*) *ccd*B-sensitive strain (e.g., DH5α, DH10B, TOP10).
8. LB agar plate with kanamycin: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L of Bacto agar, and 50 mg/L of kanamycin.
9. Liquid LB with kanamycin: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 50 mg/L of kanamycin.
10. GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific).
11. B/c vectors: Each vector contains a unique combination of bacterial and plant antibiotic resistance genes. Eudicot amiRNA B/c vectors are *pENTR-AtMIR390a-B/c* (Addgene plasmid 51,778), *pFK210B-AtMIR390a-B/c* (Addgene plasmid 51,777), *pMDC123SB-AtMIR390a-B/c* (Addgene plasmid 51,775) and *pMDC32B-AtMIR390a-B/c* (Addgene plasmid 51,778). Monocot amiRNA vectors are *pENTR-OsMIR390-B/c* (Addgene plasmid 61,468), *pH7WG2B-OsMIR390-B/c* (Addgene plasmid 61,465), *pMDC123SB-OsMIR390-B/c* (Addgene plasmid 61,466), and *pMDC32B-OsMIR390-B/c* (Addgene plasmid 61,467) [13, 14] (Table 1) (*see* **Note 2**).
12. Oligonucleotides

M13-F:	CCCAGTCACGACGTTGTAAAACGACGG
M13-R:	CAGAGCTGCCAGGAAACAGCTATGACC
attB1:	ACAAGTTTGTACAAAAAAGCAGGCT
attB2:	ACCACTTTGTACAAGAAAGCTGGGT

13. Competent cells of *Agrobacterium tumefaciens* GV3101 strain.

Table 1
***BsaI/ccdB*-based ("B/c") vectors for direct cloning of amiRNAs and syn-tasiRNAs**

Vector	Small RNA class	Bacterial antibiotic resistance	Plant antibiotic resistance	GATEWAY use	Backbone	Promoter	Terminator	Plant species
pENTR-AtMIR390a-B/c	amiRNA	Kanamycin	–	Donor	*pENTR*	–	–	–
pFK210B-AtMIR390a-B/c	amiRNA	Spectomycin	BASTA	–	*pGreen III*	*CaMV* 35S	*rbcS*	Eudicots
pMDC123SB-AtMIR390a-B/c	amiRNA	Kanamycin	BASTA	–	*pMDC123*	*CaMV* 2×35S	–	Eudicots
pMDC32B-AtMIR390a-B/c	amiRNA	Kanamycin Hygromycin	Hygromycin	–	*pMDC32*	*CaMV* 2×35S	*nos*	Eudicots
pENTR-OsMIR390-B/c	amiRNA	Kanamycin	–	Donor	*pENTR*			–
pMDC123SB-OsMIR390-B/c	amiRNA	Kanamycin	BASTA	–	*pMDC123*			Monocots
pMDC32B-OsMIR390-B/c	amiRNA	Kanamycin Hygromycin	Hygromycin	–	*pMDC32*			Monocots
pH7WG2B-OsMIR390-B/c	amiRNA	Spectinomycin	Hygromycin	–	*pH7WG2*			Monocots
pENTR-AtTAS1c-B/c	syn-tasiRNA	Kanamycin	–	Donor	*pENTR*	–	–	–
pMDC123SB-AtTAS1c-B/c	syn-tasiRNA	Kanamycin Hygromycin	BASTA	–	*pMDC123*	*CaMV* 2×35S	*nos*	*A. thaliana*
pMDC32B-AtTAS1c-B/c	syn-tasiRNA	Kanamycin Hygromycin	Hygromycin	–	*pMDC32*	*CaMV* 2×35S	*nos*	*A. thaliana*

