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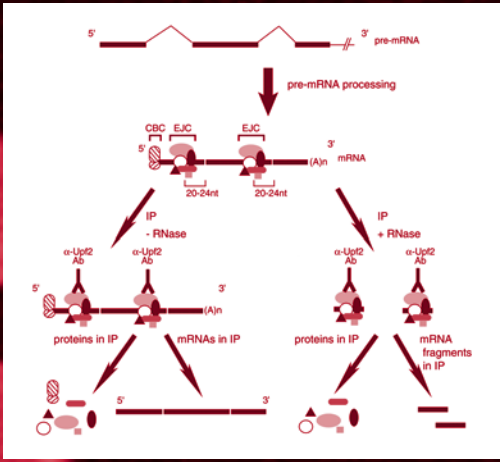

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Biochemical Dissection of RNA Silencing in Plants

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Summary

Although RNA silencing was first discovered in plants, thus far it has been studied biochemically only in animals, where it is known as RNA interference (RNAi). In animals, two components of the RNAi pathway have been identified: Dicer, a multidomain RNase III that converts long double-stranded RNA (dsRNA) into small interfering RNA (siRNA) and the RNA-induced silencing complex (RISC), as siRNA-containing protein–RNA complex that targets complementary mRNA for destruction. We have developed methods for the biochemical dissection of plant RNA silencing. In this chapter, we describe in detail how to use wheat germ extract to study two distinct Dicer-like activities, RNA-dependent RNA polymerase (RdRP), and endogenous microRNA-programmed RISC activities. These comprehensive protocols should prove useful in the further dissection of the plant RNA silencing pathway, as well as for the validation of the predicted targets of endogenous plant microRNAs.

Key Words

RNA silencing; RNAi; PTGS; siRNA; miRNA; RISC; Dicer; RdRP.

1. Introduction

RNA silencing is a universal phenomenon in eukaryotes ***(1–7)***. It describes a group of related phenomena in which aberrant or double-stranded RNA (dsRNA) triggers a dramatic reduction in either the transcription of the corresponding gene or the direct degradation of the corresponding messenger RNA (mRNA). In animals, posttranscriptional RNA degradation induced by exogenous double-stranded RNA (dsRNA) is called RNA interference (RNAi); in plants, it is called posttranscriptional gene silencing (PTGS). Biochemical approaches have been successfully used to study RNAi in *Drosophila* and mammals ***(8,9)***. In RNAi, long dsRNA is fragmented into 21–23 nt small interfering RNAs (siRNAs) by members of the Dicer family of RNase III enzymes

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(10–13). siRNAs assemble with specific proteins to form an RNA-induced silencing complex (RISC) that directs target RNA cleavage, thereby silencing gene expression ***(8,11–21)***.

In plants, endogenous gene silencing triggered by extra copies of a transgene with the same sequence is known as cosuppression ***(22)***. Cosuppression can reflect either transcriptional repression (transcriptional gene silencing [TGS]) or PTGS. PTGS is the plant RNA silencing phenomenon most closely related to RNAi ***(23)***. In IR-PTGS, dsRNA generated from the transcription of inverted repeats efficiently silences the corresponding mRNA in transgenic plants ***(24,25)***. Unlike RNAi, PTGS can also be initiated by transgenes that produce sense RNA (S-PTGS) or antisense RNA (AS-PTGS). S-PTGS and AS-PTGS initiated by highly expressed transgenes is genetically distinct from IR-PTGS, in which silencing is initiated by transgenes specifically designed to generate dsRNA ***(26)***. The unifying feature of RNAi and PTGS is the production of small RNAs of 21–25 nt in length ***(8,15,27–31)***. In contrast to the single population of small RNAs produced in *Drosophila*, two distinct populations of siRNAs, approx 21 nt and 24–25 nt, are generated in plants ***(32–34)***. The two classes of small RNAs have been proposed to play distinct functional roles in plant RNA silencing, with the long one correlating with systematic silencing and DNA methylation and the short one with local RNA silencing ***(33)***.

In addition to Dicer-like enzymes, an RNA-dependent RNA polymerase (RdRP) is involved in RNA silencing in plants, worm, *Neurospora* and *Dictyostelium*, but not in *Drosophila* or human cells ***(35–45)***. Notably, the requirement for the putative RdRP protein SGS2 (SDE1) is bypassed when silencing in plants is initiated by transgenes specifically engineered to produce dsRNA ***(26)***. RdRPs have been proposed to use aberrant RNAs as templates and copy them into complementary RNAs (cRNAs) to form dsRNA ***(23,46)***. This newly synthesized dsRNA is thought to be a substrate for Dicer-like enzymes ***(47)***. What features make an RNA aberrant remains unknown. Furthermore, a novel population of small RNAs is synthesized directly by a purified recombinant *Neurospora* RdRP (QDE1) ***(48)***. The function of these RdRP-generated small RNAs is likewise unknown.

Dicer also produces microRNAs (miRNAs), small, 21- to 24-nt single-stranded RNAs proposed to control gene expressions in eukaryotes. The first miRNAs discovered were *lin-4* and *let-7*, which regulate the timing of development in *Caenorhabditis elegans* ***(49,50)***; to date, more than a hundred miRNAs have been identified ***(51–56)***. miRNAs are encoded in genes that produce RNA transcripts that can fold into imperfect stem-loop structures. These stem-loops are the direct precursors of miRNAs and are called pre-miRNAs. Dicer cleaves each miRNA from one side or the other of the stem of the pre-miRNA ***(12,13,52–57)***. Animal and plant miRNAs have been proposed to func-

tion by different mechanisms. In animals, *lin-4* and *let-7* are thought to base pair imperfectly with sites within the 3' untranslated region of their target mRNAs, triggering translational repression of the mRNA targets ***(58,59)***. In contrast, miRNAs from plants act as natural siRNAs to mediate the cleavage of target mRNAs because of their near perfect complementarity between the miRNAs and their target RNAs ***(32,60–62)***. Regardless of how they function, miRNAs generally are associated with a common RISC-like protein complex. Current evidence suggests that the extent of complementarity between miRNAs and their target RNAs determines whether miRNAs regulate translation or direct target cleavage ***(20,63,64)***.

