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Abstract	A large body of evidence has lead to the suggestive proposal of a potential interplay between viroid-induced pathogenesis and RNA silencing regulatory mechanisms. A variety of techniques have been used to examine this interaction. This chapter outlines the use of a green fluorescent protein (GFP) sensor, containing viroid-specific sequence targets to study this phenomenon. In addition, a symptomatic transgenic line of *Nicotiana benthamiana* that expresses and processes *Hop stunt viroid* (HSVd) is used as stocks in grafting assays with the *rdr6i-Nb* lines, in which the RNA-dependent RNA polymerase 6 (*NbRDR6*) is constitutively silenced. The combination of the use of a viroid-specific GFP-sensor, the grafting assays with plants silenced for the RDR6 and the analysis of siRNAs allows one to address how to monitor viroid-specific RNA silencing and how to associate this regulatory pathway with symptom expression.

Key words (separated by '-')	Agroinfection - Agroinfiltration - Green fluorescent protein - Viroid pathogenesis - RNA-dependent RNA polymerase - RNA silencing

Chapter 22

The Interaction Between Plant Viroid-Induced Symptoms and RNA Silencing

Vicente Pallas, German Martinez, and Gustavo Gomez

Abstract

A large body of evidence has lead to the suggestive proposal of a potential interplay between viroid-induced pathogenesis and RNA silencing regulatory mechanisms. A variety of techniques have been used to examine this interaction. This chapter outlines the use of a green fluorescent protein (GFP) sensor, containing viroid-specific sequence targets to study this phenomenon. In addition, a symptomatic transgenic line of *Nicotiana benthamiana* that expresses and processes *Hop stunt viroid* (HSVd) is used as stocks in grafting assays with the *rdr6i-Nb* lines, in which the RNA-dependent RNA polymerase 6 (*NbRDR6*) is constitutively silenced. The combination of the use of a viroid-specific GFP-sensor, the grafting assays with plants silenced for the RDR6 and the analysis of siRNAs allows one to address how to monitor viroid-specific RNA silencing and how to associate this regulatory pathway with symptom expression.

Key words: Agroinfection, Agroinfiltration, Green fluorescent protein, Viroid pathogenesis, RNA-dependent RNA polymerase, RNA silencing

1. Introduction

Viroids are the smallest known plant-restricted pathogenic RNAs. Viroid genomes consist of a single-stranded, covalently closed circular, noncoding, and non-encapsidated RNA, ranging from 246 to 401 nucleotides in length (1–4). They are classified into two families; *Pospiviroidae* whose replication takes place in the nucleus, and *Avsunviroidae* that replicate and accumulate in the chloroplast (1). Given that viroids do not encode any pathogen-specific protein, their propagation and associated pathogenesis processes are fully dependent on the host biochemical machinery. This interaction activates a sequence of poorly understood events that are

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expressed as visible symptoms. It has long been accepted that the genomic RNA acts as the primary pathogenic effector via a direct interaction between specific viroid motifs and cellular factors (1, 5, 6). However, the identification of viroid-specific small RNAs in viroid-infected plants has lead to a new attractive pathogenic model based on viroid-induced RNA silencing (7).

The possibility that RNA silencing may regulate viroid-induced pathogenic processes was enunciated a few years ago (8, 9). Later, it was shown that transgenic tomato plants expressing a partial-length *Potato spindle tuber viroid* (PSTVd) RNA accumulated specific siRNAs and developed symptoms. Thus, it was proposed that the viroid-derived siRNAs might act as miRNAs to down-regulate the expression of physiologically important host genes and to induce disease-associated symptoms (7). Subsequently, independent studies reinforced this pathogenesis model, thus supporting an emergent view in which viroid-induced RNA silencing would mediate the symptom expression in infected plants (10–15). Moreover, the recent demonstration that symptoms induced by *Hop stunt viroid* in *Nicotiana benthamiana* plants depend on RDR6 activity provides additional evidence linking RNA silencing and viroid pathogenesis (16).

In this chapter, we describe the methods employed in our laboratory to: (1) analyze the viroid-induced RNA silencing activity in HSVd-infected *N. benthamiana* and cucumber plants and (2) correlate the activity of a key component of diverse RNA silencing pathways (the RDR6 enzyme) with viroid symptom expression in infected plants.

2. Materials

2.1. Plant Material

1. HSVd-Nb: in these assays, we used a symptomatic transgenic line of *N. benthamiana* that expresses and processes *Hop stunt viroid* (HSVd) (17, 18).
2. Rdr6i-Nb: this is a transgenic line of *N. benthamiana* in which the expression of the RNA-dependent RNA polymerase 6 (NbRDR*6*) is constitutively silenced (19). These plants were gently provided by the Prof. D. Baulcombe (The Sainsbury Laboratory, John Innes Centre, Norwich, UK).
3. Wild-type *N. benthamiana* plants.
4. Cucumber (*Cucumis sativus* Cv suyo).
5. The *N. benthamiana* and *C. sativus* plants described in these assays were germinated and maintained until used, in a greenhouse at 20–22°C.

2.2. Viroid Inoculation

1. Carborundum powder.
2. Inoculation buffer: PO_4Na 30 mM, pH 8.0.
3. Parafilm.
4. Razor blades.
5. Plastic bags.
6. Competent cells of *Agrobacterium tumefaciens* LBA4404 transformed with dimeric HSVd (17).
7. Luria–Bertani (LB) medium.
8. LB-KTR medium: LB supplemented with antibiotics (100 μg rifampicin/ml, 5 μg tetracycline/ml, and 50 μg kanamycin/ml).
9. Infiltration buffer: 50 mM MES [2-(*N*-morpholino)-ethane sulphonic acid] pH 5.6, 10 mM MgCl, and 100 μM acetosyringone.
10. Syringes (1 ml).

2.3. RNA Analysis

2.3.1. RNA Purification

1. Extraction buffer: 0.1 M Tris–HCl (pH 9), 0.1 M NaCl, 0.01 M EDTA, 0.1 M β-mercaptoethanol.
2. Sterilized 1-ml Eppendorf tubes.
3. Polytron.
4. Plastic bags.
5. Isopropanol.
6. miRACLE™ miRNAs isolation Kit (STRATAGENE 400813. http://www.Stratagene.com).
7. DEPC-treated water.
8. Chloroform-isoamyl alcohol (49:1).