2.3 Functional Analysis of Antiviral AmiRNAs

2.3.1 Agroinfiltration in Nicotiana benthamiana

1. *Nicotiana benthamiana*: 3–4-week-old *N. benthamiana* plants.
2. LB with antibiotics: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 mg/L rifampicin, and 50 mg/L kanamycin.
3. Sterilized stock solutions: 1 M $MgSO_4$, 1 M $CaCl_2$, 20% glucose, 1 M MES buffer (pH 5.2), and 0.1 M acetosyringone.
4. M9 solution: 6 g/L of Na_2HPO_4, 3 g/L of KH_2PO_4, 0.5 g/L NaCl, and 1 g/L NH_4Cl. Adjust pH to 5.2 with KOH and autoclave. Add 2 mL 1 M $MgSO_4$, 0.1 mL 1 M $CaCl_2$, and 10 mL 20% glucose in sterile hood.
5. Vir Induction medium: Add 5 mL 20% glucose, 5 mL 1 M MES buffer (pH 5.2), 500 μL 0.1 M acetosyringone, 50 μL 1 M $CaCl_2$, and 1 mL 1 M $MgSO_4$ to 500 mL of sterile M9 solution.
6. Infiltration solution: Add 5 mL 1 M $MgCl_2$, 5 mL 1 M MES buffer (pH 5.2), and 750 μL 0.1 M acetosyringone to 500 mL of sterile H_2O.
7. 1 mL syringes without needles.

2.3.2 Analysis of Antiviral AmiRNA Accumulation

Total RNA Extraction

1. TRIzol reagent (Invitrogen).
2. Chloroform.
3. Isopropanol.
4. Cold 75% ethanol.
5. NanoDrop (Thermo Fisher Scientific).

Northern Blot Analysis of AmiRNAs

1. 5× Tris–borate–EDTA (TBE): 54 g/L Tris base, 27.5 g/L boric acid, 20 mL 0.5 M EDTA (pH 8.0).
2. 30% polyacrylamide (37.5:1): Dissolve 29.2 g acrylamide and 0.8 g bisacrylamide in 100 mL H_2O.
3. Urea, electrophoresis grade.
4. TEMED: *N*,*N*,*N*,*N*′-tetramethylethylenediamine.
5. Ammonium persulfate: 10% aqueous solution.
6. Formamide loading buffer: 95% formamide, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF, 5 mM EDTA (pH 8.0).
7. Gel plate, an electrophoresis apparatus, and a power supply.
8. Blotting paper.
9. Whatman Nytran SuperCharge nylon membrane (Sigma-Aldrich).
10. Semidry electroblotting apparatus.
11. UV cross-linker.

12. Probe oligonucleotide: DNA or LNA oligonucleotide complementary to the amiRNA to be analyzed.
13. T4 polynucleotide kinase.
14. [^{32}P] γ-ATP, 6000 Ci/mmol, 10 mCi/mL
15. Mini Quick Spin Oligo Columns (Roche Life Science).
16. PerfectHyb™Plus buffer (Sigma-Aldrich) or equivalent hybridization solution.
17. 20× SSC (saline sodium citrate): 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0.
18. 10% SDS: 10% (w/v) sodium dodecyl sulfate in sterile H_2O.
19. Wash buffer 1: 2× SSC, 0.1% SDS. Prepare with 20× SSC, 10% SDS solution, and sterile H_2O.
20. Wash buffer 2: 0.1× SSC, 0.1% SDS. Prepare with 20× SSC, 10% SDS solution, and sterile H_2O.
21. 3× SSC: Dilute 20× SSC with sterile H_2O.

2.3.3 Virus Inoculation

1. Inoculation buffer: 50 mM potassium phosphate pH 8.0, 1% PVP (polyvinylpyrrolidone) 10, 1% PEG (polyethylene glycol) 6000 and 10 mM 2-mercaptoethanol.
2. Carborundum powder (0.013 mm).
3. Cotton swabs.

2.4 Antiviral Syn-tasiRNA Design

All of the materials required are the same as Subheading 2.1.

2.5 Antiviral Syn-tasiRNA Cloning

1. Syn-tasiRNA vectors: *pENTR-AtTAS1c-B/c* (Addgene plasmid 51774), *pMDC123SB-AtTAS1c-B/c* (Addgene plasmid 51772) and *pMDC32B-AtTAS1c-B/c* (Addgene plasmid 51773) (Table 1).
2. Other reagents listed in Subheading 2.2.

2.6 Functional Analysis of Antiviral Syn-tasiRNA

All of the materials required are the same as Subheading 2.3.

3 Methods

The main steps regarding the design, synthesis, and analysis of antiviral artificial sRNAs are summarized in Fig. 1.

3.1 Antiviral AmiRNA Design

1. Download the virus sequences (e.g., from NCBI).
2. Go to P-SAMS amiRNA Designer website (http://p-sams.carringtonlab.org/amirna/designer).