Although RNA silencing was first discovered in plants, the plant-silencing pathway has not been subject to extensive biochemical analysis in part because a suitable in vitro system was unavailable. Here, we describe an in vitro system, wheat germ extract, useful for analyzing several key features of RNA silencing in plants. Using this in vitro system, we have shown previously that the RNA silencing pathways in plants and animals are remarkably similar ***(32)***. Wheat germ extract contains Dicer-like enzymes that produce siRNAs and RNA-dependent RNA polymerases (RdRPs) that copy exogenous single-stranded RNA (ssRNA) into dsRNA. In the in vitro system, plant Dicer-like enzymes convert dsRNAs into two distinct classes of siRNAs, long and short siRNAs. Furthermore, endogenous plant miRNAs can direct mRNA degradation in wheat germ extract. In this chapter, we describe the preparation and use of wheat germ extract to dissect the RNA-silencing pathway in plants.

2. Materials

2.1. Preparation of Cell-Free Extracts

1. Raw wheat germ (e.g., Fearn Nature Fresh Raw Wheat Germ, Bread and Circus).
2. Fresh cauliflower.
3. *Drosophila melanogaster* embryo (0–2 h) lysate.
4. 1X lysis buffer: 100 m*M* potassium acetate; 30 m*M* HEPES-KOH, pH 7.4; 2 m*M* magnesium acetate.
5. 1 *M* Dithiothreitol (DTT), stored in aliquots at –20°C.
6. Complete, mini-EDTA-free protease inhibitor cocktail tablets (Roche).

2.2. Production of Transcription Templates by Polymerase Chain Reaction (PCR)

*2.2.1. DNA Primers (see **Note 1**)*

1. *Renilla reniformis* luciferase 501-bp (*Rr*-luc501) T7 template primers:
   a. Sense template primers: 5' PCR primer GCGTAATACGACTCACTATA GGAAAAACATGCAGAAAATGC and 3' PCR primer GAAGAATGGTTC AAGATATGCTG.

b. Antisense template primers: 5' PCR primer GCGTAATACGACTCACT ATAGGAATGGTTCAAGATATGCTG and 3' PCR primer GAAGAAAA ACATGCAGAAAATGC.

2. Wild-type *PHAVOLUTA* (*PHV*) T7 template primers: 5' primer containing T7 promoter GCGTAATACGACTCACTATAGGCGCCGGAACAAGTTGAAG and 3' primer GACAGTCACGGAACCAAGATG.
3. Mutant *phv* T7 template primers: First pair is 5'-*PHV* primer (the same as the wild-type *PHV* T7 template 5' primer) and CCACTGCAGTTGCGTGAA ACAGCTACGATACCAAT AGAATCCGG-ATCAGGCTTCATCCC. Second pair is 5'-*PHV* primer (the same as the wild-type *PHV* T7 template 5' primer) and GACAGTCACGGAACCAAGATGGACGATCTTTGAGGATTTCAGCGAC CTTCATGGGTTCTAAACTCACGAGGCCACAGGCACGTGCTGCTATTCCAC TGCAGTTGCGTG AAACAGC.

2.2.2. PCR Templates

1. *Renilla reniformis* luciferase (*Rr*-luc) cDNA clone as PCR template for *Rr*-luc501.
2. *Arabidopsis* flower cDNA library (CD4-6) (*Arabidopsis* Stock Center, Ohio) as PCR template for cloning wild-type *PHV*.

2.2.3. 10X PCR Buffer

100 m*M* Tris-Cl, pH 8.3; 500 m*M* KCl; 15 m*M* $MgCl_2$; 0.1% w/v gelatin.

2.2.4. Taq Polymerase

2–5 U/μL (Roche or Invitrogen).

2.3. RNA Synthesis

1. 10X T7 transcription buffer: 400 m*M* Tris-Cl, pH 7.9; 25 m*M* spermidine; 260 m*M* $MgCl_2$; 0.1% v/v Triton X-100.
2. 2X proteinase K (PK) buffer: 200 m*M* Tris-Cl, pH 7.5; 25 m*M* ethylenediamine tetraacetic acid (EDTA), pH 8.0; 300 m*M* NaCl; 2% w/v sodium dodecyl sulfate (SDS).
3. 2X Urea stop mix/loading dye: 8 *M* urea; 25 m*M* EDTA, pH 8.0; 0.025% w/v xylene cyanol FF; 0.025% w/v bromophenol blue.
4. SP6-*Pp*-luc (*Pyralis photinus* luciferase) plasmid template for RNA transcription.
5. T7 RNA polymerase (New England Biolabs).
6. SP6 RNA polymerase (New England Biolabs).
7. SequaGel System (National Diagnostics).
8. 40 U/μL RNasin (Promega).
9. Super RNasin (Ambion).

2.4. RNA Labeling

1. Radiochemicals: α-[^{32}P] adenosine triphosphate (ATP; 40 mCi/ml, 3000 Ci/mmol, ICN), α-[^{32}P] guanosine triphosphate (GTP; 40 mCi/mL, 3000 Ci/mmol, ICN), α-[^{32}P] uridine triphosphate (UTP; approx 10 mCi/mL, 25 Ci/mmol, ICN), and α-[^{32}P] ATP (6000 Ci/mmol, ≥10 mCi/mL, NEN or ICN).