2.3.2. Northern Blot Hybridization Analysis

Gel Electrophoresis

1. 10× TBE stock solution: 108 g Tris base, 55 g boric acid, 40 ml 0.5 M EDTA (pH 8.0), and distilled or deionized water to 1 l.
2. TBE 1× working solution: dilute 100 ml of 10× TBE stock with 900 ml of distilled or deionized water (dH_2O) to make 1 l.
3. Urea.
4. Denaturing buffer: 5% urea in 1× TBE 0.5% Bromophenol blue.
5. Electrophoresis apparatus (*MINI-PROTEAN* System, Bio-Rad).

RNA Blotting and Hybridization

1. Nylon membranes, positively charged.
2. UV-cross linker or UV-transilluminator.
3. Hybridization oven.

4. Maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, adjust to pH 7.5 with NaOH.
5. Blocking Reagent (Roche) 11096176001. Stock solution 10×: dissolve the blocking reagent (provided as powder) in maleic acid buffer to a final concentration 10% (w/v).
6. 20× SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.0.
7. DIG Hybridization buffer:

   For viroid mature forms. formamide 50% v/v, SSC 5×, *N*-laurylsarcosyne 0.1%, SDS 0.2%, Blocking reagent 1×.

   For small RNAs. formamide 40% v/v, SSC 5×, *N*-laurylsarcosyne 0.1%, SDS 0.2%, Blocking reagent (3×).
8. DIG-labeled (−) strand HSVd-RNA probes.
9. Washing buffer A: 2× SSC, 0.1% SDS.
10. Washing buffer B: 0.1× SSC, 0.1% SDS.

Immunological Detection

1. Blocking solution: 1× working solution: dilute 10× Blocking solution 1:10 with maleic acid buffer.
2. Antibody solution: centrifuge anti-digoxigenin-AP and dilute 1 in 10^4 in 1× Blocking solution.
3. Washing buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween 20.
4. Maleic acid buffer: 0.1 M maleic acid, 0.15 M NaCl, adjust with NaOH to pH 7.5.
5. Detection buffer: 0.1 M Tris–HCl (pH 9.5), 0.1 M NaCl.

2.4. RNA Silencing Analysis

1. Competent cells of *A. tumefaciens* strain C58C1 transformed with HSVd-GFP reporter (10).
2. Competent cells of *A. tumefaciens* C58C1 transformed with a GFP reporter gene (10).

2.4.1. Reporter Gene Expression

2.4.2. Reporter Gene Detection

1. Confocal Laser Scanning Microscopy (Leica TCS SL).
2. Microscope slides.

Fluorescence Analysis

3. Forceps with fine tips.

Western Blot Analysis

1. Electrophoresis and blotting apparatus (for Western blots).

SDS-PAGE

2. Blotting membrane (recommend PDVF).
3. 30% Acrylamide/bis-solutions, 37.5:1 mixture (Bio-Rad Cat #161-0158).
4. SDS 10% (w/v): dissolve 10 g of SDS in 90 ml of dH_2O with gentle stirring and bring to 100 ml with dH_2O. Store at room temperature (RT).

5. 1.5 M Tris–HCl, pH 8.8: dissolve 27.23 g of Tris base in 80 ml of dH_2O, adjust the pH to 8.8 with 6 N HCl. Bring the final volume to 150 ml with dH_2O, autoclave, and store at RT.
6. 0.5 M Tris–HCl, pH 6.8: dissolve 6 g of Tris base in 60 ml of dH_2O, adjust the pH to 6.8 with 6 N HCl and bring the total volume to 100 ml with dH_2O. Autoclave and store at RT.
7. Running buffer 10× (stock solution) pH 8.3: 30.3 g Tris base, 144.0 g glycine, 10.0 g SDS. Dissolve in dH_2O and bring the total volume up to 1 l with deionized water. Store at RT.
8. APS 10%: dissolve 100 mg of ammonium persulfate in 1 ml of deionized water.

Blotting and Immunological Detection

1. Protein transfer buffer (10× stock solution): dissolve 30.3 g of Tris and 144 g of glycine in 10× buffer, adjust to pH 8.3, and make up to a final volume of 1 l.
2. Protein transfer buffer 1× (working solution): 100 ml of 10× buffer, 700 ml of dH_2O, and 200 ml of methanol.
3. TBS 10× (stock solution): dissolve 80 g of NaCl in 200 ml of 1 M Tris–HCl, pH 7.6 and dilute to 1 l with distilled water.
4. Wash buffer: Tween-TBS (TBS-T): dilute 1 ml of Tween 20 in 1 l of 1× TBS.
5. Blocking buffer: 5% defatted milk powder and 2% BSA in TBS-T.
6. Primary Anti-GFP antibody (Rabbit-SIGMA G1544).
7. Secondary antibody Anti-Rabbit, peroxidase-labeled (GE-Healthcare NA931).
8. Orbital shaker.
9. Forceps with rounded, non-serrated tips.
10. X-ray film and cassettes.
11. ECL Plus™ Detection Reagents (GE-Healthcare RPN 2132): ECL Plus Solution A, 100 ml; ECL Plus Solution B, 2.5 ml.
12. Plastic bags.

3. Methods

3.1. Bioassays

3.1.1. Inoculation with Viroid RNA

1. Dilute 200 µg of viroid RNA in 200 µl of inoculation buffer.
2. Dust carborundum powder onto the cotyledons of 10- to 15-day-old cucumber plants.
3. Dispense 50 µl of inoculum mix onto each cotyledon, and gently scrape with a yellow micropipette tip.

4. Place the inoculated plants in an environmentally controlled growing chamber at 22–24°C.
5. After 4 days, move the inoculated plants to an environmentally controlled growing chamber at 28–30°C (optimal temperature for viroid-induced symptoms expression).

