Chapter 11

A Visual Reporter System for Analyzing Small RNA-Triggered Local and Systemic Silencing of an Endogenous Plant Gene

Adriana E. Cisneros and Alberto Carbonell

Abstract

Plant small RNAs (sRNAs) silence highly sequence complementary target mRNAs locally in a cell-autonomous manner but can also move long distances to induce systemic gene silencing in distal tissues. Unfortunately, the lack of appropriate experimental systems has hampered the identification of the factors determining the induction and spread of local and systemic gene silencing triggered by plant sRNAs. Here we describe a visual reporter system in which both local and systemic silencing of *Nicotiana benthamiana SULFUR* (*NbSu*) gene is triggered by the agroinfiltration of an artificial sRNA construct. Agroinfiltrated areas show strong and uniform bleaching while apical non-agroinfiltrated leaves display a characteristic near-vein chlorosis. This visual reporter system should facilitate the identification of the genetic, molecular, and/or environmental factors regulating both local and systemic silencing activities of plant sRNAs.

Key words Small RNAs, Local silencing, Systemic silencing, amiRNA, syn-tasiRNA, RNA mobility, *Nicotiana benthamiana*, Sulfur

1 Introduction

Eukaryotic small RNAs (sRNAs) are 21–24 nucleotide RNAs responsible for the sequence-specific degradation of complementary RNAs, a process known as gene silencing. In plants, some endogenous sRNA species such as microRNAs (miRNAs) are capable of moving throughout the plant and produce both short-range (cell-to-cell) and long-range (systemic) silencing effects, in addition to local (cell-autonomous) silencing of target mRNAs [1]. Unfortunately, the lack of appropriate experimental systems in plants has hampered the identification of the genetic, molecular, and/or environmental factors determining local and systemic silencing activities of sRNAs.

Artificial microRNAs (amiRNAs) and synthetic trans-acting small interfering RNAs (syn-tasiRNAs) are two classes of

Stefan de Folter (ed.), *Plant MicroRNAs: Methods and Protocols*, Methods in Molecular Biology, vol. 2900,
https://doi.org/10.1007/978-1-0716-4398-3_11,

21-nucleotide artificial sRNAs (art-sRNAs) that are computationally designed to selectively silence genes of interest with high specificity (without off-target effects). Both classes of art-sRNAs are generated by making use of the endogenous sRNAs silencing pathways. Briefly, they are produced *in planta* through the expression of a transgene including an sRNA precursor in which the endogenous miRNA and tasiRNA(s) sequences are substituted by the amiRNA or syn-tasiRNA(s) sequences, respectively. While amiRNAs are typically used to target a single gene or a few sequence-related genes, syn-tasiRNAs can be used to simultaneously target multiple sequence-unrelated genes or different sites within a single gene [2]. Importantly, recent work has shown that both classes of art-sRNAs, like some endogenous sRNAs, not only act cell-autonomously but can also move systemically and induce systemic silencing of endogenous genes [3]. In particular, an amiRNA and a syn-tasiRNA of identical sequence and targeting the magnesium chelatase subunit CHLI-encoding *SULFUR* gene from *N. benthamiana* (*NbSu*) not only silenced *NbSu* locally when expressed in leaves by agroinfiltration but also moved to apical tissues to induce the systemic silencing of *NbSu* [3]. A key feature of this system is that silencing effects are easily visualized with the naked eye (and do not require any specialized equipment) as locally silenced tissues (agroinfiltrated leaf patches) display strong and uniform bleaching while systemically silenced tissues (apical non-agroinfiltrated leaves) present severe chlorosis near the veins. Notably, this characteristic proximal-vein bleaching phenotype is induced by mobile art-sRNAs generated in the agroinfiltrated tissues and capable of moving through the phloem to distal leaves. Once discharged from the sieve elements, art-sRNAs only move 10–15 cells away, thus explaining why chlorosis derived from *NbSu* silencing remains near the veins in systemically silenced tissues [3].

Here we describe the methodology for setting up this visual reporter system to study the local and systemic silencing of *NbSu* induced by art-sRNAs in *N. benthamiana*. This reporter system should facilitate the identification of the genetic, molecular, and/or environmental factors regulating local and systemic silencing activities of sRNAs in plants.

2 Materials

2.1 Art-sRNA Design

1. Computer connected to the internet.
2. Web browser (e.g. Google Chrome, Safari, Mozilla Firefox, Internet Explorer).

2.2 Art-sRNA Cloning

1. Oligo Annealing buffer: 60 mM Tris-HCl (pH 7.5), 500 mM NaCl, 60 mM $MgCl_2$, 10 mM DTT.
2. Thermocycler or water bath.
3. Sterile H_2O.
4. T4 DNA ligase (5 U/μL, Thermo Fisher Scientific).
5. *Bsa*I (10 U/μL, New England Biolabs).
6. *pMDC32B-AtMIR390a-B/c* (plasmid 51,776; Addgene), for amiRNA cloning [4].
7. *pMDC32B-AtTAS1c-D2-B/c-AtMIR173* (plasmid 137,885; Addgene) for syn-tasiRNA cloning [5].
8. Competent cells of *Escherichia coli ccd*B-sensitive strain (e.g., DH5α, DH10B, TOP10).
9. LB agar plates with kanamycin: 20 g/L of LB, 15 g/L of Bacto Agar and 50 mg/L of kanamycin.
10. Liquid LB with kanamycin: 20 g/L of LB and 50 mg/L of kanamycin.
11. GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific).
12. Oligonucleotides: attB1 (5′ ACAAGTTTGTACAAAAAAG CAGGCT 3′) and attB2 (5′ ACCACTTTGTACAA GAAAGCTGGGT 3′).
13. Competent cells of *Agrobacterium tumefaciens* GV3101 strain.