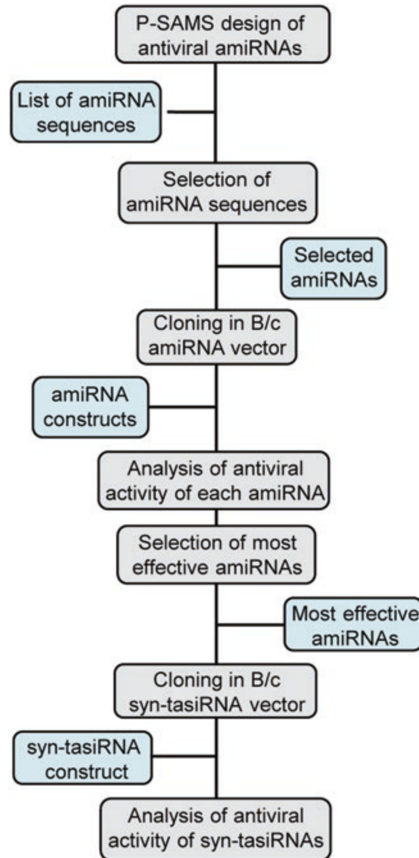

Fig. 1 Diagram of the steps for the design and synthesis of antiviral amiRNAs and syn-tasiRNAs, and for the analysis of their antiviral activity. Each step is described in light grey boxes. The product of each step is shown in light blue boxes

3. Click "Get Started" and then "Design an amiRNA" to start the design process.
4. Select the transcriptome of the species of interest and click "Yes."
5. Click "Option 2" to target an exogenous transcript such as a viral RNA.
6. Enter or paste the FASTA sequence(s) of target viral RNA(s) and click "Next" (*see* **Note 3**).
7. Click "Yes" to have the results automatically filtered based on target specificity (*see* **Note 4**).
8. Click "Submit" to submit the job.
9. Select "Click to see Results" to display the results. AmiRNAs predicted to target uniquely viral RNA(s) are output as "Optimal Results"; amiRNAs predicted to target viral RNA(s) and endogenous transcripts are output as "Sub-optimal Results."

10. Click "Download" to download the result summary including a list of amiRNA sequences together with the sequence of the two oligonucleotides required for cloning each amiRNA insert in compatible amiRNA B/c vectors (Table 1).

3.2 Antiviral AmiRNA Cloning

1. Resuspend the two oligonucleotides in sterile H_2O to a final concentration of 100 μM.
2. Assemble the oligonucleotide annealing reaction in a PCR tube as follows: 2 μL of 100 μM forward oligonucleotide, 2 μL of 100 μM reverse oligonucleotide and 46 μL of oligo annealing buffer.
3. Transfer the tube to a thermocycler set to heat the annealing reaction 5 min at 94 °C and then cool down to 20 °C at a rate of 0.05 °C/s (*see* **Note 5**).
4. Dilute the annealed oligonucleotides to a final concentration of 0.15 μM by mixing 3 μL annealed oligonucleotides and 37 μL H_2O (*see* **Note 6**).
5. Assemble the digestion–ligation reaction as follows: 50 ng B/c vector, 1 μL diluted annealed oligonucleotides, 1 μL 10× T4 DNA ligase buffer, 1 μL of 5 U/μL T4 DNA ligase, 1 μL of 10 U/μL *Bsa*I and add sterile H_2O to 10 μL. Use the amiRNA B/c vector of your choice (Table 1) (*see* **Note 7**).
6. Mix the reactions by pipetting and incubate for 5 min at 37 °C.
7. Transform 1-5 μL of the digestion–ligation product into a *ccd*B-sensitive *E. coli* strain and plate on an LB agar plate with kanamycin.
8. Pick two colonies per construct, grow in 4 mL of liquid LB with kanamycin and purify plasmids with a miniprep kit.
9. Sequence two clones per construct with appropriate oligonucleotides: M13-F and M13-R for *pENTR*-based vectors, attB1and attB2 for *pMDC32B*-, *pMDC123SB*-, *pFK210B*-, or *pH7WG2B*-based vectors.
10. Transform 0.5 μL of the purified plasmid into *A. tumefaciens* GV3101. Plate 1/10 of the culture in a LB agar plate including rifampicin and kanamycin.
11. Incubate the plate at 28 °C during 48 h.
12. Store the plate with grown colonies at 4 °C until use.