3.1.2. Grafting Inoculation

Here, we describe the method used to infect 2-week-old, wild-type, and *rdr6i N. benthamiana* scions by grafting onto 8-week-old HSVd-transgenic *N. benthamiana* stocks. Stunting is the most characteristic symptom induced by the expression of HSVd in *N. benthamiana* (17, 18). Thus, this ~~phenotypic characteristic~~ phenotype is used to determine symptom intensity in HSVd-infected plants. Grafting assays can be used to demonstrate whether or not the symptoms induced by a viroid infection are dependent on RDR6 activity, a key component of diverse RNA silencing pathways (Fig. 1). [AU1]

1. Remove the larger leaves from the stock and stake the plant with a stick.
2. Cut an oblique, 0.5–0.7-cm-deep incision in the stock stem.

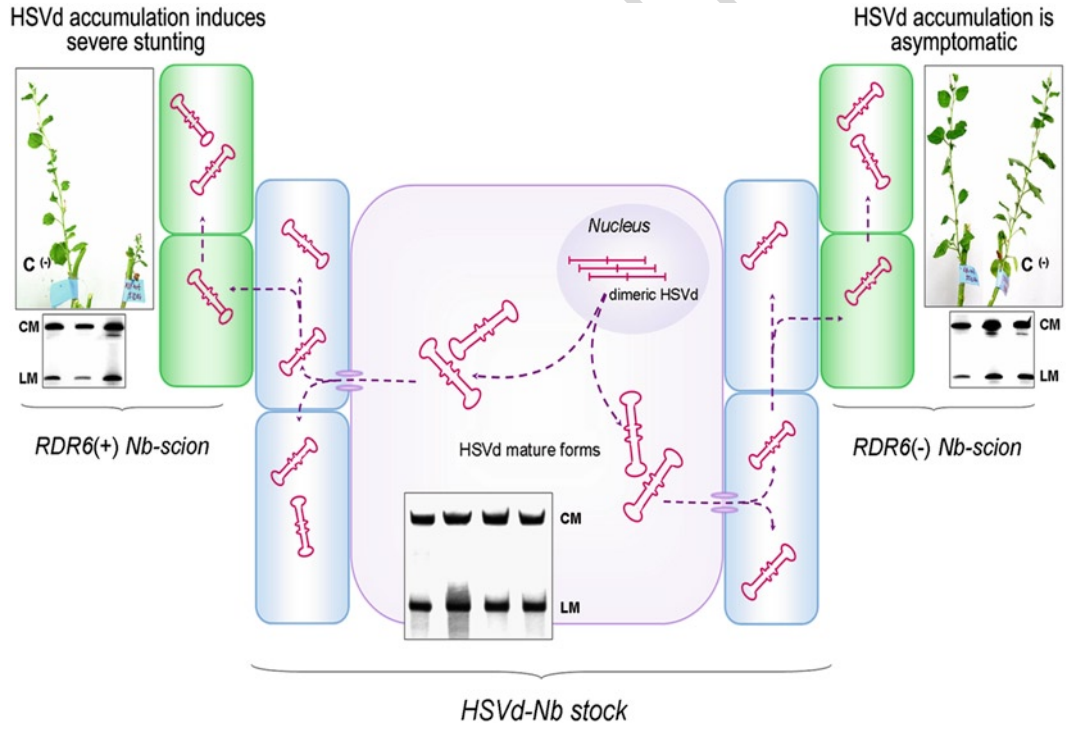

Fig. 1. Wild-type and *rdr6i-Nb* transgenic line scions were grafted onto symptomatic *HSVd/Nb* stocks which are able to process and accumulate viroid mature forms (*CM* circular monomeric forms, *LM* linear monomeric forms). At 45 days after grafting, the infected wild-type scions (*left*) showed severe stunting, reduced leaf size, and chlorosis, whereas the infected *rdr6i* scions (*right*), accumulating HSVd at a similar level, were unable to develop viroid-characteristic symptoms. These findings suggest the requirement of RDR6 activity for the development of the viroid-induced pathogenic process independently of viroid accumulation. $C^{(-)}$: *wt/Nb* and *rdr6i/Nb* scions grafted onto *wt/Nb* stocks as controls.

3. Select and cut a young growing tip with one or two leaves, from the scion.
4. Insert the scion in the stock incision and fix the graft with Parafilm.
5. Cover the grafted plant with a plastic bag and move the grafted plants to an environmentally controlled growth chamber at 22–25°C.
6. Check the grafted plant daily and make sure that the plastic bag is moist.
7. After 4 days, make 2–3 holes in the plastic bag (see Note 1).
8. After 2 days, remove the plastic bag.
9. At 10 days after grafting, move the grafted plants to an environmentally controlled growth chamber at 28–30°C (optimal temperature for the expression of viroid-induced symptoms).

3.1.3. Agroinoculation

Agroinoculation is a very easy method that permits efficient viroid-inoculation in diverse hosts. The transcription of the dimeric viroid-RNA is controlled by the CaMV 35S promoter, allowing a high accumulation of viroid-RNA inoculum in the initial phase of the infection. *A. tumefaciens* strain C58C1, transformed with the binary plasmid pMog800, carrying the 35S-HSVd-tNos cassette, is used in this case as the inoculum source.

1. Inoculate in 2 ml of liquid medium supplemented with specific antibiotics, single colonies of HSVd-transformed *A. tumefaciens* grown on agar plates. Incubate the bacterial cultures for 2 days at 28°C at 200 rpm on an orbital shaker.
2. Transfer 2 ml of the bacterial culture to an Eppendorf tube and centrifuge (at 1,500 ×*g* for 4 min) in a microfuge at RT.
3. Discard the supernatant and resuspend the bacterial pellet in 2 ml of infiltration buffer and incubate for 1–2 h at 25°C.
4. Agroinfiltrate the two basal leaves of the *N. benthamiana* plants, or the cotyledons for cucumber plants, by gentle pressure infiltration through the stomata of the lower epidermis by using a 1-ml syringe without a needle.
5. After infiltration, transfer the plants to an environmentally controlled growing chamber (28–30°C, 14 h of light).
6. At 6 days post-infiltration, remove the agroinfiltrated leaves.
7. Maintain the agroinoculated plants for 3–4 weeks under these conditions.