2.3 Local and Systemic Silencing Induced by Art-sRNAs

2.3.1 Preparation of Bacterial Cultures and Agroinfiltration in N. benthamiana

1. 3–4 week-old *N. benthamiana* plants.
2. *pMDC32B-AtMIR390a-NbSu-2* (also named *35S:amiR-NbSu-2*; plasmid 213,400; Addgene) and *pMDC32B-AtTAS1c-D2-NbSu-2-AtMIR173a* (also named *35S:syn-tasiR-NbSu-2*; plasmid 213,401; Addgene) constructs to use the visual reporter system for *NbSu* local and systemic silencing [3].
3. Liquid LB with kanamycin: 20 g/L of LB and 50 mg/L of kanamycin and 50 mg/L of rifampicin.
4. 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.
5. 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 a sterile hood before using.
6. 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.
7. 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.

8. 250 mL Erlenmeyer flasks.
9. 50 mL conical tubes.
10. Spectrophotometer and cuvettes.
11. 1 mL needle-less disposable syringes.

2.3.2 Quantification of Chlorophyll a Content

1. Acetone 80%.
2. Shaker.
3. Flat bottom 96-well plate.
4. Spectrophotometer reading absorbance at 663 nm and 647 nm like Multiskan GO microplate reader (Thermo Scientific).

2.3.3 Total RNA Isolation

1. Liquid nitrogen.
2. Mortar.
3. Trizol: 1 M guanidine thiocyanate, 1 M ammonium thiocyanate, 10 mM sodium acetate (3 M), pH 5.5, 4.9% glycerol.
4. Centrifuge for 15 mL conical tubes.
5. Cold chloroform.
6. Cold isopropanol.
7. Cold 75% Ethanol.
8. Sterile miliQ Water.
9. NanoDrop (Thermo Fisher Scientific).

2.3.4 Real-Time RT Quantitative PCR (RT-qPCR)

1. DNAse TURBO™ Kit: 10× DNAse Buffer, TURBO DNAse (2 U/μL), DNAse inactivation reagent (Invitrogen, Thermo Fisher Scientific).
2. NanoDrop (Thermo Fisher Scientific).
3. PrimeScript RT Reagent Kit: 5× PrimeScript buffer, PrimeScript enzyme mix I, Oligo dT 50 μM, Random hexamers 100 μM (Takara).
4. TB Green Premix Ex Taq (TliRNaseH Plus) Green real-time PCR master mix (Takara).
5. Oligonucleotides: *NbSu* forward (5′ TCACACCCTGCCC GATTTAT 3′), *NbSu* reverse (5′ GCAGAAAGAGCGTTCC TAGC 3′), *NbPP2A* forward (5′ GACCCTGATGTTGATGTTCGCT 3′) and *NbPP2A* reverse (5′ GAGGGATTTGAAGAGAGATTTC 3′).
6. 96-Fast PCR plate half skirt (Sarstedt).
7. QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific).
8. QuantStudio Design and Analysis Software" version 1.5.1 (Thermo Fisher Scientific).

2.3.5 Northern Blot

1. 10× Tris–borate–EDTA (TBE): 108 g/L Tris base, 55 g/L boric acid, 7.5 g/L $Na_2EDTA\ 2H_2O$.
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 (APS): 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. Oligonucleotide probe: DNA or LNA oligonucleotide complementary to the art-sRNA to be analyzed (*see* **Note 2**).
13. T4 polynucleotide kinase: T4 PNK (10 U/μL), 10x T4 polynucleotide kinase buffer A (Thermo Fisher Scientific).
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).
17. 20× SSC (saline sodium citrate): 3 M NaCl, 0.3 M sodium citrate, pH 7.0, 10% SDS: 10% (w/v) sodium dodecyl sulfate in sterile H_2O.
18. Wash buffer 1: 2× SSC, 0.1% SDS. Prepare with 20× SSC, 10% SDS solution, and sterile H_2O.
19. Wash buffer 2: 0.1× SSC, 0.1% SDS. Prepare with 20× SSC, 10% SDS solution, and sterile H_2O.
20. Wash buffer 3: 3× SSC. Dilute 20× SSC with sterile H_2O.

2.3.6 Hight-Throughput Sequencing of sRNAs

1. RNA 6000 Nano Kit (Agilent).
2. 2100 Bioanalyzer Instrument (Agilent).
3. Company for the library preparation and high-throughput sequencing of sRNAs.

3 Methods

The main steps from art-sRNA design to phenotypic and molecular analyses of local and systemic silencing triggered by art-sRNAs are presented in the flow chart of Fig. 1.