3.3 Functional Analysis of Antiviral AmiRNAs

3.3.1 Experimental Setup

The main steps of the experimental plan for the functional analysis of antiviral amiRNAs are summarized in Fig. 2 and described below. Typically, three blocks (A, B, and C) of three plants are required for the functional analysis of each antiviral amiRNA construct. It is highly recommended to compare the activity of

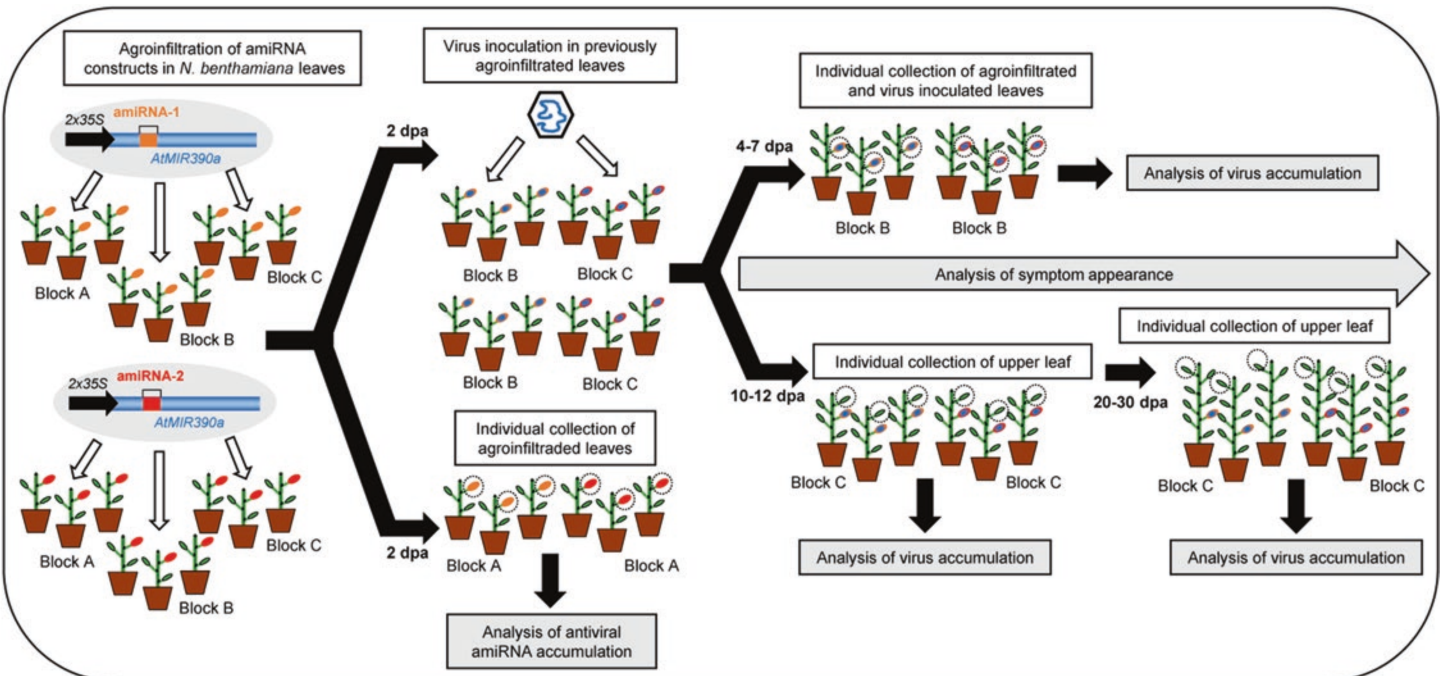

Fig. 2 Experimental plan to functionally analyze antiviral amiRNAs. Main experimental steps are described in white boxes. Main analytical steps are described in light grey boxes. “dpa” refers to days postagroinfiltration. Due to space limitations, the functional analysis plan is shown for only two amiRNA constructs, but this setup can be applied for the functional analysis of multiple amiRNA constructs in large-scale amiRNA screenings

antiviral amiRNAs with a control amiRNA targeting an exogenous sequence other than the viral RNA (*E. coli* β-glucuronidase (GUS), green fluorescent protein (GFP), etc.) (*see* **Note 8**).