3.2. RNA Analysis

3.2.1. Total RNA Purification

This protocol is a modification of the method previously reported (20). The total RNAs extracted by this method are used as inoculum to infect cucumber plants and to analyze viroid mature forms by northern blot hybridization assays.

1. Homogenize 150 mg of plant leaves in Extraction buffer at a ratio of 1:10 (w/v), in a plastic bag. ~~[How is this done?]~~ [AU2]
2. Transfer the homogenate to an Eppendorf tube and centrifuge at 12,000 × *g* for 3 min at RT.
3. Transfer 1 ml of the supernate to a fresh Eppendorf tube, add 500 µl (0.5 vol) of phenol/chloroform (1:1), and vortex vigorously for 1 min.
4. Centrifuge at 12,000 × *g* for 5 min at RT.
5. Transfer 500 µl of the aqueous phase to a fresh Eppendorf tube and add 500 µl of isopropanol.
6. Incubate at RT for 5 min. Centrifuge at 12,000 × *g* for 15 min at 4°C. Discard the supernate.
7. Air-dry the RNA pellet for 5 min and redissolve in 50 µl of sterile water (see Note 2).
8. Store the RNA at –20°C.

3.2.2. Small RNA Purification from Leaves

The procedure used to obtain small RNA-enriched total RNAs consist of two steps. Firstly, total RNAs are extracted from infected plants by means of a modification of the original TRIzol protocol adapted to 15-ml tubes. To obtain higher recoveries of small RNAs, relative to total RNA, we use a pellet of total RNA as starting material to obtain small RNA-enriched plant RNAs with a modified protocol adapted from the miRNA isolation kit (miRACLE™, STRATAGENE 400813) (steps 9–32). For the following protocol, autoclaved materials (tubes, caps, mortars, and pestles) are required. [AU3]

1. Place 2 g of plant tissue into a clean mortar, previously chilled with liquid nitrogen. Cover the tissue with liquid nitrogen and grind into a very fine powder while frozen.
2. Transfer the powdered plant tissue to a clean 15-ml tube containing 10 ml of TRIzol reagent. Mix well by vortexing and centrifuge at 12,000 × *g* for 10 min at 4°C to remove the insoluble material.
3. Transfer the clear supernate to a fresh tube. Let the samples stand for 5 min at RT.
4. Add 2 ml of chloroform, shake vigorously by hand for 15 s and allow to stand for 15 min at RT.
5. Centrifuge the samples at 8,400 × *g* for 10 min at 4°C. The mixture will separate into three phases, the upper aqueous phase containing the RNA. Transfer this aqueous phase to a fresh tube.
6. Repeat steps 4 and 5 until the white layer at the interphase disappears (this usually requires two repetitions).
7. Transfer the aqueous phase to a fresh tube and add 0.5 vol of 3 M sodium acetate, pH 5.6 and 0.5 volume of isopropanol.

8. Leave the samples for 20–30 min at RT and centrifuge at 8,400 × *g* for 15 min at 4°C.
9. Add 600 µl of miRACLE miRNA lysis buffer (provided in the kit) directly to the RNA pellet, obtained in the previous step, in order to dissolve it.
10. Add a 0.1× volume of 2 M sodium acetate (pH 4) to the lysate.
11. Add an equal volume of phenol to the lysate and mix well by inversion.
12. Add a 0.2× volume of chloroform-isoamyl alcohol (49:1) equal lysate (for example, for lysates prepared in 600 µl of lysis buffer, add 120 µl of chloroform-isoamyl alcohol). Cap the tube tightly and mix by shaking the tube vigorously for 10 s.
13. Leave the mixture on ice for 15 min.
14. Spin the mixture in a microfuge at 4°C at 16,000 × *g* for 20 min. After centrifugation, two phases should be clearly visible and some DNA and protein material may be visible at the interface.
15. Carefully transfer the upper aqueous phase, containing the RNA, to a sterile, RNase-free microcentrifuge tube. Avoid taking any material from the interphase layer. Discard the lower phase, containing phenol, proteins, and DNA. Note the volume obtained.
16. Add ethanol (96–100%) to the RNA solution to a final concentration of 35% (v/v) (for example, add 215 µl of 96–100% ethanol to 400 µl of RNA solution). Mix well by vortexing.
17. Place a miRACLE™ miRNA RNA-Binding Spin Cup in a 2-ml receptacle tube. Vigorously flex the hinge of the tube, so that it becomes flexible and the cap can be firmly seated after loading. Transfer up to 700 µl of the RNA-containing 35% ethanol solution (from step 16) to the spin cup, close the cap securely and spin the cup.
18. Spin the tube in a microcentrifuge at 16,000 × *g* for 60 s.
19. Retain the filtrate (containing the small RNAs) in the receptacle tube and transfer the filtrate to a microfuge tube (see Note 3).
20. Measure the final volume of the filtrate containing the small RNAs in 35% ethanol.
21. Add ethanol (96–100%) to the filtrate solution to a final concentration of 70% (v/v) (for example, add 700 µl of 96–100% ethanol to 600 µl of filtrate). Mix well by vortexing. This ethanol concentration will bind sRNAs to the RNA-binding spin cup.
22. Place a fresh miRACLE miRNA RNA-Binding Spin Cup in a fresh 2-ml receptacle tube. Transfer up to 700 µl of the RNA-containing 70% ethanol solution (from step 14) to the spin cup and then cap the spin cup.

