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Protocols

A Simple and Rapid Method to Detect Plant siRNAs Using Nonradioactive Probes

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Abstract. Small interfering RNAs (siRNAs) are key molecules in RNA silencing, which includes posttranscriptional gene silencing, cosuppression, quelling, and RNA interference. The presence of siRNAs indicates RNA silencing in cells. We present a method of detecting siRNAs using nonradioactive probes that involves isolating the small RNA fraction, separating siRNAs using denaturing gel electrophoresis, and performing a Northern blot analysis under low-stringency hybridization conditions. We used digoxigenin-labeled DNA probes for hybridization and detected siRNAs in petunia and rice plants exhibiting silenced phenotypes. This method is a simple and rapid way to detect siRNAs without using radioisotopes.

Key words: digoxigenin, nonradioactive probes, Northern blot analysis, PTGS, RNA silencing, siRNAs

Abbreviations: CaMV, cauliflower mosaic virus; CHS-A, chalcone synthase-A; NOS, nopaline synthase; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PTGS, posttranscriptional gene silencing; RNAi, RNA interferernce; siRNAs, small interfering RNAs.

Introduction

RNA silencing is gene silencing involving a sequence-specific degradation of RNA. It includes posttranscriptional gene silencing (PTGS), cosuppression, and RNA-mediated virus resistance in plants; RNA interference (RNAi) in animals; and silencing in fungi (quelling in *Neurospora*) and algae (Matzke et al., 2001; Plasterk, 2002). These systems are mechanistically related, as the required genes show sequence homology (Matzke et al., 2001), and small RNA molecules (21-26 nt) complementary to both strands of the silenced genes are found (Plasterk, 2002; Zamore, 2002). Small RNA molecules, known as small interfering RNAs (siRNAs), were first discovered in transgenic plants exhibiting PTGS (Hamilton and Baulcombe, 1999). Biochemical experiments with cytoplasmic extracts from *Drosophila* revealed that siRNAs are produced by processing dsRNA

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by RNaseIII-like enzymes (Tuschl et al., 1999; Zamore et al., 2000; Elbashir et al., 2001; Bernstein et al., 2001). siRNAs direct mRNA cleavage by sequence complementarity (Hammond et al., 2000); thus, the presence of siRNAs indicates the occurrence of RNA silencing in cells.

siRNAs can be detected by means of Northern blot analysis. The original method of detecting siRNAs involves isolating nucleic acids from tissues, enriching the small RNA fraction, size-fractionating the small RNA using denaturing polyacrylamide gel electrophoresis (PAGE), blotting the RNA to a membrane, hybridizing the RNA on the membrane with a labeled probe, and detecting the hybridization signal (Hamilton and Baulcombe, 1999). Probes labeled with radioisotope are prepared by in vitro transcription from plasmid DNA. Labeled RNAs are then hydrolyzed by sodium bicarbonate treatment to obtain probes averaging 50 nt before hybridization (Hamilton and Baulcombe, 1999). The use and disposal of radioisotopes in this process is costly, labor intensive, and hazardous. Therefore, we developed a nonradioactive protocol to detect siRNAs using a digoxigenin system for nucleic acid detection. Our method is quick and simple and does not require radioisotopes. It can be used to detect siRNAs in various PTGS and RNAi systems in different organisms.

Materials and Methods

Plant materials

Wild-type petunia (*Petunia hybrida*) V26 line and a transgenic line of V26 (C001; O'Dell et al., 1999) with petunia chalcone synthase-A (CHS-A) gene were used. The transgenic petunia plant expresses CHS-A transgene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (NOS) terminator. Gene silencing is observed as changes in flower color because CHS-A catalyses an essential step in the biosynthesis of anthocyanin pigments. Wild-type and silenced plants produce purple and white flowers, respectively (Figure 1) (Metzlaff et al., 1997, 2000; O'Dell et al., 1999; Kanazawa et al., 2000). A rice (*Oryza sativa*) strain exhibiting the PTGS phenotype for *glutelin* genes was also used (M. Kusaba, K. Miyahara, S. Iida, H. Fukuoka, T. Takano, H. Sassa, M. Nishimura, and T. Nishio, submitted). Nucleic acids were extracted from petal tissues of 4 petunia buds (30-45 mm) and 30 developing rice seeds.