2. T4 polynucleotide kinase (PNK, 10,000 U/mL, New England Biolabs).
3. 10X T4 polynucleotide kinase buffer: 700 m*M* Tris-HCl, pH 7.6; 100 m*M* $MgCl_2$; 50 m*M* DTT.
4. Formamide loading dye: 98% v/v deionized formamide; 10 m*M* EDTA, pH 8.0; 0.025% w/v xylene cyanol; 0.025% w/v bromophenol blue.
5. 10X Guanylyl transferase buffer: 500 m*M* Tris-Cl, pH 8.0; 60 m*M* KCl; 12 m*M* $MgCl_2$; 25 m*M* DTT.
6. *S*-adenosyl methionine (SAM) dilution buffer (5 n*M* H_2SO_4 in 10% v/v ethanol): 6.96 μL of 36 M H_2SO_4, 5 mL of absolute ethanol, and 45 mL of deionized H_2O.
7. Guanylyl transferase ***(65)***.
8. SAM (Sigma).
9. Typical RNA oligonucleotides (Dharmacon): siRNAs (sense and antisense strands) corresponding to miR165, miR166, *PHV*, and mutant *phv* target positions were miR165, UCGGACCAGGCUUCAUCCCCC and GGGAUGAAG CCUGGUCCGAGG; miR166, UCGGACCAGG CUUCAUUCCCC and GGAA UGAAGCCUGGUCCGAGA; *PHV*, CCGGACCAGGCUUCAUCCCAA and GGGAUGAAGC CUGGUCCGGAU; and mutant *phv*, CCGGAUCAGGCUU CAUCCCAA and GGGAUGAAGCCUGAUCCGGAU. The 21-nt siRNA inhibitor comprised CGUACGCGGAAUACUUCGAUU annealed with UCGAAG UAUUCCGC GUACGUG; the 25-mer siRNA inhibitor comprised AUCACG UACGCGGAAUACUUCGAUU annealed with UCGAAGUAUUCCGCGUA CGUGAUUG.

2.5. RNA Processing

1. dsRNA/RNAi processing mix: 50 μL of H_2O, 20 μL of 500 m*M* creatine monophosphate (Fluka, prepared fresh from powder), 20 μL of amino acid stock (Sigma, 1 m*M* each amino acid), 2 μL of 40 U/μL RNasin (Promega), 4 μL of 100 m*M* ATP, 1 μL of 100 m*M* GTP, 6 μL of 2 U/μL creatine phosphokinase (Cal-Biochem), prepared by diluting 2 μL of a 20 U/μL stock in 18 μL of 1X lysis buffer containing 2 m*M* DTT, 16 μL of 1 *M* potassium acetate.
2. 10X RNase digestion buffer: 3 *M* NaCl; 100 m*M* Tris-Cl, pH 7.4; 50 m*M* EDTA, pH 7.5.
3. 5X Borax/boric acid buffer (300 m*M*, pH 8.6): 150 m*M* borax (MW 381.4) and 150 m*M* boric acid (FW 61.83).

2.6. siRNA Structure Analysis

1. 1X Borax/boric acid buffer, pH 9.5: dilute 5X borax/boric acid buffer (300 m*M*, pH 8.6) in water and adjust pH with 10 *M* NaOH.
2. 200 m*M* Periodate ($NaIO_4$) solution (prepared freshly before use).
3. Phosphatase, calf intestinal (CIP, Promega or New England Biolabs).

2.7. Northern Blot Analysis

1. 20X SSPE: 3 *M* NaCl, 0.25 *M* NaH_2PO_4, and 0.02 *M* EDTA, adjust to pH 7.4.
2. 20X SSC: 3 *M* NaCl and 0.3 *M* sodium citrate, adjust to pH 7.0.

3. 50X Denhardt's reagent: 1% w/v Ficoll (type 400, Pharmacia), 1% w/v polyvinylpyrrolidone, 1% w/v bovine serum albumin (Fraction V, Sigma).
4. Formamide.
5. 10% SDS.
6. 10 mg/mL Herring sperm DNA (Invitrogen).
7. Hybond N+ membrane (Amersham Pharmacia Biotech).
8. Semidry electrotransfer apparatus.

3. Methods

3.1. Lysate Preparation

Developing cell-free systems is a key step to begin biochemical studies on RNA silencing. In RNAi, both the trigger dsRNA and the target mRNA are specifically cleaved by components of the silencing machinery. To detect these specific cleavages, the cell-free system should be free of nonspecific RNases. Special care should be taken during lysate preparation. All containers and extracting equipment should be made free of RNases by baking overnight. Wear gloves during all steps in lysate preparation.

1. Wheat germ extracts is prepared from frozen or vacuum-packed raw wheat germ (e.g., Fearn Nature Fresh Raw Wheat Germ, Bread and Circus) as described ***(66)***. The extract was centrifuged at 14,500*g* at 4°C for 25 min; the supernatant was then frozen in aliquots in liquid nitrogen and stored at –80°C.
2. For cauliflower extract, the outer layer of fresh cauliflower (Shaws Supermarket) is harvested with a razor blade and ground to a powder under liquid nitrogen in a pre-chilled mortar and pestle. Immediately transfer the powder to another prechilled mortar and pestle and homogenize it by grinding for additional 5 min on ice in 3 mL of 1X lysis buffer containing 5 m*M* DTT and EDTA-free protease inhibitor tablet (1 minitablet per 10 mL of buffer) per gram of plant tissue. The extract is centrifuged, and the supernatant is flash frozen in liquid nitrogen and stored at –70°C.
3. *Drosophila* embryo lysates are prepared as described previously ***(9)***.

3.2. Generation of T7-PCR Templates (see *Note 1)*

To study RNA cleavage in vitro, all the RNAs are generated by transcription in vitro. The easiest way to generate RNA transcription templates is to make templates by PCR. Using PCR, you can rapidly create different dsRNAs and target RNAs and introduce mutations in the RNAs. By way of example we describe here, in detail, the preparation of the RNA templates used in our recent study ***(32)***.

3.2.1. Rr-luc501 T7-PCR Templates

1. Set up PCR to amplify T7-sense-*Rr*-luc501 templates using *Rr*-luc cDNA clone as initial template, 5' PCR primer GCGTAATACGACTCACTATAGGAA

AAACATGCAGAAAATGC and 3' PCR primer GAAGAATGGTTCAAG ATATGCTG.

2. Set up PCR to amplify T7-antisense-*Rr*-luc501 templates using *Rr*-luc cDNA clone as initial template, 5' PCR primer GCGTAATACGACTCACTATAG GAATGGTTCAAGATATGCTG and 3' PCR primer GAAGAAAAACATGC AGAAAATGC.