23. Spin the mixture in a microcentrifuge at 16,000×*g* × 60 s (see Note 4).
24. Remove and retain the spin cup and discard the filtrate.
25. Add 600 µl of 1× miRACLE miRNA Low-Salt Wash Buffer (provided in the kit) to the spin cup. Centrifuge at 16,000×*g* × 60 s. Remove and retain the spin cup and discard the filtrate. Replace the spin cup in the receptacle tube.
26. Add a second 600 µl of the 1× miRACLE miRNA Low-Salt Wash Buffer to the spin cup. Centrifuge at 16,000×*g* × 60 s. Remove and retain the spin cup and discard the filtrate. Replace the spin cup in the receptacle tube.
27. Add 300 µl of the 1× miRACLE miRNA Low-Salt wash buffer to the spin cup. Centrifuge at 16,000×*g* for 30–60 s. Remove and retain the spin cup and discard the filtrate. Replace the spin cup in the receptacle tube.
28. Centrifuge at 16,000×*g* for 2 min to dry the fiber matrix.
29. Transfer the spin cup to a 1.5-ml microcentrifuge tube (provided) and discard the 2-ml receptacle tube.
30. Add 50–100 µl of miRACLE™ miRNA elution buffer (provided in the kit) directly onto the center of the fiber matrix inside the spin cup. Incubate the tube at RT for 2 min.
31. Spin the tube in a microcentrifuge at 16,000×*g* for 1 min. Retain both the spin cup and the eluate, and reload it onto the membrane.
32. Incubate the tube at RT for 2 min. Spin the tube in a microcentrifuge at 16,000×*g* for 1 min. The purified RNA, enriched for small RNAs, is in the Elution Buffer in the microcentrifuge tube. The small RNA preparation can be stored at –20°C for up to 1 month, or at –80°C for long-term storage.

3.2.3. Small RNA Purification from Phloem Exudates

To obtain the maximum amount of phloem exudates, it is highly important to use well-hydrated plants, especially for viroid-infected plants; otherwise it may be difficult to extract these exudates.

1. One day before the extraction, irrigate the plants excessively and transfer them to a less-stressful temperature (e.g. 25°C) for no less than 2 h (see Note 5).
2. Make successive cuts in the leaf petioles, or the shoot apex, and collect the phloem exudate after each cutting (to get approx. 50–100 µl of exudate) with a 10-µl pipette directly into 1 ml of TRIzol reagent (see Notes 6 and 7).
3. Before starting the RNA extraction, it is recommended that the protein pellet is dissolved by vortexing vigorously, or by using a polytron (see Note 8).
4. Leave the samples stand for 5 min at RT (to allow nucleoprotein complexes to dissociate).

5. Add 200 µl of chloroform per 1 ml of TRIzol and shake the samples vigorously for 15 s.
6. Allow the samples to stand for 15 min at RT and centrifuge the resulting mixture at 12,000 × *g* for 15 min at 4°C.
7. Transfer the upper aqueous phase to a fresh autoclaved 1.5-ml Eppendorf tube and add 250 µl of isopropanol per ml of TRIzol reagent.
8. Mix gently and allow to stand for 5–10 min at RT.
9. Centrifuge at 12,000 × *g* for 15 min at 4°C. Eventually, the RNA precipitate will form a transparent pellet on the bottom of the tube.
10. Redissolve the pellet in sterile water. The expected RNA yield from 50 to 100 µl of phloem exudates is 10–50 ng.

3.2.4. Northern Blot Hybridization Analysis

To prepare homogeneous polymerized acrylamide gels, it is very important to wash all glass plates and materials with 70% ethanol and rinse with abundant sterile water. In this protocol, we describe the use of the mini-protean system (Bio-Rad); however, these directions could be adapted to different gel-casting systems.

For Viroid Mature Forms

Gel Electrophoresis

1. Adjust the short plate on the spacer-containing plate and fix into position with two clamps on the vertical side.
2. Place the system on a stable support.
3. Prepare a 5% denaturing acrylamide premix as follows. Gently mix (to avoid bubbles) the following components in the order indicated (the following volumes are sufficient for two mini gels): 2.5 ml of acrylamide/bis-acrylamide 40% (19:1); 500 µl of 10× TBE; 10 ml of sterile water; 9.6 g of urea; 100 µl of 10% APS and 10 µl of TEMED (polymerization starts when the APS and TEMED are added).
4. Gently pour the acrylamide mix between the plates and ensure that there are no bubbles in the gel.
5. Insert a 10-well comb at the top of the gel and fill any empty space with acrylamide mix. Wait for 30 min at RT for complete polymerization.
6. Remove the comb and clamps from the gel-casting system and place the gel in the electrophoresis system.
7. Fill the tanks with cold fresh TBE 1× and flush out the sample wells using a micropipette.
8. Mix 20 µl (~5 µg) of total RNAs with denaturing buffer (1:1) and incubate for 5 min at 85°C.
9. Load the denatured samples into the sample wells and run the gel at 100 V at RT for 2 h.

10. Remove the short glass plate and place the gel carefully into ethidium bromide (1 µg/ml) staining solution. Allow to stain for 15 min, discard the staining solution and rinse the gel twice with sterile water for 5 min and visualize the gel under UV light.

RNA Blotting

1. Prepare the 1× TBE buffer. Using buffer and H_2O, chilled to 4°C, will improve heat dissipation.
2. Cut one corner of the gel to allow its orientation to be tracked.
3. Cut the membrane and the filter paper to the dimensions of the gel. Always wear gloves when handling membranes to prevent contamination. Equilibrate the gel and soak the membrane, filter paper, and fiber pads in transfer buffer (15 min).
4. Prepare the gel sandwich: place the cassette, with the gray side down, on a clean surface. Place one pre-wetted fiber pad on the gray side of the cassette and a sheet of filter paper on the fiber pad. Place the equilibrated gel on the filter paper and the pre-wetted membrane on the gel. Complete the sandwich by placing a piece of filter paper on the membrane. Add the last fiber pad (see Note 9).
5. Close the cassette firmly, being careful not to move the gel and filter paper sandwich. Lock the cassette closed with the white latch.
6. Place the cassette and add the frozen cooling unit into the module in the transfer unit and completely fill the tank with buffer.
7. Add a standard magnetic stirrer bar to help maintain even buffer temperature and ion distribution in the tank. Set the speed as fast as possible to keep ion distribution even. Run the blot for 1 h at 100 V.
8. Upon completion of the run, dismantle the blotting sandwich and remove the membrane for hybridization.