Hybridization probes

We used a petunia CHS-A gene probe, a soybean 5S RNA probe, and a rice *glutelin* gene probe. The CHS-A gene probe was prepared by amplifying a 0.44-kb sequence corresponding to the 3' end of exon 2 from petunia CHS-A cDNA by using specific primers '4246' (5'-GGCGCGATCATTATAGGTTC-3') and '2349' (5'-CCCTTCACCAGTAGTTCCTA-3'). For the 5S RNA probe, a 0.25-kb sequence of the 5S RNA gene was amplified by PCR from genomic soybean DNA (*Glycine max* cv. 'Harosoy') by using specific primers 'SOJA 5S-1' (5'-GAAGTCCTCGTGTTGCACC-3') and 'SOJA 5S-2' (5'-AGTGCTGGTATG-ATCGCACT-3'). The amplified fragment was used as a template for PCR labeling using the same primers. For the *glutelin* gene probe, a *glutelin* cDNA clone

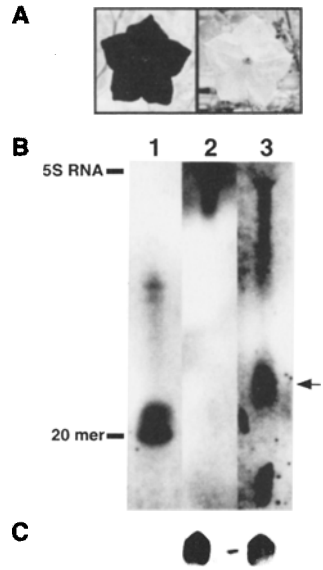

Figure 1. Northern blot analysis of low molecular weight RNA from wild-type and transgenic petunia. (A) Flower phenotypes of wild-type (left: purple) and transgenic (right: white) petunia plants. (B) Northern blot analysis of low molecular weight RNA using CHS-A gene probe. Lane 1, 20-mer CHS-A DNA primers for a size control; lane 2, small RNA fractions from wild-type petunia; lane 3, small RNA fractions from transgenic petunia. Positions of 5S RNA and 20-mer primers are shown on the left side. siRNA signals are shown by arrows on the right side. (C) Northern blot analysis of the same blot with 5S RNA gene probe in order to show that an equal amount of small RNA fraction was loaded in lanes 2 and 3.

corresponding to RG21 (Masumura et al., 1989) was used as a template for PCR labeling using universal primers.

Protocol

Solutions

- RNA extraction buffer: 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% (w/v) SDS (see Napoli et al., 1990)
- TE buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA
- RNA/DNA Mini Kit (QIAGEN)
- Gel-loading buffer: 2 × TBE, 40% (w/v) sucrose, 0.1% (w/v) bromophenol blue
- Denaturing PAGE gel: 15% polyacrylamide, 7 M Urea, 0.5 × TBE
- Prehybridization solution: 50% (v/v) formamide, 7.6% (w/v) SDS, 1% (w/v) N-laurylsarcosine, 0.6 μg yeast tRNA, 2% (w/v) blocking reagent (Roche)
- DIG Northern Starter Kit (Roche)
- Reaction mixture of PCR-labeling: 1 mM dATP, dGTP, dCTP, 0.65 mM dTTP, 0.35 mM Dig-11-dUTP (Roche), 1 × EX-Taq buffer, 5 U EX-Taq (TaKaRa), 20 pmol primers, template DNA
- Washing solution: 2 × SSC, 0.2% (w/v) SDS
- Equilibration buffer: 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, 0.02% NaN_3

Isolating total RNA and separating low molecular weight RNA from total RNA

- Freeze plant tissues with liquid nitrogen and grind them to a powder with 500 μL of RNA extraction buffer and 150 μL of TE-saturated phenol using a mortar and pestle.[1]
- Add 250 μL of chloroform to the tube and mix the suspension by vortex.
- Centrifuge the tube at 14,000 *g* for 5 min.
- Transfer the aqueous phase to a new tube.
- Precipitate nucleic acids by adding 2.2 vol of ethanol. Incubate at -20°C for 30 min.
- Centrifuge the tube at 14,000 *g* for 15 min. Discard the supernatant.
- Dissolve the pellet in 150 μL of QRL1 solution in RNA/DNA Mini Kit (QIAGEN). Separate low molecular weight RNA from DNA and higher molecular weight RNAs according to the manufacturer's instructions.[2]

Electrophoresis and transfer

- Dissolve the precipitate of low molecular weight RNA in 15 μL of formamide. Heat the tube at 65°C for 15 min and chill on ice.
- Add 1/4 vol of gel-loading buffer.
- Separate the RNAs (20-100 μg) by denaturing PAGE at 200V for 2.5 h.
- Transfer the RNAs to Hybond-N^+ membrane (Amersham Pharmacia Biotech) by capillary blotting (Sambrook and Russell, 2001).
- Rinse the membrane with 2 × SSC for 15 min and let dry. Fix RNA on the membrane by ultraviolet cross-linking.