3.2.2. PHV T7-PCR Templates

1. Wild-type *PHV* T7-PCR template: *Arabidopsis PHV* cDNA sequences containing the miR165/166 complementary sequences are amplified from an *Arabidopsis* flower cDNA library (CD4-6) by PCR using the following primer pairs: 5'-*PHV* primer, GCGTAATACGACTCACTATAGGCGCCGGAACAAGTTG AAG, and 3'-*PHV* primer, GACAGTCACGGAACCAAGATG.
2. Mutant *phv* T7-PCR template: The *Arabidopsis* G to A mutant *phv* template is initially amplified using the 5'-*PHV* primer (the same 5' primer as for wild-type *PHV*) and CCACTGCAGTTGCGTGAAACAGCTACGATACCAATAGAA TCCGGATCAGGCTTCATCCC. This PCR product is then diluted 100-fold and reamplified with the 5'-*PHV* primer (the same 5' primer as for wild-type *PHV*) and GACAGTCACGGAACCAAGATGGACGATCTTTGAGGATTTCAGCG ACCTTCATGGGTTCTAAACTCACGAGGCCACAGGCACGTGCTGCTATTCCAC TGCAGTTGCGTG AAACAGC.

3.2.3. Precipitation of T7-PCR Templates

The PCR products are precipitated directly from the reaction by the addition of 2.5 vol of absolute ethanol and 1/10 vol 3 *M* sodium acetate. The precipitate is recovered by centrifugation, washed in 70% ethanol, dried under vacuum, and redissolved in 1X T7 transcription buffer to give a stock of DNA template 10-fold more concentrated than the original PCR.

3.3. In Vitro RNA Transcription

1. Generation of uniformly ^{32}P-radiolabeled RNA: Assemble a 50-μL reaction containing 5 μL of 10X T7 buffer, 0.5 μL of 1 *M* DTT, 2.5 μL of 10 m*M* ATP, 2.5 μL of 100 m*M* UTP, 2.5 μL of 100 m*M* cytidine triphosphate (CTP), 4 μL of 100 m*M* GTP, 25 μL of α-^{32}P ATP (40 mCi/mL, 3000 Ci/mmol), 2.5 μL of (3–6 μg) PCR template (as described above), 1 μL of RNasin (40 U/μL, Promega), 2.5 μL of H_2O, and 2 μL of purified recombinant, histidine-tagged T7 RNA polymerase (6.8 mg/mL). Incubate at 37°C for 1–2 h. Stop the reaction by adding an equal volume of 8 *M* urea stop mix/loading buffer.
2. Production of nonradioactive RNA: Prepare a 100-μL reaction containing 10 μL of 10X T7 transcription buffer, 3 μL of (3–6 μg) PCR template (described above), 0.5 μL of 1 *M* DTT, 5 μL of 100 m*M* ATP, 5 μL of 100 m*M* CTP, 5 μL of 100 m*M* UTP, 8 μL of 100 m*M* GTP, 61.5 μL of H_2O and 2 μL of T7 RNA polymerase (6.8 mg/mL). Incubate at 37°C for 2 h. Stop the reaction by adding an equal volume of 8 *M* urea stop mix.

3. Production of nonradioactive capped mRNA: We use 3 μL of a 2 mg/mL stock of linearized plasmid or PCR transcription template as described above for a 100-μL reaction. From 100 m*M* stock solutions, add 5 μL each of ATP, CTP, and UTP, plus 1 μL of GTP. To this, add 10 μL of10X T7 reaction buffer (or SP6 reaction buffer if the promoter is SP6), 0.5 μL of 1 *M* DTT, 26.5 μL of water, 1 μL of RNasin (40 U/μL, Promega), 40 μL of 10 m*M* cap analog [7mG(5')ppp(5')G, New England Biolabs] and 2 μL of the appropriate recombinant bacteriophage RNA polymerase (e.g., SP6 20 U/μL or T7 6.8 mg/mL). Incubate for 2 h at 37°C. Stop the reaction by adding 7 μL of 500 m*M* EDTA. Precipitate the RNA transcripts with 0.8 *M* LiCl for 1 h on ice, centrifuge at 13,000*g* for 10 min, wash with 80% ethanol, and resuspend the pellet in 75 μL of H_2O. Examine 5 μL on a denaturing 2% agarose gel and visualize with ethidium bromide. To the remaining 70 μL, add 10 μL of 10X RQ1 DNase buffer (Promega), 5 μL of RQ1 DNase (1 U/μL, Promega), and incubate for 30 min at 37°C to eliminate any residual DNA template. Phenol/chloroform/isoamylalcohol extract and precipitate with 2.5 vol absolute ethanol. Resuspend purified transcripts in H_2O and determine concentrations by absorbance at 260 nm.

3.4. Gel Purification of In Vitro Transcribed RNA (see Note 2)

Gel purification is essential to eliminate abortive transcripts during in vitro transcription. Often, in vitro transcripts are a population of lengths. Gel purification allows selection of full-length transcripts.

1. Heat the stopped transcription reaction at 95°C for 2 min and then purify the transcribed RNA by electrophoresis on a denaturing acrylamide gel. RNA is located on gel by UV shadowing or phosphorimagery.
2. The gel portion containing RNAs is excised and the transcripts are recovered by soaking the gel slice in 1–2 mL of 2X PK buffer tumbling overnight at room temperature.
3. The elute is then phenol/chloroform/isoamylalcohol extracted and the RNA precipitated with 2.5 vol of cold absolute ethanol. The RNA precipitate is resuspended in pure, sterile H_2O.
4. To remove traces of copurifying DNA templates, use RNase-free DNase to treat the RNA at 37°C for 30 min (*see* **Subheading 3.3., step 3**). This step is only used when the template and transcript lengths are similar.
5. Phenol/chloroform/isoamylalcohol extract the RNA again to remove DNase.
6. Precipitate the DNA-free RNA with 2.5 volumes of –20°C absolute ethanol.
7. After purification, the concentration of each transcript is determined.
8. Measure the absorbance at 260 nm of 2 μL of RNA stock diluted into 198 μL of H_2O. Concentration is calculated by the following equation: C = (A260 × dilution factor)/(10313 × number of nucleotides) where C is in molar and the dilution factor in this instance is 100. This calculation assumes a 1-cm path length in the cuvette.

