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Approaches for Studying MicroRNA and Small Interfering RNA Methylation *In Vitro* and *In Vivo*

Zhiyong Yang*, Giedrius Vilkaitis†, Bin Yu*, Saulius Klimašauskas†, and Xuemei Chen*

*Department of Botany and Plant Sciences and Institute of Integrative Genome Biology, University of California—Riverside, Riverside, California

†Laboratory of Biological DNA Modification, Institute of Biotechnology, Vilnius, Lithuania

Abstract

The biogenesis of microRNAs (miRNAs) in plants is similar to that in animals, however, the processing of plant miRNAs consists of an additional step, the methylation of the miRNAs on the 3' terminal nucleotides. The enzyme that methylates *Arabidopsis* miRNAs is encoded by a gene named *HEN1*, which has been shown genetically to be required for miRNA biogenesis *in vivo*. Small interfering RNAs (siRNAs) are also methylated *in vivo* in a *HEN1*-dependent manner. Our biochemical studies demonstrated that HEN1 is a methyltransferase acting on both miRNAs and siRNAs *in vitro*. HEN1 recognizes 21 to 24 nt small RNA duplexes, which are the products of Dicer-like enzymes, and transfers a methyl group from S-adenosylmethionine (SAM) to the 2' OH of the last nucleotides of the small RNA duplexes. Here we describe methods to characterize the biochemical activities of the HEN1 protein both *in vitro* and *in vivo*, and methods to analyze the methylation status of small RNAs *in vivo*.

1. Introduction

An miRNA is a 21 to 24 nt endogenous RNA product of a non-protein-coding gene. Since the first miRNA was discovered as a regulator of developmental timing in *Caenorhabditis elegans* in 1993 (Lee *et al.*, 1993), numerous miRNAs have been identified and recognized as important regulators of gene expression in both plants and animals (reviewed in Bartel, 2004). An miRNA is derived from a long primary transcript known as pri-miRNA (Lee *et al.*, 2002). In animals, the pri-miRNA is processed by the ribonuclease III (RNase III) enzyme Drosha into a precursor miRNA (pre-miRNA) (Lee *et al.*, 2003). The pre-miRNA is subsequently processed by another RNase III enzyme, Dicer, to generate a double-stranded miRNA intermediate known as miRNA/miRNA★ (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001; Lee *et al.*, 2003). Characteristics of the miRNA/miRNA★ duplex include 5' P, 3' OH, and a 2 nt 3' overhang on each strand (Basyuk *et al.*, 2003; Lee *et al.*, 2003). In plants, both pri-miRNAs and pre-miRNAs are processed by DCL1, a dicer homolog (Kurihara and Watanabe, 2004; Park *et al.*, 2002; Reihart *et al.*, 2002). After the miRNA/miRNA★ duplex is generated, the miRNA strand of the duplex is loaded into an RNA-induced silencing complex (RISC) where it targets mRNAs through sequence complementarity to lead to their cleavage or translational repression (reviewed in Bartel, 2004).

Our work revealed another player in miRNA biogenesis in plants—HEN1. HEN1 was first identified in a genetic screen as a gene important for reproductive organ identity (Chen *et al.*, 2002). Because many aspects of the developmental defects in the *hen1-1* mutant were similar to that of *caf-1*, a mutant of *DCL1* (Chen *et al.*, 2002; Jacobsen *et al.*, 1999), we tested whether HEN1 is required for miRNA accumulation *in vivo*. Indeed, the abundance of

miRNAs is dramatically reduced in *hen1* mutants, indicating that HEN1 is required for miRNA biogenesis (Park *et al.*, 2002).

We went on to demonstrate that HEN1 is an miRNA methyltransferase. The 942 aa HEN1 protein contains a conserved SAM-binding motif in the C-terminal region and a putative double-stranded RNA binding motif in the N-terminal region. The SAM-binding motif is embedded within a larger domain showing sequence similarities to many methyltransferases (Tkaczuk *et al.*, 2006; Yu *et al.*, 2005), suggesting that HEN1 is a methyltransferase. Using purified recombinant GST-HEN1 protein, we demonstrated that HEN1 is a miRNA methyltransferase that acts on miRNA/miRNA★ in a sequence-independent manner (Yu *et al.*, 2005). HEN1 can methylate small RNA duplexes ranging from 19 to 26 nt in size but has the best efficiency on 21 to 24 nt small RNA duplexes (Yang *et al.*, 2006). In addition, it has a strict requirement for the 2 nt 3′ overhang and for the presence of both the 2′ and 3′ OH on the ribose of the 3′ terminal nucleotide, characteristic features of Dicer products (Yang *et al.*, 2006).