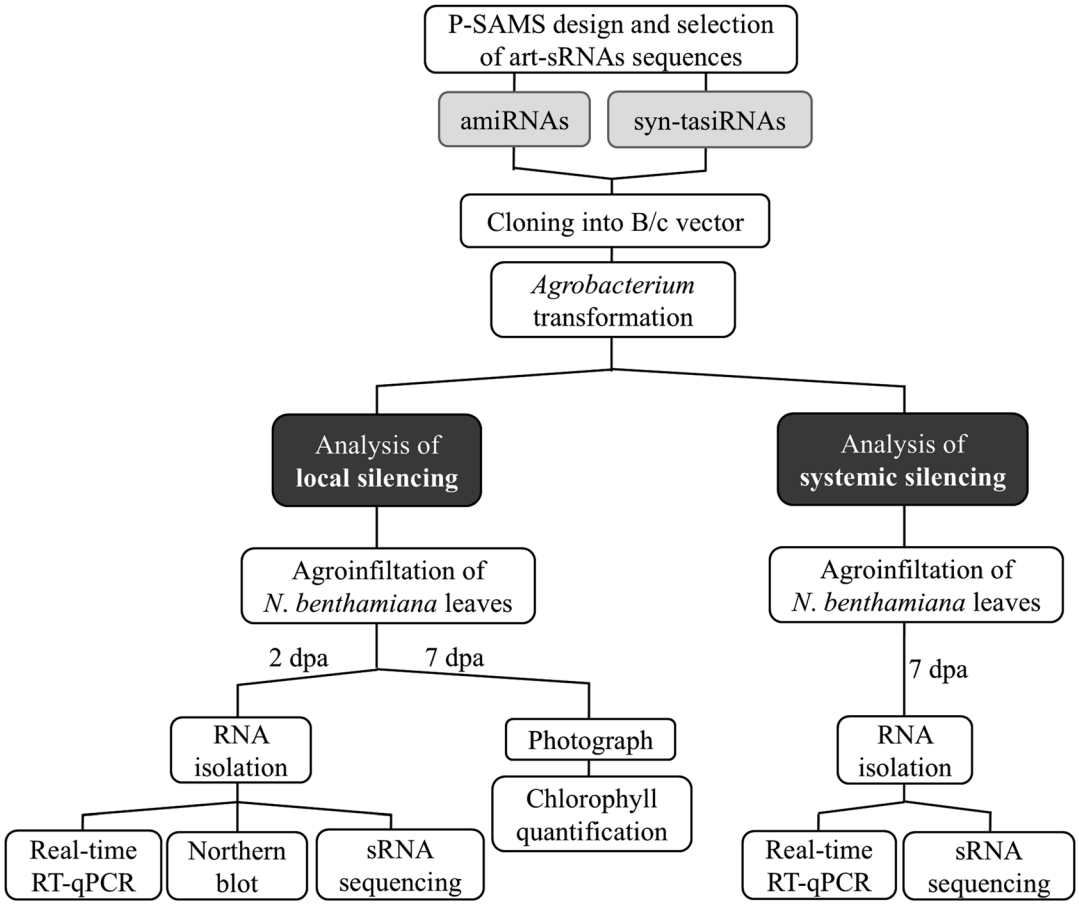

Fig. 1 Flowchart of the main steps from art-sRNA design and cloning to phenotypic and molecular analyses of both local and systemic silencing triggered by art-sRNA constructs in *N. benthamiana*

3.1 Art-sRNA Design

The Plant Small RNA Maker Suite (P-SAMS) web app [6] is used to design highly specific amiRNAs and syn-tasiRNAs with a 5′U nucleotide, a C in position 19 and with a mismatch with the target transcript at position 21. The protocol described next is intended for the design of art-sRNAs targeting transcripts from endogenous plant genes.

3.1.1 AmiRNA Design

The P-SAMS AmiRNA Designer app designs amiRNAs for gene silencing in different eudicot or monocot plant species. The app outputs the sequence of up to three optimal amiRNAs (with no off-targets) together with the sequence of the two oligonucleotides required for cloning the amiRNA in compatible B/c vectors containing the *Arabidopsis thaliana* (Arabidopsis) *MIR390a* (*AtMIR390a*) or the *Oryza sativa MIR390* (*OsMIR390*) foldbacks for use in eudicot or monocot species, respectively. Here, the necessary steps for designing an amiRNA against a *N. benthamiana* gene (*see* **Note 1**) are described next.

1. Go to P-SAMS website (http://p-sams.carringtonlab.org/).
2. Click the "P-SAMS amiRNA Designer" application button to enter the amiRNA Designer tool (https://p-sams.carringtonlab.org/amirna/designer).
3. To start the design process, click "Get Started" and then "Design an amiRNA."
4. Select the transcriptome of *N. benthamiana* and click "Yes."
5. Click "Option 1" if you have the gene ID(s) of the target transcript(s) (*see* **Note 3**).
6. Enter the gene ID(s) of the target transcript(s) and click "Next."
7. Click "Yes" to have the results automatically filtered based on target specificity.
8. Click "Submit" to submit the job.
9. Select "Click to see Results" to display the results. AmiRNAs predicted to target uniquely desired target transcript(s) are output as "Optimal Results"; amiRNAs predicted to target desired target transcript(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.

3.1.2 Syn-tasiRNA

The P-SAMS Syn-tasiRNA Designer app designs syn-tasiRNAs for gene silencing in *Arabidopsis* or other plant species if the miR173 trigger is co-expressed. The app outputs the sequence of up to three optimal syn-tasiRNAs (with no off-targets) together with the sequence of the two oligonucleotides required for cloning the amiRNA in compatible B/c vectors containing the Arabidopsis *AtTAS1c* precursor. Next are the necessary steps for designing a syn-tasiRNA against a *N. benthamiana* gene (*see* **Note 2**).