3.5. Annealing the In Vitro Transcribed ssRNAs into dsRNA

dsRNA is the substrate for Dicer-like enzymes. After annealing, it is important to check on a gel that annealing is complete and dsRNA has been generated. This is essential when the dsRNAs are radiolabeled because incomplete annealing will leave uncapped ssRNAs, which are not stable in most cell-free systems and will produce a high background in subsequent assays.

1. Equimolar amounts of the sense and antisense RNA strands are annealed by mixing them with an equal volume of 2X lysis buffer to make 0.5–1 μ*M* dsRNA.
2. The mixture is heated for 5 min at 95°C and then annealed overnight at 37°C.
3. Analyze a small portion the dsRNA on a native agarose gel. ssRNA for both sense and antisense strands of the dsRNA should be run adjacent to the dsRNA product. dsRNA, unlike ssRNA, typically runs with the same mobility as dsDNA, so we use dsDNA markers as size standard on the gel. The RNA is visualized by staining the gel with ethidium bromide or, for ^{32}P-RNAs, by drying the gel under vacuum onto a positively charged nylon membrane and detecting the RNA by phosphorimagery.

3.6. Analysis of Plant Dicer-Like Activities in Wheat Germ Extracts

Dicer-like enzymes are multidomain members of RNase III family. These enzymes cleave dsRNA into small fragments 21–25 nt in length. Therefore, Dicer-like activities can be monitored by detecting the production of small RNAs on a 15% denaturing polyacrylamide sequencing gel.

1. Assemble a typical Dicer-like activity assay as follows: for a 10-μL reaction, add 5 μL of wheat germ extract (use *Drosophila* embryo lysate as a positive control), 3 μL of dsRNA/RNAi processing mix, 1 μL of H_2O, and 1 μL of 50 n*M* uniformly [^{32}P]-labeled dsRNA (5 n*M* final concentration).
2. Incubate 1–3 h at 25°C.
3. Quench by adding 100 μL of 2X PK buffer.
4. Deproteinize by adding 10 μL of 20 mg/mL proteinase K (dissolved in water) and 1 μL of 20 mg/mL glycogen carrier (Roche) to the reaction. Incubate 15 min at 65°C.
5. Extract with phenol/chloroform/isoamylalcohol and precipitate the RNA with 3 vol of –20°C absolute ethanol. Wash the precipitate with 80% ethanol and dry the precipitate under vacuum.
6. Dissolve the pellet in 20 μL of formamide loading dye. After heating 2 min at 95°C, resolve the small RNA products produced by Dicer-like enzymes by electrophoresis on a 15%, 0.2- to 0.5-mm thick denaturing polyacrylamide sequencing gel at constant 20–25 W. Stop the electrophoresis before bromophenol blue dye runs out of the gel. 5' ^{32}P-radiolabeled synthetic RNA oligonucleotides (14, 19, 21, 25, and 34 nt) are used as size markers (*see* **Subheading 3.9.2.**, **step 1**).

3.7. Characterization of Small RNA Structures

Dicer-like activities in wheat germ extract produce two populations of small RNAs with distinct lengths, approx 21 nt and 24–25 nt. To ask whether small RNAs are *bona fide* small interfering RNAs (siRNAs) produced by Dicer-like enzymes, analyze the structure of the small RNAs. There are several criteria for siRNAs: (1) small, 21–25 nt; (2) initially double-stranded; (3) 3' 2-nt overhangs; and (4) 5' monophosphate and 3' hydroxyl termini.

3.7.1. Gel Filtration Assay

1. Scale up the Dicer assay in **Subheading 3.6.** 5–10-fold. Perform **steps 1–3** both for wheat germ extracts and Drosophila lysate (control).
2. Deproteinize the reaction at 25°C for 1 h with proteinase K without subsequent phenol/chloroform/isoamylalcohol extraction. (Phenol facilitates RNA annealing.)
3. Precipitate the RNA with 3 vol of –20°C absolute ethanol.
4. Wash the precipitate with 80% ethanol and dissolve the pellet in 100–200 μL 1X lysis buffer.
5. Immediately analyze the small RNAs by gel filtration at room temperature on a Superdex-200 HR 10/30 column (Pharmacia) at 0.75 mL/min in lysis buffer using a BioCad Sprint (PerSeptive Biosystems) or similar chromatography system, as described previously ***(19)***.
6. Collect all the fractions, add 1 μL of glycogen (20 mg/mL) to each fraction, and precipitate with 3 vol of –20°C absolute ethanol.
7. Analyze each fraction by electrophoresis on a 15% denaturing polyacrylamide sequencing gel together with size markers.
8. Compare the small RNAs with *Drosophila* Dicer products. Single and dsRNA (siRNA duplex) markers should also be used to calibrate the column.

3.7.2. RNase Protection Assay for Analyzing dsRNA With 3' 2-nt Overhangs

RNase protection assay uses RNase A and T1 in the presence of high salt to digest ssRNA portion but not dsRNA portion of siRNA duplexes. siRNA is dsRNA with 3' 2-nt overhangs (single-stranded 3' "tails"). Therefore, after RNase A and T1 treatment, the siRNA is shortened by 1–2 nt, which is readily detected on a 15% denaturing polyacrylamide sequencing gel.

1. Process uniformly [^{32}P]-labeled dsRNA in wheat germ extracts as in **Subheading 3.7.1.**, **steps 1** and **2**.
2. Wash the precipitate with 80% ethanol and dissolve the pellet in 1X RNase digestion buffer by dilution of 10X RNase digestion buffer (*see* **Subheading 2.5.**). Set up a 10-μL reaction containing 5 μL of RNAs in 1X RNase digestion buffer, 0.5 μL 10X RNase digestion buffer (add this before RNase A and T1 to avoid diluting the salt concentration so as to prevent the dsRNA being digested by the RNase), and varying amounts of RNase A and T1. The amount of RNase A and T1 should be

titrated before hand using a reaction containing radiolabeled synthetic siRNA duplex (*see* **Subheading 3.9.2.**, **step 1**) incubated in the presence of nonradioactive total wheat RNA isolated from 5 μL of wheat germ extract.

