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Methylation as a Crucial Step in Plant microRNA Biogenesis

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Abstract

Methylation on the base or the ribose is prevalent in eukaryotic ribosomal RNAs (rRNAs) and is thought to be crucial for ribosome biogenesis and function. Artificially introduced 2′-*O*-methyl groups in small interfering RNAs (siRNAs) can stabilize siRNAs in serum without affecting their activities in RNA interference in mammalian cells. Here, we show that plant microRNAs (miRNAs) have a naturally occurring methyl group on the ribose of the last nucleotide. Whereas methylation of rRNAs depends on guide RNAs, the methyltransferase protein HEN1 is sufficient to methylate miRNA/miRNA* duplexes. Our studies uncover a new and crucial step in plant miRNA biogenesis and have profound implications in the function of miRNAs.

MicroRNAs are 20- to 24-nucleotide (nt) RNAs that serve as sequence-specific regulators of gene expression in diverse eukaryotic organisms. miRNA/miRNA* duplexes, products of Dicer processing of pre-miRNAs, are structurally similar to small interfering RNAs (siRNAs) in that they have 2-nt overhangs, as well as a 5′ phosphate (P) and a 3′ OH (1). miRNA accumulation in *Arabidopsis* requires a protein named HEN1 (2). *In hen1* mutants, miRNAs are reduced in abundance, and when detectable, are heterogeneous in size (2–5). The HEN1 protein has an N-terminal putative double-stranded RNA–binding motif and a C-terminal region that is conserved among many bacterial, fungal, and metazoan proteins [(2), fig. S1a]. Embedded within this region is a recognizable *S*-adenosyl methionine (SAM)–binding motif (fig. S1a), which suggests that HEN1 may be a miRNA methyltransferase (6, 7).

To test whether HEN1 is a miRNA methyltransferase, we expressed the full-length HEN1 protein fused to glutathione *S*-transferase (GST) in *Escherichia coli* and performed a methylation reaction. In vitro synthesized miR173 and miR173* RNA oligonucleotides with 5′ P and 3′ OH were annealed to generate the miR173/miR173* duplex. Purified GST or GST-HEN1 (fig. S1b) was incubated with the miR173/miR173* duplex in the presence

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Supporting Online Material

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Materials and Methods

Figs. S1 to S6

References and Notes

of ^{14}C-labeled SAM. GST-HEN1 indeed resulted in the methylation of the miRNA/miRNA* duplex, but GST alone did not (Fig. 1A). GST-HEN1m2, in which the potential SAM-binding motif in the conserved C-terminal domain of HEN1 was mutated (fig. S1), was unable to methylate miR173/miR173* (Fig. 1A).

We next determined the substrate specificity of HEN1 by testing various molecules as potential substrates in the in vitro reaction. An in vitro transcribed RNA corresponding to the putative pre-miR173 or *E. coli* tRNA was not methylated by GST-HEN1 (Fig. 1B, lanes 1 and 6). Unlike miR173/miR173*, single-stranded miR173 or miR173*, or a DNA duplex with sequences identical to miR173/miR173*, was not methylated by GST-HEN1 (Fig. 1B, lanes 2 to 5). GST-HEN1 could also methylate miR167a/miR167a* (Fig. 1B, lane 7), another miRNA/miRNA* duplex, which suggests that HEN1 does not have sequence specific recognition properties. In addition, two siRNA duplexes with perfect sequence complementarity failed to be methylated by GST-HEN1 (8). These results indicate that HEN1 is a methyltransferase and that HEN1 recognizes and acts on miRNA/miRNA* duplexes in vitro. However, although HEN1 is insufficient to methylate siRNA duplexes in vitro, other factors may facilitate the methylation of siRNAs in vivo. In fact, *HEN1* is required for the accumulation of siRNAs from sense transgenes (5).