1. Go to P-SAMS website (http://p-sams.carringtonlab.org/).
2. Click the "P-SAMS syn-tasiRNA Designer" application button to enter the syn-tasiRNA Designer tool (https://p-sams.carringtonlab.org/syntasi/designer).
3. To start the design process, click "Get Started" and then "Design syn-tasiRNAs."
4. Steps from **4** to **8** are as in Subheading 3.1.1.
9. Select "Click to see Results" to display results.
10. Navigate through the "Detailed Results" tab to display the list of "Optimal Results" and "Suboptimal Results" for each gene set. Syn-tasiRNAs predicted to target uniquely desired target

transcript(s) are output as "Optimal Results"; syn-tasiRNAs predicted to target desired target transcript(s) are output as "Suboptimal Results." Each result contains the syn-tasiRNA sequence and a summary of the target prediction analysis (score, target coordinates, target sequence, syn-tasiRNA/target base pairing and target description.

3.2 Art-sRNA Cloning

The selected amiRNA and/or syn-tasiRNA sequences are cloned into a compatible *pMDC32B*-based B/c binary vector as follows.

3.2.1 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 described below:
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 3**).
4. Dilute the annealed oligonucleotides to a final concentration of 0.15 μM as described below (*see* **Note 4**):

Annealed oligonucleotides	**3 μL**
H_20	37 μL

5. Assemble the digestion-ligation reaction as described below:

***pMDC32B-AtMIR390a-B/c* vector (50 ng)**	**X μL**
Diluted annealed oligonucleotides	1 μL
10× T4 DNA ligase buffer	1 μL
T4 DNA ligase (5 U/μL)	1 μL
*Bsa*I (10 U/μL)	1 μL
Sterile H_20	To 10 μL

6. Mix the reactions by pipetting and incubate for 5 min at 37 °C (*see* **Note 5**).
7. Transform 1–5 μL of the digestion-ligation into a *ccd*B-sensitive *Escherichia coli* strain (*see* **Note 6**). Plate all the cultures in a LB agar plate containing 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 the oligonucleotides attB1 and attB2.

10. Transform 0.5 μl of the purified plasmid into *A. tumefaciens* GV3101. Plate 1/10 of the culture in a LB agar plate containing rifampicin and kanamycin.
11. Incubate the plate at 28 °C for 48 h.
12. Store the plate with grown colonies at 4 °C until use.

3.2.2 Syn-tasiRNA Cloning

1. Follow the steps from **1** to **4** as in Subheading 3.2.1.
2. Assemble the digestion-ligation reaction as described below:

***pMDC32B-AtTAS1c-D2-B/c-AtMIR173* vector (50 ng)**	**X μL**
Diluted annealed oligonucleotides	1 μL
10× T4 DNA ligase buffer	1 μL
T4 DNA ligase (5 U/μL)	1 μL
*Bsa*I (10 U/μL)	1 μL
Sterile H_20	To 10 μL

3. Proceed with **Steps 6–12** as in Subheading 3.2.1.

3.3 Analysis of the Local Silencing Induced by Art-sRNAs

The main steps involved in the analysis of *NbSu* local silencing induced by amiR-NbSu-2 or syn-tasiR-NbSu-2 are represented in Fig. 2, and the detailed methodologies are described next (*see* **Notes 7** and **8**).

3.3.1 Preparation of Bacterial Cultures for Agroinfiltration

1. For each art-sRNA 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 OD600 ~ 0.5.
6. Centrifuge 45 mL of culture for 15 min at 5000 × g in a 50 mL conical tube.
7. Remove the supernatant and resuspend it in an equal volume of the Vir Induction medium.
8. Transfer back to flasks and shake overnight (~14 h) at 28 °C.
9. Measure OD600 of the overnight culture.
10. Calculate the volume of the infiltration solution to resuspend the pellet to an OD600 ~ 1.1.