Notes:

1. To detect rice *glutelin* siRNAs, grind 30 developing seeds in 2 mL of 10 × TE buffer (100 mM Tris-HCl [pH 7.5], 10 mM EDTA) with a mortar and pestle. Extract nucleic acids once with 2 mL of phenol-chloroform-isoamylalcohol (25:24:1).
2. Separating low molecular weight RNA from DNA and higher molecular weight RNAs can be replaced by precipitating high molecular weight nucleic acids using polyethylene glycol (PEG) as follows:
   - Dissolve the pellet of nucleic acids in 300 μL of TE buffer.
   - Add an equal volume of PEG precipitation solution (20% PEG [MW = 8000], 2 M NaCl), mix, and keep on ice for at least 30 min.
   - Centrifuge the tube at 14,000 *g* for 15 min. Transfer the supernatant to a new tube.
   - Precipitate small RNAs by adding an equal amount of 2-propanol.

Hybridization and chemiluminescent detection

- Label the probe with digoxigenin using PCR consisting of 3 steps: denaturation at 94°C for 5 min; 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min (for reaction mixture, see above).
- Prehybridize the fixed membrane in prehybridization solution at 40°C for 1 h.[1]

- Denature the labeled probe by heating and chilling on ice.
- Add probe to the prehybridization solution.
- Perform the hybridization at 40°C for 12 h.
- Wash the membrane twice with washing solution at 50°C for 15 min.
- Perform the signal detection process, including blocking the membrane, reacting hybridized probes with Anti-Digoxigenin-AP Fab fragments, and washing the membrane, according to manufacturer's instructions (DIG Northern Starter Kit, Roche).
- Equibrate the membrane in equibration buffer.
- Soak the membrane in CDP-Star solution (Applied Biosystems) and expose it to x-ray film (Fuji film).

Notes:

1. Hybridization and detection were performed according to the protocol of Masuta et al. (1998) with some modifications.

Results and Discussion

We determined the presence or absence of siRNAs in transgenic petunias that showed a silenced flower color phenotype. We analyzed wild-type petunias as a negative control. Small RNA fractions extracted from these plants were separated by PAGE, blotted to nylon membranes, and hybridized with a labeled probe. Hybridization signals were detected in the silenced plants, but not in the wild-type plants (Figure 1). The hybridized RNAs migrated in the gel slightly more slowly than a 20-mer DNA oligonucleotide, indicating that the signals corresponded to siRNAs. At the hybridization stringency that allowed siRNA detection (see below), nonspecific hybridization signals appeared in the lower mobility regions, as observed in other studies (Hamilton and Baulcombe, 1999; Mette et al., 2000). The siRNA signals in Figure 1 did not appear as clear bands, probably because of the presence of polysaccharides in the small RNA fraction that affected RNA migration during electrophoresis. Alternatively, CHS-A mRNA might not be fully degraded to 21-26 nt RNA and thus appears as smeared bands. Hybridization signals of RNA more mobile than 5S RNA and less mobile than siRNAs were also detected in the silenced plants (Figure 1). These may come from intermediate products of RNA degradation produced in cells or during RNA extraction or PAGE. Regarding CHS-A transcripts in petunia, Metzlaff et al. (2000) actually detected a number of short poly(A)$^+$ RNA molecules that are thought to be the cleaved products of transcripts in silenced cells.