The requirement of HEN1 for miRNA biogenesis *in vivo* and the *in vitro* biochemical activities of HEN1 suggest that plant miRNAs are methylated *in vivo*. We have demonstrated that this is indeed the case—plant miRNAs carry a methyl group on the 3′ terminal nucleotides (Yu *et al.*, 2005). Another class of small RNAs similar to miRNAs in structure and in their biogenesis is siRNAs. In contrast to miRNAs that are derived from single-stranded precursors, siRNAs are generated by Dicer-like proteins from long double-stranded RNAs. Several types of endogenous siRNAs have been found in *Arabidopsis*, such as trans-acting siRNAs, nat-siRNAs, and heterochromatic siRNAs (reviewed in Brodersen and Voinnet, 2006; Vaucheret, 2006). In addition to the endogenous siRNAs, viruses and transgenes also give rise to siRNAs (reviewed in Brodersen and Voinnet, 2006; Vaucheret, 2006). We and others have shown that all types of siRNAs (except nat-siRNAs that have not been tested) are methylated *in vivo* in a HEN1-dependent manner (Akbergenov *et al.*, 2006; Ebhardt *et al.*, 2005; Li *et al.*, 2005). Our biochemical studies with recombinant HEN1 protein also demonstrated that HEN1 is a siRNA methyltransferase (Yang *et al.*, 2006).

In this chapter, we describe molecular and biochemical methods used to delineate the activity of HEN1 as a small RNA methyltransferase *in vitro*. We report an enzymatic assay with HEN1 immunoprecipitated from plant extracts to confirm its *in vivo* activity. We also describe methods used to determine the methylation status of small RNAs *in vivo*.

2. Expression and Purification of Recombinant HEN1 Proteins

2.1. Generation of expression vectors

A modified version of the HEN1 cDNA (such that a hotspot for *Escherichia coli* transposon insertion was mutated [Yu *et al.*, 2005]) was used to generate fusion protein constructs. The HEN1 coding region was amplified with primers HEN1p4 (5′-ccggaattctcaagatcagctcttttctttctacatctctt-3′; the introduced EcoRI site is in italics) and HEN1p6 (5′-ccggaattcatatggccgtgtg-gggaagc-3′; the introduced EcoRI site is in italics) and cloned as an EcoRI fragment into pGEX-2TK for GST-HEN1 fusion protein expression in *E. coli*.

To fuse HEN1 with a shorter N-terminal peptide (MGSSHHHHHHSSGLVPRGSH) containing a His₆-tag followed by a thrombin cleavage site, the *HEN1* cDNA was subcloned into the pET-15b vector plasmid (Novagen) in two steps. First, the 1.7-kb NdeI fragment from pGEX-2TK-HEN1 was cloned in the vector plasmid through the NdeI site. In the next step, a full-length *HEN1* gene was reconstituted by replacing the Bsu15I-XhoI fragment of the resulting plasmid with the 2.2-kb Bsu15I-EcoRI fragment from pGEX-2TK-HEN1 (the

XhoI and EcoRI sites were blunt-ended by T4 DNA polymerase fill-in treatment prior to ligation).