3.3.2 Agroinfiltration of N. benthamiana Leaves with Antiviral AmiRNA Constructs

1. For each amiRNA construct to be analyzed, pick a single colony of *A. tumefaciens* transformed with the corresponding construct using a toothpick.
2. Transfer to a culture tube that contains 5 mL of LB with rifampicin and kanamycin.
3. Incubate the starter culture at 28 °C for 24 h on a shaking incubator.
4. Transfer 1–5 mL of the starter culture to 50 mL of LB containing kanamycin in a 250 mL flask.
5. Shake for 4–6 h at 28 °C until OD_{600} ~0.55.
6. Centrifuge 45 mL of culture for 15 min at 5000 × *g* in a 50 mL conical tube.
7. Remove the supernatant and resuspend in equal volume of Vir Induction medium.
8. Transfer back to flasks and shake overnight (~14 h) at 28 °C.
9. Measure OD_{600} of the overnight culture.
10. Calculate the volume of Infiltration solution to resuspend the pellet to an OD_{600} ~1.1.
11. Centrifuge the culture for 15 min at 5000 × *g* in a 50 mL conical tube.
12. Remove the supernatant and resuspend pellet in calculated volume of Infiltration solution.
13. Measure OD_{600}, and normalize the culture to a final OD_{600} of 1.0.
14. Mix the amiRNA culture with an equal volume of a *A. tumefaciens* culture including an empty vector or control construct such as *pMDC32-GUS* [17].
15. Infiltrate the whole abaxial surface of the fourth emerged leaf of nine 3–4-week-old *N. benthamiana* plants (Fig. 2).
16. Two days postagroinfiltration (dpa), collect independently the infiltrated leaf of each of the three plants of block A (Fig. 2). Store tissue at −80 °C until needed.

3.3.3 Analysis of Antiviral AmiRNA Accumulation

This step is recommended to confirm that amiRNAs are being expressed in planta.

Total RNA Extraction

1. Place frozen leaf in a labeled mortar and pour 30–50 mL liquid nitrogen.
2. Lightly crush leaves after liquid nitrogen has completely evaporated; grind vigorously until tissue is powdered.

3. Add 5 mL of TRIzol and continue homogenization to mix partially frozen sample.
4. Transfer homogenized sample to a 15 mL conical tube.
5. Centrifuge at 7000 × *g* for 10 min at 4 °C to pellet cell debris.
6. Transfer supernatant to a new 15 mL conical tube.
7. Add 3 mL of chloroform and shake vigorously for 20 s.
8. Incubate at room temperature for 3 min.
9. Centrifuge at 7000 × *g* for 10 min at 4 °C to separate phases.
10. Transfer aqueous phase to new 15 mL conical tube.
11. Add 5 mL cold isopropanol.
12. Invert several times and incubate for 10 min at room temperature.
13. Centrifuge at 10,000 × *g* for 10 min at 4 °C to pellet RNA.
14. Wash the pellet with cold 75% ethanol by adding the ethanol, inverting twice and pouring off.
15. Air-dry for 2 min and remove residual ethanol by pipetting.
16. Air-dry for 2 min and resuspend the pellet in 100–200 μL sterile H_2O.
17. Transfer to a 1.5 mL microcentrifuge tube.
18. Quantify RNA concentration with a NanoDrop.

Northern Blot Analysis of AmiRNAs

Confirm the accumulation of amiRNA in planta by Northern blot hybridization following standard protocols. The following are some specifications.

1. Prepare a 17% polyacrylamide gel containing 7 M Urea in 0.5× TBE. Mix 17 mL of 30% polyacrylamide (acrylamide–bisacrylamide, 37.5:1), 12.6 g of urea, 1.5 mL of 5× TBE, and 2 mL H_2O. Mix thoroughly by inversion. Do not shake or vortex as this incorporates oxygen to the solution which inhibits polymerization. Heat to 65 °C for 10 min to dissolve the urea. Stir for additional 5 min on the bench to allow final resuspension of urea, and keep 20 min on ice to chill the solution. Add 25 μL TEMED and mix by inversion. Add 150 μL of 10% ammonium persulfate and mix quickly by inversion. Pour the gel and allow it to polymerize for at least 30 min.
2. Prerun the gel at 180 V in 0.5× TBE for 1 h. Rinse wells before loading samples as urea accumulates at the bottom of the wells.
3. Add an equal amount of formamide loading buffer to the total RNA. Heat the samples for 10 min at 65 °C and immediately quench on ice briefly.
4. Load 10–20 μg of total RNA. Run at 180 V in 0.5× TBE until the bromophenol blue reaches the bottom of the gel (~4 h).