3. Stop the RNase protection reaction by adding 1 μL of 10% SDS and treat with 1 μL of 40 mg/mL proteinase K at 25°C for 1 h.
4. Precipitate the RNase protection products and analyze them by electrophoresis on a 15% denaturing polyacrylamide sequencing gel as described previously. In wheat germ extract, the result should be that each of the two populations of wheat small RNAs are shortened by RNase. Positive controls, synthetic siRNA duplexes should also be shortened by 1–2 nt.

3.7.3. Analysis of 5' and 3' End Structures

Dicer products, like all RNase III products, have 5' monophosphate and 3' hydroxyl termini. To test for this type of end structure, we analyzed the siRNAs using CIP, PNK, and periodate treatment followed by β-elimination ***(67)***. CIP can remove all terminal phosphates (tri-, di-, or monophosphate at 5' or 3' end) from any RNA. PNK adds the γ-phosphate of ATP to a free hydroxyl group at the 5'-end of nucleic acids. If an RNA has a single monophosphate terminus, it should have its original mobility restored when treated sequentially with CIP then PNK. If the input RNA has a 5' triphosphate terminus, for example, the untreated RNA will have a faster gel mobility than the same RNA treated with CIP then PNK. Unambiguous assignment of a monophosphate to the 5' end requires analysis of 3' end structures using periodate-treatment/β-elimination. Periodate only reacts with RNAs containing vicinal diols, which are oxidized to aldehydes. That is, adjacent 2' and 3' hydroxyl groups must be present ***(67)***. The aldehydes are then reacted with base (borax/boric acid) to catalyze β-elimination, resulting in an RNA one nucleotide shorter at its 3' end but with a 3'-monophosphate bearing two negative charges in place of the singly charged phosphodiester bond ***(67)***. Therefore, an RNA must have 5' monophosphate and 3' hydroxyl termini if it both is able to be β-eliminated after periodate treatment and has a slower mobility when treated with CIP, but its original mobility when the CIP-treated RNA is treated with PNK and ATP.

3.7.3.1. Analysis of 5' End Structure

1. Scale up and process uniformly ^{32}P-labeled dsRNA in wheat germ extract as for the assay for Dicer-like activities (**Subheading 3.6.**, **steps 1–6**).
2. Purify the small RNAs from a denaturing polyacrylamide gel. Next, the RNA is dissolved in water. Aliquot the RNA for CIP (Promega) and PNK (Promega) treatments.
3. For CIP treatment, the small RNAs are reacted with 1–2 U of CIP in a 10-μL volume at 37°C for 0.5–1 h. Stop the reaction by adding 10 μL of formamide loading buffer.

4. For CIP plus PNK treatment, the small RNAs are first treated with CIP as above but stop the reaction by treating with 100 μL of 2X PK buffer, 1 μL of glycogen (20 mg/mL), and 10 μL of proteinase K at 65°C for 15 min. Phenol/chloroform/isoamylalcohol extract the reaction and precipitate the RNAs as described previously. The RNAs are then treated with PNK 1 h at 37°C in 10 μL (1 μL 10X T4 polynucleotide kinase buffer, 1 μL 10,000 U/mL PNK, 1 μL 10–20 m*M* ATP, and 7 μL H_2O). Stop the reactions by adding 10 μL of formamide loading buffer.
5. As a control, the small RNAs are treated directly with PNK, without CIP treatment.
6. Heat the various small RNAs in formamide loading buffer at 95°C for 2 min: no treatment, CIP-treated, CIP plus PNK-treated, and PNK only. Analyze the relative gel mobilities of the RNAs by electrophoresis on a 15% denaturing polyacrylamide sequencing gel.

3.7.3.2. 3' End Analysis

1. Process uniformly [^{32}P]-labeled dsRNA in wheat germ extracts and purify the RNA (*see* **Subheading 3.6., steps 1–5**).
2. Dissolve RNA pellets in 13.5 μL of H_2O, 4 μL of 5X borax/boric acid buffer (pH 8.6), and 2.5 μL of 200 m*M* $NaIO_4$.
3. Incubate 10 min at 25°C.
4. Add 2 μL of glycerol to the reactions to quench unreacted $NaIO_4$.
5. Dry under vacuum.
6. Dissolve the pellet in 50 μL of 1X borax/boric acid buffer (pH 9.5).
7. Incubate 90 min at 45°C (β-elimination reaction).
8. Add 3 μL of 5 *M* NaCl and 150 μL of ethanol and incubate on ice for 2 h.
9. Collect the pellet by centrifugation. Wash the pellet with 80% ethanol.
10. Dissolve the pellet in 20 μL of H_2O.
11. Analyze 5 μL of RNA plus 5 μL of 8 *M* urea stop mix by electrophoresis through a 15% denaturing polyacrylamide sequencing gel as described above. Include a control without periodate treatment and β-elimination.

3.8. Testing Dicer-Like Activities for Susceptibility to Product Inhibition

Wheat germ extracts contain two distinct Dicer-like activities that produce two different lengths of small RNAs, 21 nt and 24–25 nt. To distinguish whether these two different siRNAs are generated by distinct Dicer-like enzyme, we use synthetic siRNA duplexes of the corresponding size as Dicer inhibitors. If the enzyme has high affinity for its small RNA products, the synthetic siRNA duplex will act as an inhibitor. Conversely, if the enzyme has low affinity for its product, the synthetic siRNAs will not inhibit the enzyme reaction. Results from our studies showed that the enzyme responsible for the production of 24–25 nt siRNA has high affinity for its siRNA products, whereas the enzyme that produces the approx 21 nt siRNAs does not. We concluded that wheat germ extracts contain at least two distinct Dicer-like enzymes ***(32)***. To test whether

Dicer-like enzymes are inhibited by their products, assay the conversion of a fixed amount of uniformly radiolabeled long dsRNA into siRNA in the presence of increasing concentrations of unlabeled synthetic siRNA duplexes.