2.2. Expression and purification of GST-tagged HEN1 protein

The *E. coli* strain BL21-CodonPlus(DE3)-RIL (Stratagene) was transformed with pGEX-2TK-HEN1. A single colony of the transformed *E. coli* was inoculated into 5 ml LB with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol. After 16 h at 37° with vigorous shaking at 250 rpm, the 5-ml culture was transferred into 500 ml 2×YT medium containing 100 µg/ml ampicillin. Incubation was continued at 37° with vigorous shaking until the OD₆₀₀ reached 0.6 to 0.9. The expression of the GST-HEN1 protein was induced by adding 500 µl of 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) so that the final concentration of IPTG was 1 mM. The culture was then grown at 30° with shaking at 250 rpm for 3 to 4 h. Cells were pelleted by centrifugation at 5000g for 10 min at 4°. The supernatant was discarded and the cell pellet (kept on ice) was resuspended in approximately 20 ml of cold PBS Plus buffer (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, 1 mM DTT, 1× Protease Inhibitor Tablet without EDTA (Roche), pH 7.5). The resuspended cells were disrupted using a sonicator with a microtip (Branson 450; output at 30%). Six 10-sec sonications were performed with a 1-min interval on ice between each sonication. Twenty percent Triton X-100 was added to the sonicated solution to reach a 0.6% final concentration and mixed gently. The slurry was centrifuged at 17,000g for 20 min at 4°. The supernatant was transferred to a new tube and centrifuged again for another 10 min. The second 17,000g supernatant was utilized for protein purification with a column containing pre-prepared Glutathione Sepharose 4B Matrix (Amersham Pharmacia Biosciences). Glutathione Sepharose 4B beads were washed with PBS buffer three times to remove the 20% ethanol storage solution. Then 1-ml beads were packed into a disposable polypropylene column. The 17,000g supernatant was transferred into the column and allowed to slowly pass through the beads. The beads were washed with at least 10 bed vol of PBS. The GST-HEN1 protein was eluted with 10-mM reduced glutathione in 50 mM Tris-HCl buffer (pH 8.0). One-ml fractions were collected. Protein concentrations were determined using the Bradford reagent (BioRad) with BSA as a standard.

2.3. Expression and preparation of His-tagged HEN1

The protease-deficient *E. coli* strain BL21-CodonPlus(DE3)-RIL (Stratagene) was transformed with the recombinant pET-15b-HEN1 plasmid, plated on LB agar containing 100 µg/ml ampicillin and 30-µg/ml chloramphenicol, and incubated at 37°. A single colony was inoculated into 5 ml of liquid LB with the antibiotics and incubated in a shaker overnight at 37°. Five hundred ml of LB medium containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol was inoculated with a 0.005 to 0.0025 vol of the overnight culture and was cultivated at 37° until it reached a density of OD₆₀₀ = 0.6 to 0.8. Protein expression was induced by adding IPTG to a final concentration of 0.1 mM followed by incubation at 16° overnight. Cells were harvested by centrifugation at 4000g for 10 min (the pellet can be stored at -70° for future use). The pellet was washed with ice-cold PBS, resuspended in 25 ml of Phosphate Buffer (20 mM Na₂HPO₄, 500 mM NaCl, pH 7.5) supplemented with 0.5% (v/v) Triton X-100, 5 mM 2-mercaptoethanol, and protease inhibitors (500 µM PMSF, 10 µM E-64, 1 µM pepstatin A), and sonicated. Lysed cells were centrifuged at 39,000g for 20 min at 4°, and the supernatant was collected and passed through a 0.45 µm pore filter. The hexahistidine tagged HEN1 protein was purified on a nickel-loaded agarose resin (Amersham Pharmacia Biosciences) according to manufacturer's recommendations. Briefly, after loading the lysate, the column was washed with Phosphate Buffer and then with 0.01-M imidazole/Phosphate Buffer until no UV absorbing material was detectable in the eluate. The desired protein was eluted with a 0.01 to 0.5 M concentration gradient of imidazole in PBS. All purification steps were performed at 4° or on ice. Purest fractions were pooled and

dialyzed twice against Storage Buffer [20 mM Tris-HCl, 50 mM NaCl, 0.01% (v/v) Triton X-100, 2 mM DTT, pH 7.5] containing 5% glycerol, then against Storage Buffer containing 50% glycerol, and stored at -20° . The concentration and purity of the proteins were assessed using SDS-PAGE analysis (Fig. 8.1) or a colorimetric Bradford assay. The yield of the HEN1 protein obtained from 500 ml of bacterial culture was 2.5 to 5 mg.