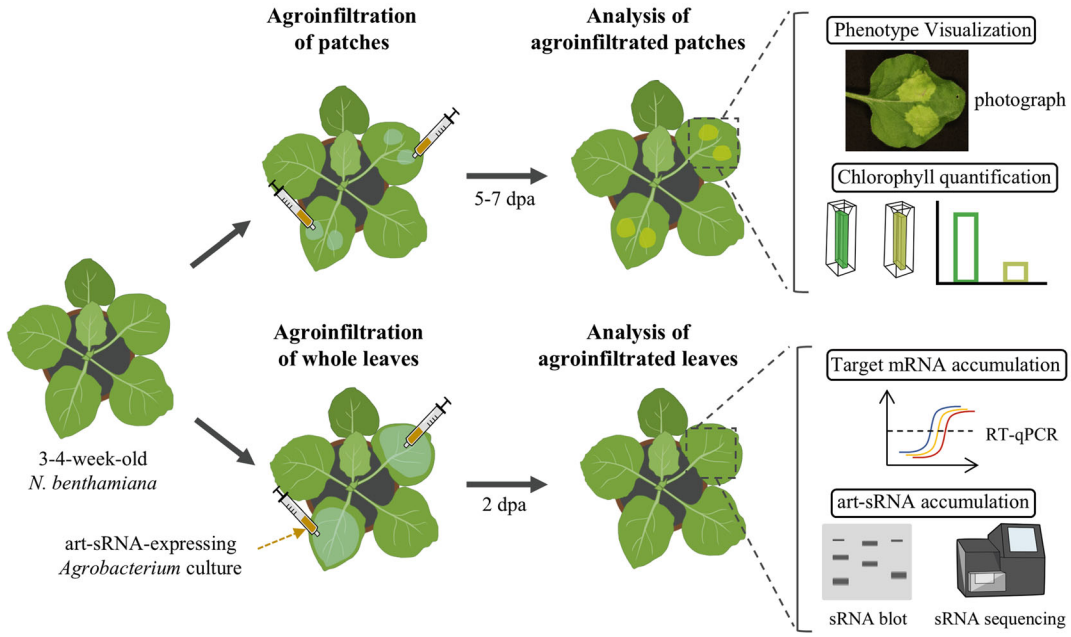

Fig. 2 Analysis of local silencing of *NbSu* by art-sRNAs in *N. benthamiana*. The third and fourth leaves of 3–4-week-old plants are agroinfiltrated. Upper panel, patches are agroinfiltrated to visualize silencing phenotypically and quantify chlorophyll content in silenced (bleached) areas at 5–7 days post-agroinfiltration. Lower panel, full leaves are agroinfiltrated and collected 2 dpa to isolate total RNA for assessing target mRNA levels and art-sRNA accumulation by real-time RT-qPCR and Northern blot/sRNA high-throughput sequencing, respectively

11. Centrifuge the culture for 10 min at 5000 × g in a 50 mL conical tube.
12. Remove the supernatant and resuspend the pellet in the calculated volume of the infiltration solution.
13. Measure OD600 and normalize the culture to a final OD600 of 1.0.
14. Mix the art-sRNA culture with an equal volume of a *A. tumefaciens* culture including an empty vector or control construct such as *pMDC32-GUS* [7].

3.3.2 Agroinfiltration of N. benthamiana *Leaves for Visual Observation of Local Silencing and Chlorophyll Content Analysis*

A first set of *N. benthamiana* plants is agroinfiltrated with the art-sRNA construct for visual analysis of local silencing. The degree of silencing induced by the art-sRNA can be determined biochemically by measuring the content of chlorophyll *a* in the agroinfiltrated tissue (Fig. 2, upper panel).

1. Agroinfiltrate two patches in the central region of the second and third leaves of three 3–4-week-old *N. benthamiana* plants.
2. When agroinfiltrated patches show strong bleaching (typically 5–7 days post-agroinfiltration, dpa), take a photograph of the corresponding leaf to record silencing phenotypes.

3. Collect 4 discs (50 mg) of the agroinfiltrated patches from each individual plant with a 1.5 mL Eppendorf tube.
4. Add leaf discs into a 13 mL culture tube containing 5 mL of 80% (v/v) acetone.
5. Shake at moderate speed for 16–24 h in the dark (*see* **Note 9**).
6. Transfer 100 μL of supernatant to a flat bottom 96-well plate containing 100 μL of 80% acetone (*see* **Notes 10** and **11**).
7. Add 200 μL of 80% acetone in several wells for blank measurements.
8. Use a spectrophotometer to measure sample's absorbance at 663 nm and 647 nm.
9. Chlorophyll *a* is calculated with the following formula:
   Chlorophyll *a* (mg/L of extract) = 12.21 × $\text{Absorbance}_{663\text{nm}}$ − 2.81 × $\text{Absorbance}_{647\text{nm}}$.

3.3.3 Agroinfiltration of N. benthamiana Leaves for Art-sRNA and Target mRNA Analysis

A second set of *N. benthamiana* plants is agroinfiltrated with the art-sRNA construct for analyzing target mRNA levels, art-sRNA accumulation and accuracy of art-sRNA precursor processing by RT-qPCR, Northern blot and high-throughput sequencing, respectively (Fig. 2, lower panel).

1. Agroinfiltrate the whole surface of the second and third leaves of three 3–4-week-old *N. benthamiana* plants.
2. Collect and combine the two agroinfiltrated leaves from each individual plant and snap-freeze them (*see* **Note 12**).
3. Place the tissue in a mortar and pour 30–50 mL of liquid nitrogen.
4. Lightly crush leaves after liquid nitrogen has completely evaporated; grind vigorously until the tissue is powdered.
5. Add 5 mL of trizol and continue homogenization to mix the partially frozen sample.
6. Transfer the homogenized sample to a 15 mL conical tube.
7. Centrifuge at 7000 × g for 10 min at 4 °C to pellet cell debris.
8. Transfer the supernatant to a new 15 mL conical tube.
9. Add 3 mL of chloroform and shake vigorously for 20 s.
10. Incubate at room temperature for 3 min.
11. Centrifuge at 7000 × g for 10 min at 4 °C to separate phases.
12. Transfer the aqueous phase to a new 15 mL conical tube.
13. Add 5 mL cold isopropanol.
14. Invert several times and incubate for 10 min at room temperature.
15. Centrifuge at 10,000 × g for 10 min at 4 °C to pellet RNA.
16. Wash the pellet with cold 75% ethanol by adding the ethanol, inverting twice and pouring off.

17. Air-dry for 2 min and remove residual ethanol by pipetting.
18. Air-dry for 2 min and resuspend the pellet in 100–200 μL sterile H_2O.
19. Transfer to a 1.5 mL Eppendorf tube.
20. Quantify RNA concentration with a NanoDrop.
21. Store at −80 °C until needed.