5. Assemble the blot sandwich with blotting paper (three sheets, prewetted with 0.5× TBE), gel, a sheet of positively charged nylon membrane, and another three sheets of blotting paper prewetted with 0.5× TBE in a semidry chamber. Transfer for 30 min at 1 mA per cm^2 of membrane. Cross-link RNA to the membrane at 0.12 J/cm^2 using a UV cross-linker. Store membranes between two sheets of filter paper until use.
6. Prepare the following reaction mix to radiolabel the probe oligonucleotide (*see* **Note 9**): 1 μL 10 μM probe oligonucleotide, 1 μL 10× polynucleotide kinase buffer, 3 μL sterile H_2O, 4 μL [^{32}P] γ-ATP (6000 Ci/mmol; 10 mCi/mL), and 1 μL T4 polynucleotide kinase.
7. Incubate for 60 min at 37 °C. Purify probe on spin columns according to manufacturer's instructions. Quantify the incorporated radioactivity (CPM/μL).
8. Place the membrane (RNA-side-up) in a hybridization tube and prehybridize with rotation for at least 5 min at 38–42 °C (*see* **Note 10**) in 5 mL of hybridization buffer.
9. Mix 1,000,000–2,000,000 CPM of probe with 200 μL of PerfectHyb™Plus buffer, incubate for 2 min at 95 °C, and transfer immediately to ice briefly.
10. Replace the hybridization solution, add the probe to the hybridization tube and incubate for 12–16 h at 38–42 °C (*see* **Note 10**).
11. Remove hybridization solution and wash the membrane five times with preheated wash solutions as follows: wash buffer 1 thorough rinse, wash buffer 1 for 5 min with rotation at 38–42 °C, wash buffer 1 for 20 min with rotation at 55 °C, wash buffer 1 for 20 min with rotation at 55 °C, wash buffer 2 for 20 min with rotation at 55 °C, and wash buffer 2 for 30 min with rotation at 55 °C (*see* **Note 11**).
12. Rinse membrane briefly in 3× SSC, then air-dry briefly and cover in transparent plastic wrap.
13. Autoradiograph.

3.3.4 Virus Inoculation

The mechanical inoculation of plants from blocks B and C is described next.

1. From a previously obtained virus infected tissue, prepare a crude extract in 20 volumes of Inoculation buffer.
2. Deposit several drops (5 μL each) of 10% carborundum in Inoculation buffer on leaf surface. Note that the number of drops will depend on the leaf size, although one or two drops per leave should be enough.
3. Dip a cotton swab in the tube containing the infected-plant extract.

4. Apply the extract to the whole surface of the leaf that was previously agroinfiltrated 2 days before with the artificial sRNA construct (Fig. 2) (*see* **Note 12**).

3.3.5 Analysis of Symptom Appearance

After virus inoculation, plants must be monitored for viral symptoms appearance (Fig. 2). Depending on the virus, symptoms can appear on inoculated and/or upper noninoculated tissue. The day of symptom appearance in either tissue must be annotated.

3.3.6 Analysis of Virus Accumulation

Virus presence or absence can be determined through different types of assays including ELISA, Western blot, Northern blot, or quantitative RT-PCR (*see* **Note 13**). Two time points are recommended for virus detection: (1) an early time point a few days after the controls show viral symptoms in upper noninoculated tissues (e.g., 10–12 dpa if symptoms appear 7–9 dpa in controls), and (2) a late time point (20–30 dpa) to analyze if the plant is definitely free of virus close to the end of its life cycle (Fig. 2). Note that dpa time is always 2 days ahead of virus inoculation.