3. Small RNA Methyltransferase Assays with Recombinant HEN1 Proteins

3.1. Methyltransferase assays as monitored by the incorporation of [^{14}C]-methyl groups

Single-stranded RNA oligonucleotides corresponding to miR173, miR173★, or other miRNAs or miRNA★s were purchased from Integrated DNA Technologies, Dharmacon RNA Technologies, or Metabion in high-performance liquid chromatography (HPLC) purified forms. The RNA oligonucleotides were dissolved in the annealing buffer (50 mM Tris/HCl, pH 7.6, 100 mM KCl, and 2.5 mM MgCl_2). The concentrations of the RNAs were determined by UV spectrometry. Equal molar amounts of the miRNA and miRNA★ strands were mixed together. Annealing was performed by heating the mixture for 5 min at 95° , slowly cooling it to 37° , followed by incubation for 2 h at 37° , and 1 h at room temperature in a thermal cycler.

A 100- μl methyltransferase reaction was set up for each pair of annealed RNAs. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM MgCl_2 , 0.1 mM EDTA, 2 mM DTT, 5% glycerol, 2 μl RNasin (40 U/ μl ; Promega), 0.5 μCi *S*-adenosyl-L-[methyl- ^{14}C] methionine ([^{14}C]-SAM) (58.0 mCi/mmol; Amersham Pharmacia Biosciences), 5 μg purified protein, and 1 nmol RNA substrate. After incubation at 37° for 2 h, the reaction was stopped by adding 100- μl 2 $\times$ proteinase K solution (100-mM Tris-HCl, pH 8.0, 10-mM EDTA, 150-mM NaCl, 2% SDS, and 0.4-mg/ml proteinase K) followed by incubation at 65° for 15 min. The reaction was then extracted with phenol/chloroform. To precipitate the small RNAs, 1 μl glycogen (RNase-free, 5 mg/ml), 0.1 vol of 3-*M* NaOAc (pH 5.2), and 2.5 vol of ice-cold 100% ethanol were added to the reaction. The mixture was stored at -80° for 2 h and centrifuged at 4° for 30 min. The pellet was washed with 100- μl 70% cold ethanol. The RNAs in the pellet were dissolved with 1 $\times$ RNA loading buffer, heated at 95° for 5 min, immediately put on ice, and loaded onto a 15% polyacrylamide gel with 7-*M* urea. After electrophoresis, the gel was treated with an autoradiography enhancer (En³hance from Perkin Elmer) following manufacturer's instructions and exposed to X-ray film at -80° . Because the reaction results in the transfer of the ^{14}C -methyl group from SAM to the miRNAs, the miRNAs become ^{14}C -labeled after the reaction (Yu *et al.*, 2005).

3.2. Methyltransferase assays as monitored by the incorporation of [^3H]-methyl groups

[methyl- ^3H]-SAM can also be employed as the cofactor in the methylation reaction, and the enzymatically transferred methyl groups can be quantified in a liquid scintillation counter. As compared to ^{14}C label, tritium offers a 1000-fold higher specific activity and thus a higher sensitivity of detection in a scintillation counter. Therefore, the accessible concentration range can be expanded down by 2 to 3 orders of magnitude. This method is well suited for monitoring the reaction in a time-dependent manner for kinetic studies of methyltransferases (Vilkaitis *et al.*, 2001).

Enzymatic methylation velocities were monitored by measuring the incorporation of tritiated methyl groups from [methyl- ^3H]-SAM (Amersham Pharmacia Biosciences) onto the miR173/miR173★ duplex in the presence of HEN1. For determining steady-state velocities, methylation reactions were carried out at 37° for 60 min in 25 μl of Reaction Buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl_2 , 2 mM DTT, 0.1 mg/ml bovine serum albumin, 0.1 U/ μl RiboLock™ ribonuclease inhibitor [Fermentas Life Sciences, added immediately before use]), which contained 0.02 to 0.1 mM HEN1, 1 to 2 μM RNA duplex,