Where does HEN1 methylate miRNA/miRNA* in vitro? miR173/miR173m1* (species 2 in Fig. 2A), a duplex without any 3′ overhangs, was a poor substrate for HEN1 (Fig. 2B, compare lane 2 with lane 1), which suggests that the 2 nt 3′ overhangs are an important feature in the substrate. In fact, HEN1 requires the 2′ OH on at least one of the last two nucleotides in the substrate because miR173d1d2/miR173*d1d2, a duplex identical to miR173/miR173* in sequence but with 2′-deoxyribose in the last two nucleotides (Fig. 2A, species 7), failed to be methylated (Fig. 2B, lane 7). Both miR173d1d2/miR173* and miR173/miR173*d1d2 (species 3 and 4 in Fig. 2A) could be methylated by HEN1 (Fig. 2B, lanes 3 and 4), which suggests that HEN1, at least in vitro, does not distinguish the miRNA strand from miRNA*. miR173d2/miR173*d1d2 (Fig. 2A, species 5), in which the penultimate nucleotide of miR173 is a deoxynucleotide, was still methylated by HEN1 (Fig. 2B, lane 5). However, miR173d1/miR173*d1d2 (Fig. 2A, species 6), in which the last nucleotide of miR173 is a deoxynucleotide at the 2′ position of the ribose, failed to be methylated by HEN1 (Fig. 2B, lane 6). Therefore, the 2′ OH on the last nucleotide is critical for the methylation reaction. The last nucleotide differs from all other nucleotides in that it also has a 3′ OH. A duplex identical to miR173/miR173*d1d2 except that the last nucleotide of miR173 is a deoxynucleotide at the 3′ position of the ribose (Fig. 2A, species 8) also failed to be methylated (Fig. 2B, lane 8), which indicated that the 3′ OH is also critical for the methylation reaction.

The OH groups on the last nucleotide may be the actual sites of methylation or may only be features necessary for substrate recognition. We tested whether methylation occurs on at least one of the OH on the last nucleotide. After incubation with GST-HEN1 in the presence of [^{14}C]SAM, the miR173/miR173* duplex was treated with sodium periodate followed by β elimination, chemical reactions that require the presence of OH groups at both 2′ and 3′ positions on the ribose of the last nucleotide. If the chemical reactions occur, the miRNA will be one nucleotide shorter and have a 3′ P (9), so that the apparent mobility would be

increased by two nucleotides (10). The effectiveness of the chemical reactions was demonstrated with control miR173 oligonucleotides with or without a 2′-*O*-methyl on its last nucleotide (fig. S2a). A mobility shift after the chemical reactions was observed for miR173 incubated with GST alone, but not for the ^{14}C-labeled miRNA resulting from the incubation with GST-HEN1 (fig. S2b), which indicated that methylation by GST-HEN1 had occurred on at least one of the two OH groups on the last nucleotide of miR173.

Are plant miRNAs methylated on the ribose of the last nucleotide as predicted from our in vitro results? We first performed the oxidation and β elimination reactions to evaluate whether the 2′ or 3′ OH on the 3′-most nucleotides is blocked in plant miRNAs. Total RNAs were treated with the chemicals, and specific miRNAs were detected by filter hybridization. In vitro synthesized miR173-OH (without any methyl groups) or miR173-2′me (with a 2′-*O*-methyl on its last nucleotide) was mixed with total RNAs from *dcl1*-9, which does not have detectable levels of miR173 (2), to show that the treatments were effective (Fig. 3A). The endogenous miR173 in wild type did not show any mobility shift (Fig. 3B). The miR173-OH standard was also included in the total RNA from wild type to demonstrate that the treatment was sufficient to induce the mobility shift (Fig. 3B). Similar results were obtained for miR167, miR172, miR159, and miR163 (Fig. 3C). Therefore, the last nucleotide of *Arabidopsis* miRNAs is blocked at the 2′-*O*-ribose, 3′-*O*-ribose, or both positions. Furthermore, for each miRNA species, any unmethylated miRNAs, if present, were below the limits of detection (fig. S3). In the *hen1-1* mutant, the heterogeneous miRNAs, when detectable, showed a mobility shift after the oxidation and β elimination reactions (Fig. 3C), which indicates that HEN1 is responsible for blocking the OH group(s) in vivo.