Blot Hybridization

1. Place the membrane on Whatman 3MM-paper and UV-crosslink the membrane (0.18 J × 2.5 min). [AU4]
2. After the UV-crosslinking, rinse the membrane briefly with transfer buffer and allow it to air-dry.
3. Pre-warm an appropriate volume of 1× DIG Hybridization buffer (10–15 ml/100 cm^2 membrane) to the hybridization temperature (68°C).
4. Pre-hybridize the membrane with 1× DIG Hybridization buffer for 60 min with gentle agitation in an appropriate container (see Note 10).

5. Denature the DIG-labeled RNA probe by boiling for 5 min and cooling rapidly on ice.
6. Add the denatured DIG-labeled RNA probe (100 ng/ml) to the pre-warmed 1× DIG Hybridization buffer (3.5 ml/100 cm^2 membrane) and mix.
7. Discard the pre-hybridization solution and add the probe/hybridization mixture to the membrane.
8. Incubate overnight at 68°C with gentle agitation (see Note 11).
9. Discard the probe and wash the membrane twice for 5 min in wash buffer A at RT with constant agitation.
10. Wash the membrane twice for 15 min in wash buffer B (pre-warmed to washing temperature) at 68°C under constant agitation.

Immunological Detection

1. After hybridization and stringency washes, rinse the membrane briefly (1–5 min) in washing buffer.
2. Incubate for 30 min in 100 ml of blocking solution.
3. Incubate for 30 min in 50 ml of antibody solution.
4. Wash 2× 15 min in 100 ml of washing buffer.
5. Equilibrate 2–5 min in 100 ml of detection buffer.
6. Place the membrane (with RNA side facing up) on a development folder (or hybridization bag) and quickly apply approx. 1 ml of CSDP solution.
7. Cover the membrane with the second sheet of the folder to spread the substrate evenly, and without air bubbles, over the membrane.
8. Incubate for 5 min at RT.
9. Squeeze out the excess liquid and seal the edges of the development folder around the damp membrane (see Note 12).
10. Expose to an imaging device for 5–20 min or to X-ray film for 15–25 min at RT (see Note 13). Representative northern blot assays for viroid mature forms are shown in the Figs. 1 and 2.

For Viroid-Derived Small RNAs

Gel Electrophoresis

1. Prepare a 20% denaturing acrylamide premix. Mix gently (to avoid bubbles) the following components in the order shown (indicated volumes allow for the preparation of two mini gels): 10 ml of acrylamide/bis-acrylamide 40% (19:1); 500 µl of 10× TBE; 2.5 ml of sterile water; 9.6 g of urea; 100 µl of APS (10%) and 10 µl of TEMED (polymerization starts when the APS and TEMED are added).
2. Mix 50 µl (~5 µg) of small-enriched RNA with 10 mg of urea and incubate for 5 min at 85°C.

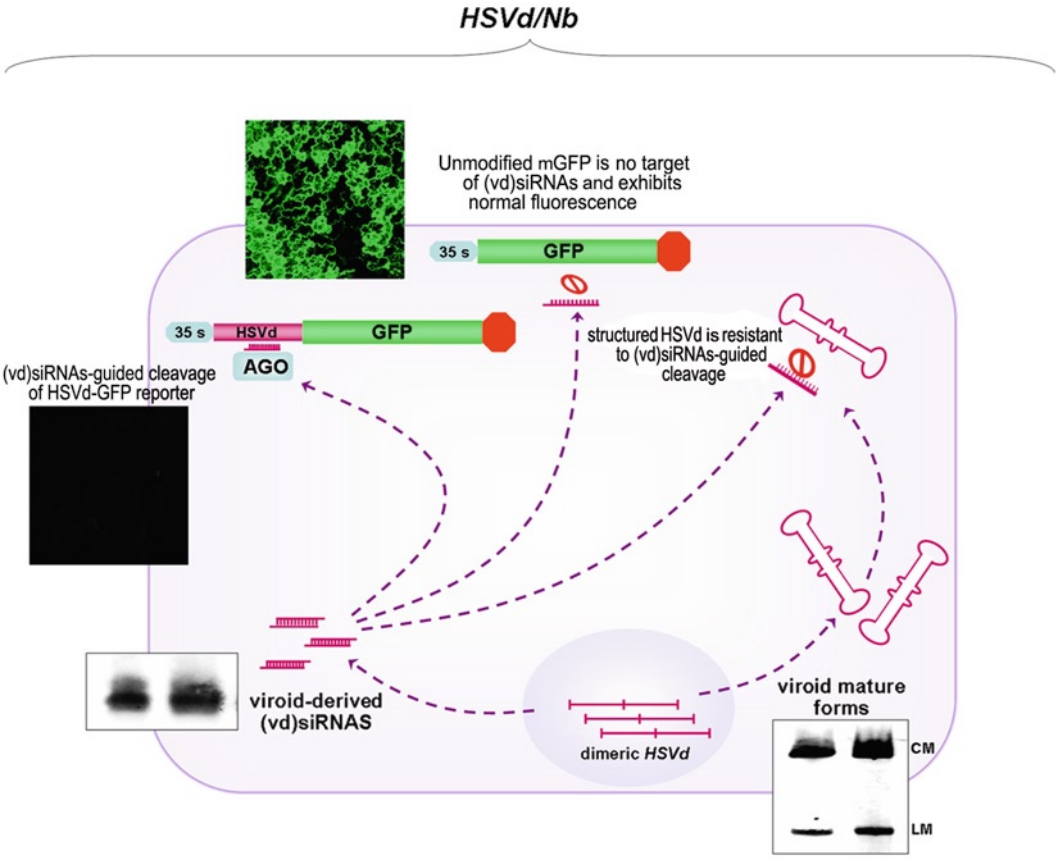

Fig. 2. The *HSVd-Nb* plants expressing and processing a dimeric HSVd-RNA accumulate high levels of HSVd mature forms (*CM* circular monomeric forms, *LM* linear monomeric forms) and viroid-derived (vd) siRNAs. *Left*: *HSVd/Nb* plants were agroinfiltrated with HSVd-GFP and GFP constructs. The expression of the HSVd-GFP reporter gene was suppressed in this transgenic plant, unlike the unmodified GFP that showed normal fluorescence, indicating that (vd) siRNAs are functional and able to guide the specific cleavage of the full-length unstructured HSVd-RNA sequence. *GFP* green fluorescent protein.