3.4 Antiviral Syn-tasiRNA Design

1. Select 2–5 amiRNA sequences with high antiviral activity based on the amiRNA functional analysis described above (*see* Subheading 3.3). These sequences will be expressed as syn-tasiRNAs in a single construct.
2. Go to P-SAMS syn-tasiRNA Designer website (http://p-sams.carringtonlab.org/syntasi/designer) [15].
3. Click "Get Started" to start the design process.
4. Click "Generate Oligos."
5. Enter 2–5 syn-tasiRNA sequences.
6. Click "Submit" to submit the job.
7. Select "Click to see Results" to display results.
8. Click "Download" to download the sequence of the syn-tasiRNAs and the sequence of the two oligonucleotides required for cloning the syn-tasiRNA cassette in compatible syn-tasiRNA B/c vectors (Table 1).

3.5 Antiviral Syn-tasiRNA Cloning

Follow the steps described in Subheading 3.2 using the syn-tasiRNA vector of your choice (Table 1).

3.6 Functional Analysis of Antiviral Syn-tasiRNA

Follow the experimental plan described in Fig. 2 to functionally analyze the antiviral activity of the antiviral syn-tasiRNA construct. As noted for the antiviral amiRNA functional analysis (*see* Subheading 3.3.1), it is highly recommended to compare the activity of the antiviral syn-tasiRNA construct with a control syn-tasiRNA including several syn-tasiRNA sequences targeting an artificial transcript (e.g. GUS, GFP, etc.) (*see* **Note 14**).

4 Notes

1. Prepare Oligo Annealing Buffer, make 1 mL aliquots and store at −20 °C.
2. B/c vectors must be ordered at Addgene website (http://www.addgene.org/).
3. Multiple viral sequences can be entered, for example, when several sequence variants of the same virus are required to be targeted simultaneously.
4. This option allows the design of specific amiRNAs that will exclusively target viral RNA(s) without affecting endogenous transcripts.
5. Alternatively, the annealing reaction can be done in a water bath or thermoblock by heating during 5 min at 94 °C and then turning off the apparatus. Let the reaction to cool down until it reaches room temperature.
6. Do not store the diluted oligonucleotides.
7. If amiRNA inserts are cloned in *pENTR*-based B/c vectors, then the amiRNA cassette can be transfer through GATEWAY recombination to a destination vector such as *pMDC32* or *pMDC99*.
8. As for antiviral amiRNAs, use P-SAMS amiRNA Designer to design a highly specific amiRNA targeting an exogenous sequence other than the viral RNA. Confirm that the selected amiRNA does not extensively base pair with viral RNA(s) to avoid undesired silencing of the virus.
9. Oligonucleotide sequence is antisense to the artificial sRNA that is analyzed. Start by using a DNA probe for artificial sRNA detection as DNA oligonucleotides are cheap and work well most of the times. However, if sRNA detection with a DNA probe fails then use an LNA probe. Order the LNA oligonucleotide with every other three nucleotides locked, including the first one (e.g., an LNA probe to detect *A. thaliana* miR172 is A + TGC + AGC + ATC + ATC + AAG + ATT + CT, where the + indicates the locked nucleotide).
10. If using an LNA probe prehybridize and hybridize at ~20 °C below the calculated dissociation temperature (T_d) [T_d (°C) = 4(G + C) + 2(A + T)] for the corresponding ^{32}P-labeled oligonucleotide.
11. This works in the majority of cases. If there is still a background problem, proceed to an additional incubation of the membrane with 0.1× SSC/0.1% SDS for 60 min at 50 °C, or with 0.1× SSC/1% SDS for 60 min at 50 °C.
12. Use the cotton swab as a pencil to gently “paint” the whole surface of the leaf.

13. The choice of a particular assay will depend on the virus to be detected. For instance, ELISA has been optimized for *Tomato spotted wilt virus* detection [18].
14. The control syn-tasiRNA construct can be designed using P-SAMS syn-tasiRNA Designer (http://p-sams.carringtonlab.org/syntasi/designer).

Acknowledgments

This work was supported by grants from Ministerio de Ciencia, Innovación y Universidades (MCIU, Spain), Agencia Estatal de Investigación (AEI, Spain) and Fondo Europeo de Desarrollo Regional (FEDER, European Union) [RTI2018-095118-A-100 and RYC-2017-21648 to A.C.; BIO2017-83184-R to J.-A.D.].

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