and 20 μM [*methyl*- ^3H]-SAM. Commercial [*methyl*- ^3H]-SAM with two different specific activities can be purchased (0.5 Ci/mmol at 0.5 mCi/ml or 15 Ci/mmol at 1 mCi/ml). If desired, 4.7 Ci/mmol [*methyl*- ^3H]-SAM could be prepared by mixing the two at a 1:6(v/v) ratio. The reactions were quenched by adding a 100-fold or higher excess of cold SAM (Sigma) and proteinase K (Fermentas Life Sciences) to a final concentration of 1 mg/ml in Stop Buffer (2 $\times$ buffer: 20 mM Tris-HCl, pH 7.8, 1% SDS, 20 mM EDTA, 20 mM NaCl; store at room temperature). After 15-min incubation at 55°, duplicate or triplicate samples were spotted onto 2.3-cm DE-81 filters (Whatman), washed four times with 0.05-*M* sodium phosphate buffer, pH 7.0, then two times with water, two times with ethanol, and finally once with acetone. Carefully dried filters were counted for radioactivity in 4 ml of scintillation cocktail (Rotiszint@eco plus, Carl Roth GmbH; CytoScint™, Fisher Biotech, or equivalent) using a liquid scintillation spectrometer. Background counts obtained from samples lacking dsRNA were subtracted. Figure 8.2 shows an example of the reaction time course analysis for the His₆-HEN1 methyltransferase. The method described above can be used for kinetic analyses of HEN1 and its derivatives.

In cases when accurate absolute numbers are required or when a significant variation in repetitive experiments is observed, it may be helpful to monitor (and to normalize the tritium counts for) the extent of recovery of the RNA duplex on DE-81 filters during the multiple wash procedures (typically 50 to 90%) (Vilkaitis *et al.*, 2001). This is achieved by adding small amounts of high specific activity ^{32}P -labeled dsRNA (~1000 dpm/sample). Because the energy spectra for the isotopes are very different, they can be measured independently using appropriate settings for spectral windows (0 to 400 for ^3H and 600 to 1000 for ^{32}P in Beckman scintillation counters) (Yang *et al.*, 2003).

3.3. Methyltransferase assays as monitored by β -elimination

The methyltransferase reactions can also be monitored by the appearance of the products in the absence of radioactivity. The products can be distinguished from the substrates because the products, not the substrates, are resistant to sodium periodate treatment followed by β -elimination. Periodate is specific for cis-diols, which are only found in the 3'-terminal nucleotide when both of its adjacent 2' and 3' hydroxyl groups are unmodified. Sodium periodate (oxidation reaction) cleaves the vicinal hydroxyl groups to a dialdehyde. Then the dialdehyde is treated with borate at pH 9.5 (β -elimination reaction) to result in a 1-nt shorter RNA with a 3'-monophosphate (Alefelder *et al.*, 1998). The RNA will move faster in gel electrophoresis by approximately 1.5 nt than the RNA prior to the treatment. Figure 8.3 shows the difference in electrophoretic mobility of an *in vitro* synthesized RNA oligonucleotide with two free hydroxyl groups on the terminal ribose. The product of HEN1-catalyzed reaction has a 2'-O-methyl group on the ribose of the last nucleotide (Yang *et al.*, 2006) and is therefore resistant to the chemical treatments. Monitoring the products this way allows the methyltransferase reactions to be performed in the presence of cold SAM or no added SAM (to assay SAM copurification with recombinant HEN1).

After the methyltransferase reactions (as described above), one pmol of miRNAs was dissolved in 7.5 μl of DEPC-treated H₂O and mixed with 10 μl 2 $\times$ borax/boric acid buffer (0.12 *M*, pH 8.6). Then 2.5- μl sodium periodate (200 mM) was added. The reaction was incubated in the dark at room temperature for 30 min. Two- μl 100% glycerol was added to the reaction and incubation was continued in the dark for 10 min at room temperature. The miRNAs were then precipitated and dissolved in 20 μl 1 $\times$ borax/boric acid buffer (0.06 *M*, pH 9.5), incubated for 90 min at 45°, and then precipitated again. The pellet was washed with ice-cold 70% ethanol, dissolved in 10 μl 1 $\times$ RNA loading buffer, and analyzed by Northern blotting (see following).

This assay can also be used for the quantitative detection of endogenous SAM in HEN1 preparations. The cofactor SAM is often strongly associated with methyltransferases and may be retained in preparations even after several steps of chromatographic purification (Kumar *et al.*, 1992). Substantial amounts of bound SAM (over 10 to 20 mol%) may interfere with certain biochemical and kinetic experiments. Several rounds of dialysis against a suitable buffer are usually sufficient to remove most of the bound cofactor. The detection of endogenous SAM is based on *in vitro* enzymatic reactions, in which increasing amounts of a methyltransferase are incubated with a fixed amount of its substrate without added SAM (Kumar *et al.*, 1992). For example, methylation reactions can be performed with 0.2 μ M miR173/miR173★ duplex and several concentrations of GST-HEN1 in the range 0.2 to 3 μ M.