1. Process uniformly [^{32}P]-labeled dsRNA in wheat germ extracts (with fly embryo lysate as a control) as **Subheading 3.6.** but in the presence of increasing amount (0–800 n*M*) of the cold synthetic siRNA duplexes.
2. Work up the processed RNAs as described above and dissolve the purified RNA in 10 μL of formamide loading buffer as described above.
3. Analyze the two populations of small radioactive RNAs produced by electrophoresis on a 15% denaturing polyacrylamide sequencing gel. Plot the amount of siRNA produced vs inhibitor siRNA duplex concentration. In our experiments, the data fit well to a single-exponential, and we used τ to compare the sensitivity of different enzymes to inhibitor or the effectiveness of different lengths of siRNA duplexes.

3.9. Analysis of RdRP Activity

RdRP is an enzyme that uses RNA as template to synthesize complementary RNA (cRNA). We have shown that wheat RdRPs can synthesize both primed and unprimed cRNA ***(32)***. In addition, RdRPs can also produce a novel population of small RNAs of approx 24–25 nt in length. These RdRP-related small RNAs can be the products of a specific wheat Dicer-like enzyme that uses the dsRNA synthesized by wheat RdRP or produced directly by the RdRP itself ***(48)***.

When assaying for RdRP activity in crude extracts, special care should be taken in interpretation of the results. In crude extracts, terminal transferase activities can label RNA templates directly using the input radionucleotides. Such terminal transferase activity is present in *Drosophila* embryo lysate. To detect authentic RdRP products, control reactions with 3' deoxynucleotides should be performed. When 3' deoxynucleotides are incorporated into RNA, RNA synthesis is terminated because of the lack of a 3' hydroxyl. Terminal transferases require only a single type of NTP rather than all four, so they are not necessarily blocked by a single 3' dNTP in the presence of the other three NTPs. This labeled template RNA is easily misinterpreted as an RdRP product. Using a radiolabeled primer to assay RdRP is a more reliable way to exclude terminal transferase activity. However, in our hands, such primer extension reactions are inefficient. Primer extension also may reflect reverse transcriptase activity, so control experiment using 2', 3' ddNTPs should be performed to verify that an RdRP, not an RNA-dependent DNA polymerase is being detected. Resistance to RNase-free, DNase treatment can also establish that the RNA primer was used to initiate RNA synthesis. It is important to note that undialyzed *Drosophila* embryo lysates contain high concentration of endogenous nucleotides, making RdRP activities impossible to detect using exogenous

radioactive NTPs. Using a radiolabeled primer can overcome this problem. Wheat germ extracts are typically gel-filtered, and therefore contain low concentration of endogenous NTPs.

3.9.1. Analyzing Unprimed RdRP Products

1. Set up a 10-μL reaction containing 5 μL of wheat germ extract (or fly embryo lysate as a control), 100 μ*M* GTP, 100 μ*M* CTP, 500 μ*M* ATP, 20 μ*M* UTP, 5 μCi of α-[^{32}P]UTP (25 Ci/mmol, approx 10 mCi/mL), 10 m*M* creatine phosphate, 10 μg/mL creatine phosphokinase, 5 m*M* DTT, 0.2 U/μL Super-RNasin (Ambion), and various concentrations of 7-methyl-G- or A-capped RNA template.
2. After incubation at 25°C for 3 h, deproteinize the reaction with proteinase K in 200 μL of 2X proteinase K buffer at 65°C for 15 min.
3. After phenol/chloroform/isoamylalcohol extraction, precipitate the aqueous phase with three volumes of cold ethanol, then resuspend in 10 μL of 2X formamide loading buffer.
4. Resolve the labeled RdRP products on 7 or 10% polyacrylamide sequencing gel.

3.9.2. Analyzing Primed RdRP Products

1. Label antisense and control sense RNA primers with PNK and γ-[^{32}P]ATP:
   In a 10-μL reaction, add 10 pmol RNA oligo, 1 μL of γ-[^{32}P]ATP, 1 μL 10X PNK buffer, and 1 μL of PNK. Incubate the reaction at 37°C for 1 h and stop by passing through a G-25 spin column (Roche). Finally gel-purify the labeled RNA oligo as described above. It is important that all traces of γ-[^{32}P]ATP be removed, as contaminating γ-[^{32}P]ATP can be used by enzymes in crude extracts to 5' radiolabel abundant, endogenous RNAs.
2. For primed RdRP product assays, capped RNAs are preincubated with single-stranded radiolabled 21-nt antisense RNA primer, sense RNA primer or siRNA duplexes at room temperature for 10 min before the remaining reaction components are added.
3. Process the reactions as for analyzing unprimed RdRP products (*see* **Subheading 3.9.1.**, **steps 1–4**).

3.10. Detection of Endogenous miRNAs in Wheat Germ Extracts (see *Notes 3 and 4*)

Although wheat germ extracts can produce siRNAs, the siRNAs seem unable to direct target RNA cleavage (unpublished data). There are several explanations: (1) wheat germ extracts might lack cofactors that are essential for RISC assembly; (2) RISC components are limited; or (3) RISC components are fully occupied by endogenous miRNAs. In wheat germ extract, endogenous miRNAs assembled in RISC complexes can direct target RNA cleavage. Unlike animal miRNAs, plant miRNAs target specific mRNA cleavage because of their extensive complementarity to specific mRNAs. We choose the wheat ortholog of *Arabidopsis* miR165, miR165/166, and an Arabidopsis target mRNA,

PHABULUTA (*PHV*), to test this idea and give here a protocol for these experiments. We also describe procedures for demonstrating that a miRNA can cleave its wild-type target mRNA but not a mutant RNA ***(32)***.