To determine the nature and number of modifications that are present in plant miRNAs, we used affinity purification to isolate miR173 from *Arabidopsis* total RNAs with a biotinylated antisense DNA oligonucleotide in order to perform mass spectrometry to determine the molecular mass of this miRNA. miR173 was chosen because no related miRNA genes are present in the genome. The final RNA fraction from the purification contained miR173 and was free of contamination from other miRNA species (fig. S4, a and c). The fraction, however, did contain trace amounts of larger RNAs that comigrated with tRNAs or rRNAs (fig. S4d) and some antisense DNA that was used as the affinity probe (fig. S4b). The amount of the DNA was expected to be below the limits of detection by our mass spectrometry analysis. In addition, we designed the DNA oligonucleotide with two extra nucleotides, so that the expected molecular mass would differ greatly from that of miR173. In the mass spectrometry analysis of the final purified RNA fraction, a large peak of unresolved molecular mass was detected and probably corresponds to the contaminating large RNAs in the fraction (fig. S5a). In the resolvable mass range (less than 8000 dalton), only one major peak with a mass of 7118.9 dalton was found (Fig. 4A; fig. S5a). This peak could not be the DNA oligonucleotide because the expected molecular mass of the DNA is 7758.3 dalton. Therefore, this peak must correspond to the in vivo miR173 species. The mass of the in vivo miR173 is larger than the expected mass of miR173 standard without any modifications (fig. S5b) by 13.6 dalton and is close to that of the miR173 standard with one methyl group (Fig. 4B). The deviation of the measured mass of the in vivo miR173 from the expected mass of the 1 methyl standard fell well within the experimental error (0.025%),

which was measured with miR173 standards harboring 0, 1, or 2 methyl groups (Fig. 4B; fig. S5, b and c). Therefore, we conclude that miR173 from *Arabidopsis* has one methyl group, which appears to be the only form of modification on the bulk of miR173. This and results of the β elimination reactions indicated that the methyl group is either at the 2′ or the 3′-*O*-ribose position of the last nucleotide.

Although proteins homologous to HEN1 along its entire length are not found in non-plant organisms, ones homologous to the C-terminal methyltransferase domain of HEN1 are present in diverse organisms (2). We examined three miRNAs from *C. elegans* and four from *Drosophila* and found that they have free 2′-OH and 3′-OH groups on their last nucleotides (fig. S6). *Drosophila let-7* was previously shown to have free 2′ and 3′ OH on its last nucleotide (9). Therefore, animal miRNAs may not be modified as they are in plants. However, it cannot be ruled out that methylation of animal miRNAs occurs in a small temporal or spatial window during development.

We envision several potential functions of the methyl group in plant miRNAs. First, it may protect miRNAs by attenuating enzymes such as exonucleases that target the 3′ end of miRNAs. This would be consistent with the reduced abundance of miRNAs in *hen1* mutants. Second, the methyl group may facilitate the recognition of miRNAs by plant argonaute proteins in RNA-induced silencing complex (RISC) assembly. In fact, the 2′ OH of the last nucleotide is a site of contact by the PAZ domain in animal argonaute proteins (11, 12), and 2′-*O*-methyl on the last nucleotide decreases the binding affinity by the PAZ domain (12). Perhaps plant argonaute proteins, unlike their animal counterparts, prefer methylated miRNAs. It is noteworthy that previous efforts to program RISC with exogenously introduced miRNAs in plant extracts have been unsuccessful (13). We suspect this lack of success was due to the inefficient incorporation of unmethylated miRNAs into RISC. Third, the methylation may limit the ability of plant miRNAs to serve as primers for RNA-dependent RNA polymerase (RdRP). Plant miRNAs show extensive complementarity to their target mRNAs, which raises the possibility that miRNAs may serve as primers to allow RdRP to generate dsRNA using the target mRNA as a template. The presence of the methyl group may reduce the efficiency of such a reaction. Note that miRNA-mediated cleavage of target mRNAs can lead to transitive RNA silencing dependent on an RdRP (14) and that HEN1 is required for sense transgene silencing that also depends on an RdRP (15, 16). However, the generation of dsRNAs by RdRP in these processes does not necessarily have to be primed by small RNAs (13).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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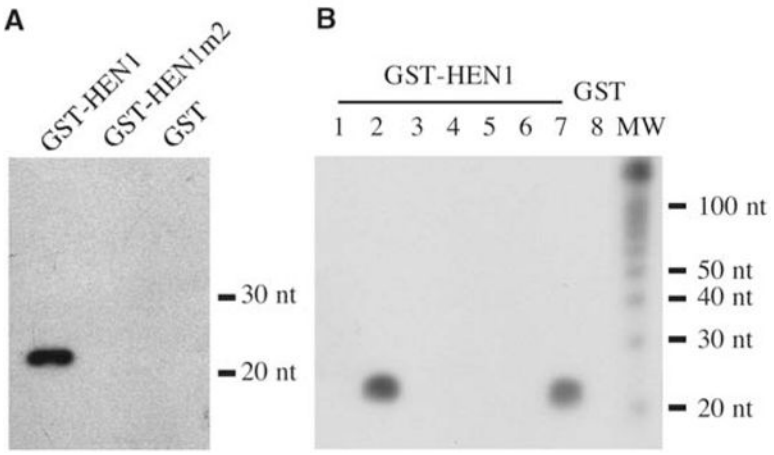