Reactions are incubated and analyzed using the β -elimination assay as described previously to determine the concentration of product [Product] formed. Methyltransferase concentrations [Methyltransferase] that give $0.1 < [\text{Product}]/[\text{Substrate}] < 0.9$ are selected to calculate the mol% of bound SAM according to $\text{SAM\%} = [\text{Product}]/[\text{Methyltransferase}] \times 100\%$.

4. Reverse-Phase HPLC Analysis to Determine the Position of the Methyl Group in Products of HEN1-Catalyzed Reactions

Using the aforementioned β -elimination reactions, we showed that at least one of the two OH groups on the 3' end of small RNAs is blocked after methylation, suggesting that a methyl group is on either the 2' OH, the 3' OH, or both positions (Yu *et al.*, 2005). One way to determine the position of the methyl group is to analyze the terminal nucleoside by HPLC. To simplify our analysis, we designed an RNA oligonucleotide, miR173★C, by changing the last nucleotide of miR173★ from G to C so that the terminal nucleotides of both strands in the miR173/miR173★C duplex are C. Because both strands are methylated, the G-to-C change ensures that only methylated cytidine be present after the reaction.

We annealed miR173 to miR173★C and performed the GST-HEN1 methylation reaction and a control reaction in which GST instead of GST-HEN1 was included. Approximately 200 μ g of annealed miR173/miR173★C duplex was incubated with purified GST-HEN1 or GST in the presence of 1-mM cold SAM at 37° for 2 h. After the reaction, the miRNAs were extracted and precipitated, as described earlier.

The precipitated miR173/miR173★C duplex was dissolved in a 45- μ l sodium acetate buffer (20 mM; pH 5.3) containing 5 mM ZnCl₂ and 50 mM NaCl, and then digested with 5 U of nuclease P1 (1000 U/ml; USB) for 60 min at 37°. Nuclease P1 is specific for single-stranded RNA and is therefore expected to fully digest the 2 nt 3' overhang in the duplex. After digestion, 10 μ l 1-M Tris-HCl (pH 8.0) was added to bring the pH back to 8.0. Then 1 U of calf intestine alkaline phosphatase (Roche) was added, and the reaction was allowed to proceed for 30 min at 37°. Following the dephosphorylation reaction, all of the miR173/miR173★C hydrolysate was subjected to reverse-phase HPLC with a Phenomenex Luna C18 (250 $\times$ 4.60 mm) column at a flow rate of 0.8 ml/min. The mobile phase was 50-mM triethylamine acetate (pH 7.6) and 2% acetonitrile (ACN). A gradient was used in which the concentration of ACN was gradually increased starting at 15 min. The program was as follows: 0 to 15 min: 2% ACN; 15 to 20 min: linear increase from 2 to 100% ACN; 20 to 25 min: 100% ACN; 25 to 30 min: linear decrease from 100 to 2% ACN. This HPLC scheme allowed the separation of 2'-O-methyl C from 3'-O-methyl C as well as from other nucleosides. Our analyses showed that HEN1-catalyzed methylation of miRNAs occurs on the 2' OH on the ribose of the 3' terminal nucleotides (Yang *et al.*, 2006).

5. Immunoprecipitation and HEN1 Activity Assay

5.1. 35S::HA-HEN1 construction and plant transformation

A *HEN1* genomic fragment encompassing the entire coding region plus introns was cloned into the binary plant transformation vector pPZP211 to generate pPZP211-HEN1. Then a cassette containing the 35S promoter, a translational leader, and a 3× HA epitope was cloned into pPZP211-HEN1, resulting in a translational fusion of 3× HA to the N-terminus of HEN1. This construct, when introduced into the *hen1-1* background, was able to rescue the *hen1-1* morphological defects, suggesting HA-HEN1 fusion was functional. Western blots on total protein extracts from 35S::HA-HEN1 *hen1-1* inflorescences confirmed the accumulation of HA-HEN1 (data not shown).