3.3.4 Analysis of Target mRNA Accumulation by Real-Time Quantitative PCR

The degree of silencing induced by the art-sRNA can also be determined molecularly by measuring the accumulation of target mRNA in the agroinfiltrated tissue as follows.

1. Assemble the DNAse I treatment reaction as described below:

Total RNA (2 μg)	X μL
10× DNAse I buffer	1.2 μL
DNAse I	1 μL
Sterile H_20	To 12 μL

2. Incubate the reactions at 37 °C for 30 min.
3. Add 2 μL of inactivation buffer to each sample (*see* **Note 13**).
4. Incubate for 5 min at room temperature.
5. Centrifuge at 10,000 × g for 2 min.
6. Transfer 9 μL of the supernatant to a new 1.5 mL Eppendorf tube.
7. Quantify RNA concentration with a NanoDrop.
8. Assemble the cDNA synthesis reaction as described below:

Total RNA treated with DNAse I (500 ng)	X μL
5× PrimeScript buffer	2 μL
Oligo dT primer (50 uM)	0.5 μL
Random hexamers (100 uM)	0.5 μL
PrimeScript RT enzyme mix I	0.5 μL
Sterile H_20	To 10 μL

9. Incubate the reaction tubes under the following conditions:

15 min at 37 °C
5 s at 85 °C
Hold at 4 °C

10. Transfer the tubes back to 4 °C.

TB Green Premix Ex Taq (2×)	10 μL
Forward primer	0.4 μL
Reverse primer	0.4 μL
ROX II reference dye (50×)	0.4 μL
1:5 diluted cDNAs	2 μL
Sterile H_20	To 20 μL

11. Prepare the real-time quantitative PCR mix as follows:
12. Set the qPCR reaction as follows:

Hold stage	
50 °C	2 min
95 °C	10 min
PCR stage	
95 °C	15 s
60 °C	1 min
Melt curve stage	
95 °C	15 s
60 °C	1 min
95 °C	15 s

13. Target mRNA accumulation is calculated relative to a *N. benthamiana* reference gene such as *NbPP2A* using the delta cycle threshold comparative method of "Quant Studio Design and Analysis Software" version 1.5.1 (Thermo Fisher Scientific).

3.3.5 Analysis of Art-sRNA Accumulation by Northern Blot

Typically, target mRNA silencing degree positively correlates with art-sRNA accumulation, which can be analyzed by Northern blot analysis of total RNAs isolated from agroinfiltrated tissues as follows.

1. Prepare a 17% polyacrylamide gel as follows:

30% polyacrylamide	17 mL
Urea	12.6 g
10× TBE	1.5 mL
Sterile H_20	2 mL

2. Mix thoroughly by inversion (*see* **Note 14**).
3. Heat to 65 °C for 15 min to dissolve urea and keep 20 min on ice.
4. Add 25 μL of TEMED and mix by inversion. Add 150 μL of 10% APS and mix quickly by inversion.
5. Pour the gel and allow to polymerize for 30 min.
6. Pre-run 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.
7. 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.
8. 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).
9. 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.
10. Transfer for 30 min at 1 mA per cm^2 of membrane (typically 400 mA).
11. Cross-link RNA to the membrane at 0.12 J/cm^2 (typically 1200 J) using a UV cross-linker.
12. Store membranes between two sheets of filter paper until use.
13. Prepare the following reaction mix to radiolabel the probe oligonucleotide:

Probe oligonucleotide (10 μM)	**1 μL**
10× polynucleotide kinase buffer A.	1 μL
Sterile H_20	3 μL
[32P] γ-ATP	4 μL
T4 polynucleotide kinase	1 μL

14. Incubate for 60 min at 37 °C. Add 40 μL of sterile H_20 and purify the probe on spin columns according to the manufacturer's instructions.
15. Place the membrane (RNA-side-up) in a hybridization tube and prehybridize with rotation for at least 5 min at 38–42 °C in 5 mL of PerfectHyb™Plus buffer.
16. Mix 25 μL of the probe with 200 μL of PerfectHyb™Plus buffer and add to the hybridization tube. Incubate for 1–24 h at 42 °C.
17. Remove the hybridization solution and wash the membrane five times with preheated wash solutions as follows:

Washing buffer 1	Thorough rinse
Washing buffer 1	5 min at 42 °C
Washing buffer 1	20 min at 55 °C
Washing buffer 1	20 min at 55 °C
Washing buffer 2	20 min at 55 °C
Washing buffer 2	30 min at 55 °C

18. Rinse the membrane briefly in washing buffer 3, then air-dry briefly and cover in transparent plastic wrap.
19. Autoradiograph.

3.3.6 Small RNA High-Throughput Sequencing and Bioinformatic Analyses

For the genome-wide identification of art-sRNAs species accumulating in vivo as well as for analyzing art-sRNA processing accuracy sRNA libraries can be prepared from total RNAs of agroinfiltrated tissues and sequenced as follows.