Fig. 1.

HEN1 has miRNA methyltransferase activity in vitro. The RNA substrates were incubated with the proteins and resolved by electrophoresis, and the presence of ^{14}C-labeled RNA was detected by autoradiography. (**A**) miRNA methyltransferase assay using the miR173/miR173* duplex as the substrate. (**B**) Various RNA substrates were tested for methylation by GST-HEN1. The substrates used are the putative pre-miR173 (lane 1), miR173/miR173* duplex (lane 2), miR173 (lane 3), miR173* (lane 4), a DNA duplex identical in sequence to miR173/miR173* (lane 5), *E. coli* tRNA (lane 6), and miR167a/miR167a* duplex (lane 7). Lane 8, miR173/miR173* was incubated with GST.

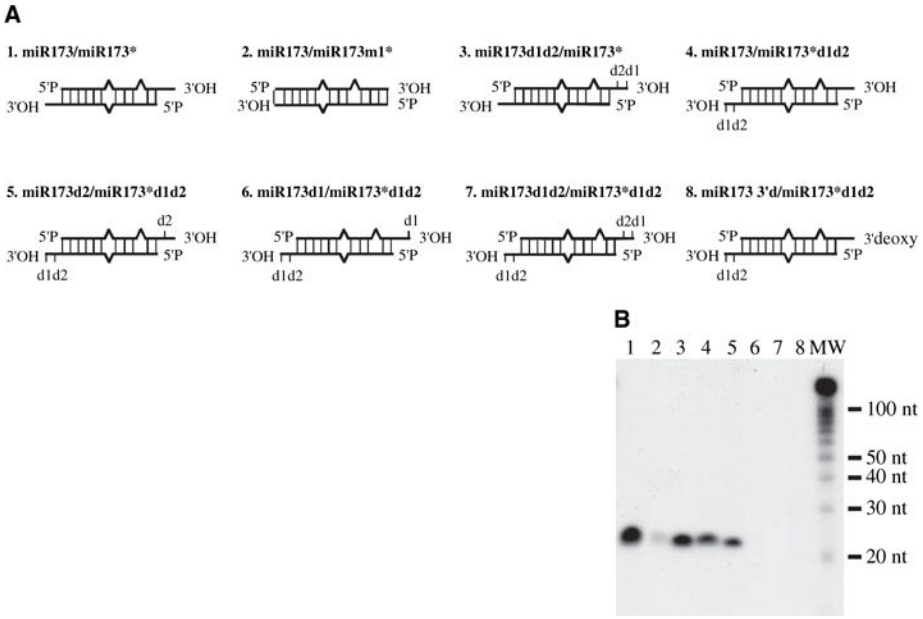

Fig. 2.