We also detected siRNAs in rice plants exhibiting the PTGS phenotypes of *glutelin* genes in endosperm cells (Figure 2). In this case, 2 bands, likely corresponding to short and long siRNAs (Hamilton et al., 2002), were clearly detected. Although we detected siRNAs by subjecting 20 μg of the small RNA fraction from rice endosperm and petunia flowers to PAGE, the amount of RNA required to detect siRNAs may be reduced. As a positive hybridization control, we used a mixture of 2 DNA oligonucleotides, which were used as primers for PCR labeling. We detected hybridization signals from 20 pmol of oligonucleotide DNA (10

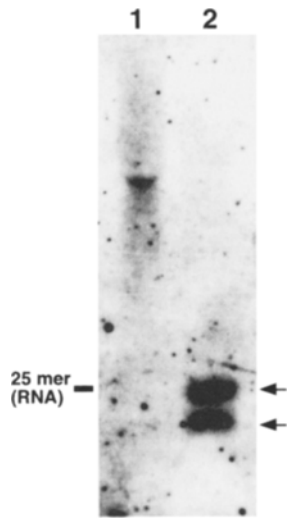

Figure 2. Northern blot analysis of low molecular weight RNA from wild-type strain and a strain that shows a silenced phenotype of rice *glutelin* genes. Lane 1, small RNA fractions from wild-type strain of rice; lane 2, small RNA fractions from silenced strain of rice. Position of a 25 nt synthetic RNA is shown on the left side. siRNA signals are shown by arrows on the right side.

pmol of each) on the membrane, suggesting that our method can detect siRNAs from small amounts of RNA.

We modified existing methods of detecting siRNAs to simplify the protocol. Hamilton and Baulcombe (1999) originally used in vitro transcribed radiolabeled RNA probes, which allowed them to distinguish sense and antisense RNAs. The presence of both sense and antisense siRNA strands has been shown in various PTGS/RNAi systems (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Mette et al., 2000; Parrish et al., 2000; Yang et al., 2000; Sijen et al., 2001). siRNAs have also been detected using radiolabeled DNA probes (Ketting et al., 2001; Llave et al., 2002). We prepared probes by PCR, incorporating digoxigenin-labeled dUTPs into DNA strands. Similar results were obtained when DNA was labeled using a random priming reaction (data not shown). We also used digoxigenin-labeled RNA probes prepared by means of in vitro transcription. Stronger hybridization signals were detected using RNA probes, although the background level was often higher (data not shown). We used the labeled probes for hybridization without hydrolyzing them. Although not needed in our case, hydrolysis produces probes averaging 50 nt (Hamilton and Baulcombe, 1999) and may facilitate the detections of siRNAs. Using a radiolabeled probe, Sijen et al. (2001) obtained a result similar to ours in detecting siRNAs in petunia. We believe there are few disadvantages in using nonradiolabeled probes for siRNA detection.

In our protocol, the small RNA fraction was enriched using an ion-exchange column. This was necessary because of the limited RNA solubility and the volume of RNA solution that can be applied to electrophoresis. This process could be replaced by precipitating high molecular weight nucleic acids using 10% PEG

(MW = 8000) and 1 M NaCl and precipitating the small RNA fraction using 2-propanol. After PAGE fractionation, previous methods blotted the RNA to membranes using an apparatus such as the Bio-Rad Trans-Blot SD semidry transfer cell (Hamilton and Baulcombe, personal communication; Mette et al., 2000; Llave et al., 2002). We blotted the RNA to membranes using a conventional capillary transfer method (Sambrook and Russell, 2001). Visualizing RNA in the gel using ethidium bromide staining before and after capillary blotting indicated that the small RNA fraction was fully transferred from the gel within 5 h.

The stringency of the hybridization and posthybridization washes needed to be lower than in the standard protocol for Northern blot analysis recommended in the DIG Northern Starter Kit (Roche). We optimized the stringency by reducing the hybridization temperature and washing the membrane without changing the stringency of the hybridization or wash solutions. Hybridization was performed at 40°C, which is lower than the temperature recommended for standard Northern blot analysis (50°C). Hybridization signals were detected when the final membrane wash was done in 2 × SSC and 0.2% SDS at 50°C, whereas no signal was detected with high-stringency washes in 0.2 × SSC and 0.2% SDS at 50°C. siRNA signals were detected by exposure to x-ray film for 1 h at room temperature. Digoxigenin-labeled probes can be stored for long periods, facilitating repeated experiments. In addition, using nonradiolabeled probes avoids problems that accompany radiolabeled probes. Although the extraction of nucleic acids in the first step of this protocol is based on the method used to isolate RNA from plant tissues (Napoli et al., 1990), the remainder of the process can be applied to RNA from any source. Our method is applicable to detecting siRNAs in a variety of organisms, once nucleic acids have been properly extracted from tissues.

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