1. Determine the Total RNA purity, integrity and quantity of each total RNA sample with a 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit.
2. Prepare sRNA libraries and submit them for high-throughput sequencing (*see* **Note 15**).
3. After receiving the adaptor-removed clean reads, use fastx-collapser [8] to collapse identical reads into a single sequence, while maintaining read counts.
4. Map each clean and unique read against the sequence of interest (e.g., the precursor of the overexpressed art-sRNA), not allowing mismatches or gaps.
5. To determine the processing accuracy of amiRNA foldbacks or syn-tasiRNA transcripts, quantify the proportion of 19 to 24 nt sRNAs (+) reads mapping within ±4 nucleotides of the 5′ end of the amiRNA guide or the DCL4 processing position 3'D2 (+) (*see* **Note 15)** respectively [4].
6. To determine the phasing of the art-sRNA construct, calculate the proportion of 21-nucelotide sRNAs (+) reads in each register relative to the art-sRNA cleavage site for all 21-nucleotide positions downstream of the cleavage site [4, 9].

3.4 Analysis of the Systemic Silencing Induced by Art-sRNAs

The main steps involved in the analysis of *NbSu* systemic silencing induced by amiR-NbSu-2 or syn-tasiR-NbSu-2 are represented in Fig. 3, and the detailed methodologies are described next (*see* **Note 16**).

3.4.1 Agroinfiltration of Art-sRNA Constructs in N. benthamiana

1. Prepare the bacterial cultures including the art-sRNA constructs as described in Subheading 3.2.1.

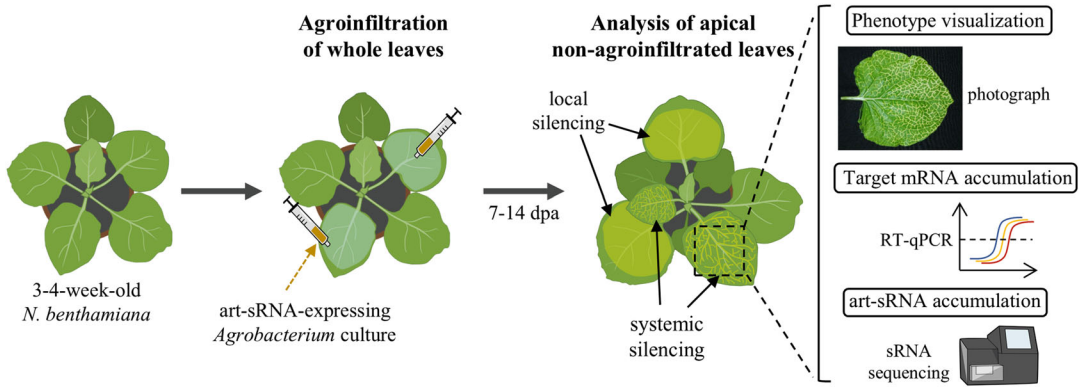

Fig. 3 Analysis of systemic silencing of *NbSu* by art-sRNAs in *N. benthamiana*. The third and fourth leaves of 3- to 4-week-old plants are agroinfiltrated in the whole surface. Near-vein chlorosis in apical non-agroinfiltrated leaves characteristic of *NbSu* systemic silencing appears at 6–7 days post-agroinfiltration (dpa), and the degree of chlorosis usually intensifies up to 14 dpa and then remains stable for weeks. Leaves displaying systemic silencing phenotype are collected for total RNA purification to assess target mRNA levels and art-sRNA accumulation by real-time RT-qPCR and Northern blot/sRNA high-throughput sequencing, respectively. Local silencing in agroinfiltrated leaves remain also visible for weeks

2. Agroinfiltrate the whole surface of the second and third leaves of three 3–4-week-old *N. benthamiana* plants (*see* **Notes 17** and **18**).
3. Wait until 7–14 dpa to visualize *NbSu* systemic silencing characterized by the appearance of strong bleaching near the veins of apical non-agroinfiltrated leaves (*see* **Note 19**).

3.4.2 Total RNA Purification and Target mRNA Quantification

Target mRNA accumulation will be analyzed as follows to assess systemic silencing efficiency.

1. Collect two apical leaves displaying the systemic silencing phenotype of near-vein chlorosis at 7–14 dpa.
2. Proceed to total RNA isolation as described in Subheading 3.3.3.
3. Proceed to RT-qPCR analysis of *NbSu* levels as described in Subheading 3.3.4.

3.4.3 Analysis of Art-sRNA Accumulation

Generate sRNA libraries and submit for high-throughput sequencing as described in 3.3.5 to detect mobile art-sRNAs in apical tissues displaying systemic silencing (*see* **Note 20**).

4 Notes

1. P-SAMS amiRNA Designer was used to design amiR-NbSu-2 (5′ UGUAUGACUCCCGGAAUUCCA 3′) for silencing *NbSu* in *N. benthamiana* [3]. The amiRNA insert was

introduced in *pMDC32B-AtMIR390a-B/c* vector to generate the *pMDC32B-AtMIR390a-NbSu-2* (also named *35S:amiR-NbSu-2*) construct (plasmid 213,400; Addgene) [3].