Methylation of miRNA/miRNA* in vitro requires the two free OH groups on the 3′-most nucleotides. The substrates diagrammed in (**A**) were assayed for methylation by GST-HEN1 in (**B**). The numbers of the molecules in (A) correspond to the lane numbers in (B). In the diagrams, the top strand is miR173 (22 nt), which is 1 nt longer than miR173*, the bottom strand. The two mismatches in the duplex are represented by the bulges. Species 2 in (A) differs from species 1, in that the miR173m1* strand is longer by 2 nt at its 5′ end but shorter by 2 nt at its 3′ end than miR173*. In species 3 to 8, d1 and d2 represent a deoxyribose at the 2′ carbon of the last and penultimate nucleotide, respectively. Species 8 has a deoxyribose at the 3′ carbon of the last nucleotide.

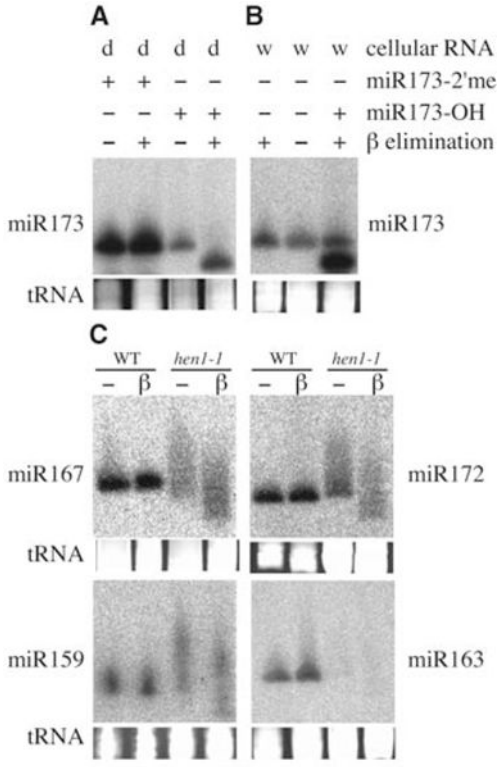

Fig. 3.

One or both OH groups on the ribose of the last nucleotide of *Arabidopsis* miRNAs are blocked. Total RNAs were treated with periodate followed by β elimination and then subjected to RNA filter hybridization with probes against various miRNAs (as indicated next to the blots). The region of the stained gel corresponding to where tRNAs migrate is shown below the hybridization images to indicate the amount of total RNAs used. (**A**) In vitro synthesized miR173-OH or miR173-2′me was added to total RNA from *dcl1-9* (d) and treated with the chemicals. miR173-OH showed the expected mobility shift but miR173-2′me was resistant to the chemical modification reactions. (**B**) miR173 from wild-type total RNA (w) is resistant to the chemical modification reactions. (**C**) Total RNA from wild type (wt) or *hen1-1* was either subjected (β) or not (−) to the chemical modification reactions and probed for various miRNAs.

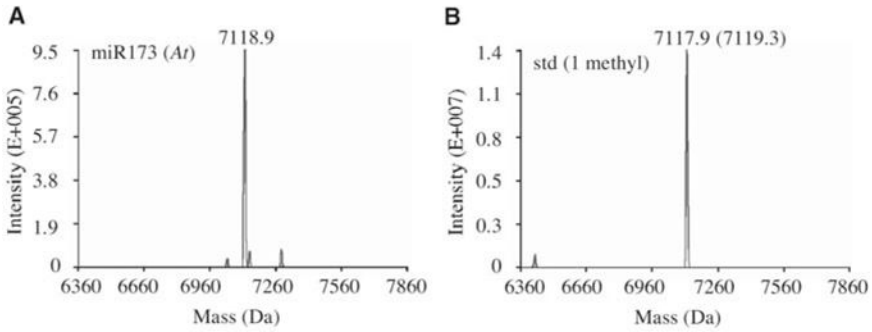

Fig. 4.

Mass spectrometry analysis of miR173 isolated from *Arabidopsis* (**A**) and a miR173 standard with one methyl group (**B**). The position of a peak along the *x* axis represents the molecular mass of the species. Signal intensity approximately reflects the amount of the RNA in each peak. The expected mass of the standard is indicated by the number in parentheses. The measured mass is indicated by the numbers directly above the peaks.