3. Load the denatured samples into the sample wells and run the gel for 4 h at 85 V, at RT.
4. Remove the gel from the electrophoresis system.
5. Remove the short glass plate and carefully place the gel in an ethidium bromide staining solution. Stain for 15 min and carefully discard the staining solution. Rinse the gel twice with sterile water for 5 min and visualize under UV light.
6. Cut off the top of the gel (containing the high-molecular-weight RNAs) and discard.

RNA Blotting

Follow the identical procedure to that described above for the blotting of total RNAs, except that the blotting is only run for 2 h at 65 V.

Hybridization

1. Place the membrane on Whatman 3MM-paper and UV-crosslink the membrane (0.18 J × 2.30 min).
2. After the UV-crosslinking, rinse the membrane briefly with transfer buffer and allow it to air-dry.
3. Pre-warm an appropriate volume of DIG Hybridization buffer (10–15 ml/100 cm^2 membrane) at 68°C (see Note 14).
4. Pre-hybridize the membrane with DIG Hybridization buffer for 2 h with gentle agitation in an appropriate container at 68°C.
5. Denature the DIG-labeled RNA probe by boiling for 5 min and rapidly cooling on ice.
6. Add the denatured DIG-labeled RNA probe (100 ng/ml) to pre-warmed DIG Hybridization buffer (3.5 ml/100 cm^2 membrane) and mix.
7. Discard the pre-hybridization solution and add the probe/hybridization mixture to the membrane.
8. Incubate overnight at 32°C with gentle agitation (see Note 15).
9. Wash the membrane twice for 15 min in wash buffer A at RT under constant agitation.
10. Wash the membrane twice for 15 min in wash buffer A (pre-warmed to the wash temperature) at 35°C under constant agitation.

Immunological Detection

Follow the identical procedure to that described above for immunological detection in northern blot assays of total RNAs. A representative detection by northern blot analysis of viroid-derived siRNAs is shown in the Fig. 2.

3.3. RNA Silencing Analysis

3.3.1. Reporter Gene Expression

To analyze the viroid-induced RNA silencing activity, we designed a transcriptionally fused 5′ HSVd-GFP silencing reporter gene used to agroinfiltrate HSVd-Nb and HSVd-infected Wt-Nb plants. In these plants, the mature forms of the HSVd could resist the HSVd-induced RNA silencing-mediated degradation, whereas the identical full-length HSVd sequences fused to the mRNA GFP could not. Consequently, the HSVd-GFP construct could be used as a reporter of the HSVd-specific RNA silencing activity, in the symptomatic plants. Although here we describe the use of a sensor system to detect the HSVd-induced RNA silencing, similar reporter constructs can be used to analyze the RNA silencing induced by other nuclear viroids.

Agroinfiltration

Binary pMog800 plasmids, carrying the 35S-HSVd-GFP-tNos or 35S:GFP:t-Nos cassettes, are transformed into *A. tumefaciens* strain C58C1 containing the virulence helper plasmid pCH32 (21).

1. Two days prior to agroinfiltration, inoculate 2 ml of liquid medium, supplemented with specific antibiotics, with single colonies of HSVd-GFP- and HSVd-transformed *A. tumefaciens* grown on agar plates. Incubate the bacterial cultures for 2 days at 28°C at 200 rpm on an orbital shaker.
2. Two days later, start new bacterial cultures by inoculating fresh medium with the old suspension cultures (1/200 ratio, v/v). Grow the new cultures under the same conditions for an additional day.
3. After 24 h, transfer 2 ml of each bacterial culture to Eppendorf tubes and pellet the cells by centrifugation (1,500 × *g* for 4 min) in a microfuge at RT.
4. Discard the supernatant and resuspend the cell pellet in 2 ml of infiltration buffer. Dilute the bacterial suspensions with infiltration buffer to a final OD_{600} of 0.2 and incubate for 1–2 h at 25°C.
5. Agroinfiltrate the two basal leaves of *N. benthamiana* plants by gentle pressure infiltration through the stomata of the lower epidermis by using a 1-ml syringe without a needle.
6. After infiltration, transfer the plants into an environmentally controlled growing chamber (28°C, 14 h of light).

3.3.2. Reporter Detection

By Confocal Microscopy

1. Three days after agroinfiltration, cut approx. 5.5 mm^2 leaf discs (three per leaf) and mount in water under glass cover slips for microscopy.
2. Analyze the GFP expression with a confocal laser scanning microscope, with excitation at 488 nm and emission at 510–560 nm. An example of the potential results is shown in Fig. 2.

By Western Blot Analysis

These instructions assume the use of a Bio-Rad Mini Protean gel system. However, they are easily adaptable to other gel formats.

Gel Electrophoresis

1. Adjust the short plate on the spacer-containing plate and fix lock into position with two clamps on the vertical sides.
2. Place the system on a stable support.
3. Prepare a 12% SDS-PAGE premix. Mix gently (to avoid bubbles) the following components, in the order shown (the indicated amounts are sufficient for preparing two 20 ml mini gels): 8 ml of acrylamide/bis-acrylamide (30%); 5 ml of 1.5 M Tris (pH 8.8); 6.6 ml of sterile water; 200 µl of 10% APS; and 10 µl of TEMED (polymerization starts when APS and TEMED are added).
4. Gently pour the acrylamide mix between the gel plates, leaving space for a stacking gel, and ensure that are there are no bubbles

in the gel. Carefully overlay with 500 µl of water. The gel should polymerize in about 30 min.