3.10.1. Quantitative Northern Detection of Wheat miRNAs in Wheat Germ Extracts

1. Synthesize sense and antisense miR165 or corresponding DNA oligonucleotides as probes and controls.
2. Proteinase K, treat 30 μL of wheat germ extract in 200 μL of 2X PK buffer at 65°C for 0.5–1 h.
3. Precipitate the total RNAs as above, then dissolve the RNA pellet in 10–20 μL of formamide loading buffer.
4. Heat the sample 2 min at 95°C and load on a 15% 2 mm-thick polyacrylamide sequencing gel together with synthetic sense miRNA or corresponding DNA oligonucleotides as a positive control. Make a dilution series of the corresponding synthetic miR165 to serve as concentration standards.
5. Transfer the small RNAs from the gel to Hybond N+ (Amersham Pharmacia Biotech) membrane using a semidry electrotransfer apparatus.
6. Rinse the membrane with 2X saline/sodium phosphate/EDTA (SSPE), pH 7.4.
7. UV-crosslink the membrane in UV-Stratalinker 2400.
8. Prehybridize the membrane for at least 2 h at 42°C in 50% formamide, 5' SSPE (pH 7.4), 5' Denhardt's reagent, 0.5% SDS, denatured herring sperm DNA (160 μg/mL).
9. Radiolabel antisense miR165 RNA or corresponding DNA oligo with PNK and γ-[^{32}P]ATP (ICN).
10. Purify the radiolabeled probe by passing through G-25 spin column (Roche).
11. Carefully add the purified probe to the hybridization solution without directly touching the membrane and continue the hybridization overnight.
12. Discard the hybridization solution into radioactive waste and rinse the membrane immediately with 2X saline sodium citrate (SSC) and 0.1% SDS at 42°C.
13. Wash the membrane twice more, 20 min per wash, at 42°C in 2X SSC and 0.1% SDS. Check the background signal after each wash.
14. Wrap the membrane with saran and exposure overnight under film.

3.10.2. Detection of miRNA-Programmed RISC Activity

1. Preparation of cap-radiolabeled partial PHV mRNA containing the miRNA 165/166 target region.
   a. PCR generate T7-*PHV* transcription template as described above.
   b. Transcribe uncapped *PHV* RNA using T7 RNA polymerase as described above.
   c. After gel purification, 10 pmol of the uncapped mRNA target (in 1.75 μL of H_2O) is added to 5 μL of α-[^{32}P]GTP (3000 Ci/mmol, 40 mCi/mL, custom order, ICN), 1 μL of 10X guanylyl transferase buffer, 0.25 μL of 40 U/μL RNasin (Promega), 1 μL of 10 m*M* *S*-adenosyl methionine (SAM, Sigma) in SAM dilution buffer, and 0.5 μL of recombinant, heterodimeric vaccinia virus guanylyl transferase.

d. Incubate for 2 h at 37°C, adjust the reaction to 100 μL with H_2O and pass through a G-50 spin column (Roche).
e. Gel-purify the radiolabeled target as mentioned previously.

2. Assay for miRNA-directed target RNA cleavage.
   a. Set up reactions as for wheat Dicer-like activity assay (**Subheading 3.6.**) except use 5 n*M* purified, cap-radiolabeled wild-type *PHV* or mutant *phv* RNA targets instead of the uniformly [^{32}P]-labeled dsRNA.
   b. Incubate the reaction 0–80 min at 25°C.
   c. In parallel, RNAi control reactions, as previously described ***(30)***, should be conducted in fly embryo lysate, with a synthetic siRNA duplex in which one strand is the miRNA. It is useful to use as a control an siRNA duplex with perfect complementarity to the target region corresponding to the miRNA.
   d. Deproteinize and precipitate the reactions, then analyze the cleaved products on a 5% polyacrylamide sequencing gel. Include appropriate size markers. We typically use the 100-nt RNA ladder (Ambion) labeled with Guanylyl transferase, α-[^{32}P]GTP, and SAM.

4. Notes

1. When designing primers for generating PCR DNA templates for the transcription of RNA, the 5' PCR primer should include at its 5' end the T7 promoter sequence. In principle, T3 or SP6 could be used. The T7 promoter is followed by 15–20 bases of sequence complementary to the desired target RNA sequence. In addition to the final, transcribed G of the T7 promoter, one or two additional guanosines should be included immediately after the promoter sequence to ensure efficient transcription. A 20 nt, 3' PCR primer is designed to be complementary to the target sequence 200–1000 bases downstream from the 5' sequences. Two separate primer pairs are used to generate templates for the transcription of sense and antisense RNA strands.
2. Polyacrylamide/urea gel purification of transcribed RNA is appropriate for RNA transcripts ≤600 nt. However, longer RNA is difficult to recover in good yield from polyacrylamide gels. Instead, we use LiCl to precipitate the transcribed RNAs as described in **Subheading 3.3.**, **step 3**.
3. Plant miRNAs were initially discovered in *Arabidopsis*. Many miRNAs are conserved between *Arabidopsis* and rice. To assess the presence of miRNAs in wheat germ, we perform Northern analysis using synthetic 21 nt RNA or DNA probes complementary to individual *Arabidopsis* miRNAs. Our results show that at least nine orthologs of *Arabidopsis* miRNAs are present in wheat germ extracts.
4. *PHV* (*PHAVOLUTA*), like *PHB* (*PHABULOSA*), is a member of homeodomain, leucine-zipper transcription factors, which were initially discovered in *Arabidopsis*. *PHB* is abundant on the adaxial face of leaf primordial but not on the abaxial face ***(68)*** and has been proposed to specify adaxial fates during leaf development. Dominant gain-of-function *Arabidopsis* mutants with in-frame or point mutations in the putative sterol/lipid-binding domains show loss of leaf

asymmetry and express mutant *phb* transcript on both adaxial and abaxial region in the leaf primordium ***(68)***. miRNA 165 was predicted to pair with the region in *PHB* mRNAs where the mutations are found ***(61)***. Therefore, the wild-type *PHB* mRNA was proposed to be cleaved by a protein–RNA complex containing miR165 ***(61)***. The mutant *phb* mRNA was proposed to be resistant to such cleavage because of its reduced complementarity to the miRNA.

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