5.2. HA-HEN1 immunoprecipitation and enzymatic activity assay

Inflorescences (5 g) from 35S::HA-HEN1 *hen1-1* or the *hen1-1* control *Arabidopsis* were ground in liquid nitrogen to a fine powder. The powder was added to 30 ml IP buffer (40 mM HEPES/KOH, pH 7.4, 150 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 1 mM PMSF, 1× Complete Protease Inhibitor without EDTA [Roche]). The slurry was filtered through three layers of miracloth (Calbiochem), incubated on a rotating wheel at 4° for 30 min, and then centrifuged for 30 min at 17,000g at 4°. The supernatant was transferred to a new centrifuge tube and spun for another 20 min. The supernatant was again transferred to a new tube. A 20-μl fraction was removed from the supernatant, mixed with 20 μl 2× SDS sample buffer, and boiled for 5 min so that it served as a total input control. One hundred-μl anti-HA affinity matrix (Roche) was added to the rest of the supernatant. The mixture was incubated on a rotating wheel for 3 h at 4°. The beads were precipitated by spinning at 500 g for 5 min and were washed three times with PBS containing 1× Protease Inhibitor Tablet. The beads were then equilibrated with methyltransferase assay buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 5% glycerol, and 1 mM DTT). For the methyltransferase assay, 50 μl of beads was used for each assay using the same method as described previously (with ¹⁴C incorporation as the measure for activity). For SDS-PAGE, the affinity-bound proteins were eluted from the beads by boiling for 5 min in 2× SDS sample buffer before loading onto a 10% SDS-polyacrylamide gel (Fig. 8.4A). As shown in Fig. 8.4B, the HA-HEN1 immunoprecipitate from plant extracts was able to methylate the miR173/miR173★ duplex. This confirms that HEN1 has miRNA methyltransferase activity *in vivo*.

6. Analysis of the *In Vivo* Methylation Status of miRNAs and siRNAs

We developed two methods to evaluate the methylation status of miRNAs and siRNAs *in vivo*. First, we combined β-elimination of total plant RNAs with small RNA Northern blots to show that plant miRNAs lack at least one free OH on the 3° terminal nucleotides. Second, we isolated sufficient amounts of miR173 by affinity purification and subjected the purified miR173 to mass spectrometry to determine its molecular weight. The latter approach showed that miR173 is approximately 14 Daltons larger than expected, consistent with its carrying a methyl group (Yu *et al.*, 2005). Although the second method is more definitive in the determination of the methylation status of *in vivo* small RNAs, it cannot be routinely used due to the requirement for large amounts of purified RNAs. In addition, it is also limited to those small RNAs without related species *in vivo* because the affinity purification step cannot effectively distinguish related species, and the presence of related species complicates the mass spectrometry analysis. Since this approach cannot be routinely used, it is not discussed further here. Instead, we present a detailed protocol for the first method (β-elimination followed by Northern blotting).

6.1. Periodate treatment and β -elimination

Total RNA was extracted from liquid nitrogen–frozen plant tissues using Tri Reagent (Molecular Research Center, Inc.) according to manufacturer's instructions. Periodate treatment and β -elimination were performed as previously described (Alefelder *et al.*, 1998) with some modifications. Approximately 50 μ g of total RNA was dissolved in 37.5 μ l DEPC-treated water and mixed with 50- μ l 2 $\times$ borax/boric acid buffer (0.12 M, pH 8.6). Then 12.5 μ l sodium periodate (200 mM in water) was added. The solution was mixed quickly and incubated in the dark at room temperature for 30 min. One hundred percent glycerol (10 μ l) was added to the reaction to quench unreacted sodium periodate by incubation for an additional 10 min in the dark at room temperature. The RNA was precipitated and dissolved in 100 μ l 1 $\times$ borax/boric acid buffer (0.06 M, pH 9.5), incubated for 90 min at 45°, and then precipitated with ethanol. The pellet was washed with 70% ethanol and dissolved in 10 μ l 1 $\times$ RNA loading buffer. The treated total RNA was analyzed by small RNA Northern blotting.