5. Pour off the water.
6. Prepare the stacking gel (8 ml) by mixing: 1.3 ml of acrylamide/bis-acrylamide 30%; 1.0 ml of 1.0 M Tris (pH 6.8); 5.5 ml of sterile water; 80 µl SDS 10%; 80 µl of APS 10%; and 5 µl of TEMED (polymerization starts when APS and TEMED are added).
7. Use about 3 ml of this to quickly rinse the top of the gel and then pour the stacking gel mix and insert the comb. The stacking gel should polymerize within 20–30 min.
8. Prepare the running buffer by diluting 100 ml of the 10× running buffer with 900 ml of water.
9. Add the running buffer to the upper and lower chambers of the gel unit.
10. Once the stacking gel has set, carefully remove the comb and use a micropipette to wash the wells with running buffer.
11. Complete the assembly of the gel unit and connect it to a power supply.
12. Mix 45 µl of protein extract with 5 µl of sample buffer. Denature the sample (3 min at 95°C). Centrifuge for 15 s at maximum speed.
13. Load the denatured samples in the well and run at RT for 2 h at 85 V.

Blotting

1. After electrophoresis is complete, turn off the power supply and remove the tank lid and carefully lift out the Inner Chamber Assembly. Pour off, and discard, the running buffer (see Note 16).
2. Open the cams of the Clamping Frame. Pull the Electrode Assembly out of the Clamping Frame and remove the Gel Cassette Sandwiches.
3. Remove the gels from the Gel Cassette Sandwich by gently separating the two plates of the gel cassette. The green, wedge-shaped, plastic Gel Releaser may be used to help pry the glass plates apart (see Note 17).
4. Run the sharp edge of the Gel Releaser, or a razor blade, along each spacer to separate the gel from the spacer. Remove the gel by floating it off the glass plate by immersing the gel and plate under transfer solution and agitating gently until the gel separates from the plate.
5. Fill the Bio-Ice cooling unit with water and store it in your laboratory freezer at –20°C until ready to use. After use, return the cooling unit to the freezer for storage.

6. Prepare the protein-transfer buffer (see Note 18).
7. Cut the PDVF membrane and the filter paper to the dimensions of the gel. Always wear gloves when handling membranes to prevent contamination. Soak the PDVF membrane in methanol.
8. Equilibrate the gel and soak the membrane, filter paper, and fiber pads in transfer buffer for 5 min.
9. Prepare the gel sandwich: place the cassette, with the gray side down, on a clean surface. Place one pre-wetted fiber pad on the gray side of the cassette and a sheet of filter paper on the fiber pad. Place the equilibrated gel on the filter paper and the pre-wetted membrane on the gel. Complete the sandwich by placing a piece of filter paper on the membrane. Add the last fiber pad (see Note 9).
10. Close the cassette firmly, being careful not to move the gel and filter paper sandwich. Lock the cassette closed with the white latch.
11. Place the cassette in module.
12. Add the frozen Bio-Ice cooling unit. Place the cassette in the tank and completely fill with buffer.
13. Add a standard stir bar to help maintain even buffer temperature and ion distribution in the tank. Set the speed as fast as possible to keep ion distribution even.
14. Put on the lid, plug the cables into the power supply, and run the blot for 1 h at 100 V.
15. Upon completion of the run, dismantle the blotting sandwich and remove the membrane for development.

Blot Development

1. Incubate the membrane in 50 ml of blocking buffer for 1 h at RT on a rocking platform.
2. Discard the blocking buffer and add a 1:10,000 dilution of the anti-GFP in TBST/2% BSA for 7–9 h at 4°C on a rocking platform.
3. Remove the primary antibody and wash the membrane three times for 10 min each with 50 ml of TBS-T.
4. Prepare fresh secondary antibody, at a 1:10,000-fold dilution in blocking buffer, and add it to the membrane. Incubate for 60 min at RT.
5. Discard the secondary antibody and wash the membrane three times, for 15 min each, with TBS-T.
6. During the final wash, 2-ml aliquots of each portion of the ECL reagent are warmed separately to RT and the remaining steps are done in a dark room under safe light conditions. Once the final wash is removed from the blot, mix the ECL reagents

and then immediately add them to the blot. Incubate at RT for 5 min to ensure even coverage.

7. Remove the blot from the ECL reagents and place it between the two pieces of plastic that has been cut to the size of an X-ray film cassette.
8. Place the bag, containing the membrane, in an X-ray film cassette with film for a suitable exposure time, typically for 3–7 min.

4. Notes

1. The holes in the plastic bag permit a gradual adaptation to environmental conditions.
2. The pellet dissolution can be facilitated by incubation at 55°C.
3. If it is not possible to load the entire sample in the spin cup in step 9, reseat the same spin cup in the receptacle tube and repeat steps 9 and 10, combining all of the filtrates into a single tube.
4. If the sample volume is greater than 700 µl, repeat steps 22 and 23.
5. Better results are obtained with an overnight exposure.
6. Younger leaves from the apex, and the apex itself, are the preferred plant tissue to carry out phloem extraction. Before cutting the petiole, clean the cut surface with sterilized filter paper to avoid cellular contamination.
7. In infected plants, it is difficult to obtain more than 0.5–1 µl of phloem by cutting. Exudates form a whitish pellet due to protein precipitation.
8. The removable parts of the polytron must be cleaned with SDS 10% and ethanol, after each use.
9. Remove any air bubbles which may have formed. Use a tube to gently roll out the air bubbles.
10. Membranes should move freely, especially if several membranes are used in the same pre-hybridization solution.
11. DIG Hybridization buffer containing DIG-labeled probe can be stored at –20°C and be reused when freshly denatured at 65°C for 10 min.
12. Ensure that the membrane does not dry out during exposure. Otherwise a dark background will be obtained.

13. Luminescence continues for at least 24 h. The signal increases in the first few hours. Multiple exposures can be taken to achieve the desired signal strength.
14. To avoid background signal, it is important to use the DIG Hybridization buffer specific to small RNAs northern blots.
15. In principle, DIG Hybridization buffer containing DIG-labeled probe can be stored at –20°C and reused when freshly denatured at 65°C for 10 min. In northern blot assays to detect small RNAs, we do not recommend reusage of DIG Hybridization buffer containing RNA probes, due to the increase of the background signal.
16. Always pour off the buffer before opening the cams to avoid spilling the buffer.
17. To remove the gel from a Ready Gel Cassette, first slice the tape along the sides of the Ready Gel Cassette where the inner glass plate meets the outer plastic plate.
18. Using buffer chilled to 4°C will improve heat dissipation.

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