2. The syn-tasiRNA syn-tasiR-NbSu-2 (5′ UGUAUGA CUCCCGGAAUUCCA 3′, identical sequence to amiR-NbSu-2) for silencing *NbSu* in *N. benthamiana* was introduced in *pMDC32B-AtTAS1c-D2-B/c* vector to generate the *pMDC32B-AtTAS1c-D2-NbSu-2* (also named *35S:syn-tasiR-NbSu-2*) construct (plasmid 213,401; Addgene) [3].
3. Alternatively, the annealing reaction can be done in a water bath or thermoblock by heating for 5 min at 94 °C and then turning off the apparatus. Let the reaction cool down until it reaches room temperature.
4. Do not store the diluted oligonucleotides.
5. The incubation time of the digestion-ligation reaction can be increased up to 30 min if necessary.
6. The digestion-ligation reaction can be transferred to a spin column for nucleic acid purification. This optional step usually increases the number of colonies obtained after *E. coli* transformation.
7. The same methodology can be applied to analyze the local silencing of other *N. benthamiana* genes whose silencing leads to visible leaf bleaching such as *NbDXS* (*1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE*) [10] or *NbPDS* (*PHYTOENE DESATURASE*).
8. The visual reporter system for analyzing *NbSu* local silencing could be used, for instance, to study the sequence determinants in sRNA precursors required for accurate processing and efficient release of art-sRNAs.
9. It is critical to maintain the tubes in total absence of light as chlorophyll is light sensitive.
10. This is a 1:2 dilution adjusted for chlorophyll analysis of *N. benthamiana* leaves; it is possible that under different conditions of other species, a different dilution is required (or even undiluted). In any case, make sure that the absorbances are lower than 0.8.
11. It is recommended to analyze at least two technical replicates per biological replicate.
12. Collected leaves can be stored at −80 °C until needed.
13. It is recommended to vortex samples softly during the incubation time as the inactivation buffer tends to accumulate in the bottom of the tube.
14. Do not shake or vortex as it incorporates air bubbles into the solution and inhibits polymerization.

15. Several companies (e.g., BGI, https://www.bgi.com) offer the service of preparation of sRNA libraries and high-throughput sequencing.
16. The visual reporter system for analyzing *NbSu* systemic silencing could be used, for instance, to study the genetic or environmental requirements for SS induction and spread, if *N. benthamiana* mutant lines are examined or different abiotic stresses are applied during plant growth, respectively.
17. To trigger the systemic response, the infiltrated area must cover at least the basal half of the leaf (as close to the petiole as possible).
18. The degree of induced SS can also be improved by increasing the optical density of *A. tumefaciens* cultures prior to infiltration. For instance, we have agroinfiltrated the art-sRNA culture with an OD = 1 with good results.
19. Near-vein chlorosis in apical leaves can appear as soon as 6–7 dpa. The degree of chlorosis typically intensifies up to 14 dpa and then remains stable for weeks.
20. To detect mobile art-sRNAs in apical tissues displaying systemic silencing, it is recommended using high-throughput sequencing over northern blot as mobile art-sRNAs accumulate to low levels.

Acknowledgments

This work was supported by grants or fellowships from Ministerio de Ciencia, Innovación y Universidades (MCIU, Spain), Agencia Estatal de Investigación (AEI, Spain) and Fondo Europeo de Desarrollo Regional (FEDER, European Union) [PID2021-122186OB-I00 and RYC-2017-21 648 to A.C. and PRE2019-088439 to A.E.C.].

References

1. Liu L, Chen X (2018) Intercellular and systemic trafficking of RNAs in plants. Nat Plants 4:869–878. https://doi.org/10.1038/s41477-018-0288-5
2. Carbonell A (2017) Artificial small RNA-based strategies for effective and specific gene silencing in plants. In: Dalmay T (ed) Plant gene silencing: mechanisms and applications. CABI Publishing, pp 110–127
3. Cisneros AE, de la Torre-Montaña A, Carbonell A (2022) Systemic silencing of an endogenous plant gene by two classes of mobile 21-nucleotide artificial small RNAs. Plant J 110:1166–1181. https://doi.org/10.1111/tpj.15730
4. Carbonell A, Takeda A, Fahlgren N, Johnson SC, Cuperus JT, Carrington JC (2014) New generation of artificial MicroRNA and synthetic trans-acting small interfering RNA vectors for efficient gene silencing in Arabidopsis. Plant Physiol 165:15–29. https://doi.org/10.1104/pp.113.234989
5. López-Dolz L, Spada M, Daròs J-A, Carbonell A (2020) Fine-tune control of targeted RNAi efficacy by plant artificial small RNAs. Nucleic Acids Res 48:6234–6250. https://doi.org/10.1093/nar/gkaa343
6. Fahlgren N, Hill ST, Carrington JC, Carbonell A (2016) P-SAMS: a web site for plant artificial microRNA and synthetic trans-acting small

interfering RNA design. Bioinformatics 32: 157–158. https://doi.org/10.1093/bioinformatics/btv534

7. Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, Chapman EJ, Fahlgren N, Allen E, Carrington JC (2008) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell 133:128–141. https://doi.org/10.1016/j.cell.2008.02.033
8. Hannon GJ (2010) FASTX-Toolkit (RRID: SCR_005534). http://hannonlab.cshl.edu/fastx_toolkit
9. Cuperus JT, Carbonell A, Fahlgren N, Garcia-Ruiz H, Burke RT, Takeda A, Sullivan CM, Gilbert SD, Montgomery TA, Carrington JC (2010) Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in Arabidopsis. Nat Struct Mol Biol 17:997–1003. https://doi.org/10.1038/nsmb.1866
10. Cisneros AE, Martín-García T, Primc A, Kuziuta W, Sánchez-Vicente J, Aragonés V, Daròs J-A, Carbonell A (2023) Transgene-free, virus-based gene silencing in plants by artificial microRNAs derived from minimal precursors. Nucleic Acids Res 51:10719–10736. https://doi.org/10.1093/nar/gkad747