6.2. Small RNA Northern blotting

The treated total RNA and control total RNA without periodate treatment were loaded on a 15% polyacrylamide gel containing 7-M urea. The RNAs were transferred to Zeta-probe GT membranes (BioRad) using semi-dry transfer equipment (Owl Separation Systems) for 1 h at 10 V. After electro-blotting, RNAs were fixed to the membrane by ultraviolet (UV) cross-linking for 1 min followed by baking in a vacuum oven at 80° for 1 h. The membrane was prehybridized with UltraHyb-oligo hybridization buffer (Ambion) for 2 h at 40°. During the prehybridization, a 5' end-labeled probe was prepared as follows: 5 μ l 10 $\times$ T4 DNA polynucleotide kinase buffer (New England Biolabs), 5 μ l T4 DNA polynucleotide kinase (New England Biolabs), 0.5 μ l 100- μ M DNA oligonucleotide (antisense strand), 5 μ l gamma [³²P]-ATP (6000 Ci/mmol; PerkinElmer), and 34.5 μ l H₂O were mixed and incubated at 37° for 1 h. The oligonucleotide was isolated from the free [³²P]-ATP by passing the reaction mixture through a MinSpin G-25 Column (Amersham Pharmacia Biosciences). The membrane was hybridized with the 5' end-labeled antisense oligonucleotide probe for approximately 20 h at 40°. Then the membrane was washed three times with 2 $\times$ SSC/0.5% SDS at 40°. The radioactive signals were visualized and quantified with a PhosphorImager.

The expected results are such that a shift in mobility by about 1.5 nt should be observed when a small RNA is not methylated and that no shift be observed when a small RNA is methylated. However, it should be noted that an *in vitro* synthesized oligonucleotide without methylation should be spiked into total RNA as a control for the completeness of the chemical reactions. In addition, resistance to the chemical reactions only indicates that one of the two OH groups is blocked without reference to the nature of the modification on the small RNAs.

7. Concluding Remarks

The biogenesis of miRNAs and siRNAs in plants involves a critical methylation step catalyzed by the methyltransferase HEN1. HEN1 uses small RNA duplexes, products of Dicer-mediated processing of precursor molecules, as its substrates. HEN1 introduces one methyl group onto the 2' OH of the 3' terminal nucleotide on each strand of the duplex. *In vivo*, all miRNAs and siRNAs in *Arabidopsis* are methylated in a HEN1-dependent manner.

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Figure 8.1.

SDS-polyacrylamide gel electrophoresis of affinity-purified recombinant GST-HEN1 (lane1) and His₆-HEN1 (lane 2). Protein staining with Coomassie Brilliant Blue R-250 indicates a greater than 90% purity of both recombinant methyltransferases. The sizes of molecular mass markers are indicated on the left.

Figure 8.2.

A time course of dsRNA methylation catalyzed by the His₆-HEN1 methyltransferase. Experiment was performed at 37° in Reaction Buffer containing 2- μ M miR173/miR173★ RNA, 20- μ M [*methyl*-³H]-SAM (4.7 Ci/mmol), and 0.1- μ M methyltransferase. Duplicate aliquots were removed for analysis at specified time points and processed as described.

Figure 8.3.

β -elimination of an RNA oligonucleotide with two free OH groups on the 3' terminal ribose. The RNA was treated (+) or untreated (–) with the chemicals for β -elimination, resolved on a 15% polyacrylamide gel with urea, blotted to a membrane, and hybridized with an antisense probe.

Figure 8.4.

Immunoprecipitation (IP) of HEN1 followed by a methyltransferase assay. IP was performed from inflorescence tissues of *35S::HA-HEN1 hen1-1* (lane 1) or *hen1-1* (lane 2). A, Western blotting with anti-HEN1 polyclonal antibodies. Ten- μ l beads from the IP were mixed with 10- μ l 2 $\times$ SDS sample buffer, boiled, and loaded on a 10% SDS-polyacrylamide gel for Western blotting with anti-HEN1 polyclonal antibodies. Sizes of molecular mass markers are indicated on the left. B, A HEN1 methyltransferase assay with the immunoprecipitates from *35S::HA-HEN1 hen1-1* (lane 1) and *hen1-1* (lane 2). The miR173/miR173 $\star$ duplex was used as the substrate and [*methy*- 14 C] SAM was used as the cofactor.